Centro de Investigación en Alimentación y Desarrollo, A.C.

LAS PROLAMINAS DEL MAIZ INDUCEN RESPUESTA INMUNE CELULAR EN ALGUNOS PACIENTES CON ENFERMEDAD CELIACA

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por:

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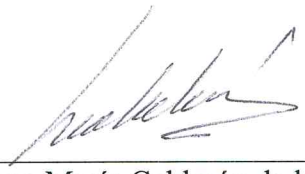

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CONTENIDO

Nota de la numeración: los número romanos y arábigos en la parte central inferior corresponden a los que se señalan en la tabla de contenido. Los números situados en la parte superior derecha o izquierda corresponden a la numeración de cada artículo según el formato de envío o la numeración propia de la revista donde fue publicado

RESUMEN

La enfermedad celiaca (EC) es un desorden sistémico mediado inmunológicamente en individuos predispuestos y es inducido por gliadinas del trigo, principalmente después de deamidadas por la transglutaminasa tisular (TG) del intestino. En raros casos, el maíz induce los síntomas por lo que se hipotetiza que las zeinas, aunque diferentes a gliadinas pero deamidables por la TG, son capaces de inducir una respuesta celular similar en algunos pacientes celiacos. Nuestro objetivo fue demostrar que las zeínas inducen una respuesta inmune celular y la reacción pro-inflamatoria que la precede. Se midió respuesta pro-inflamatoria y permeabilidad de monocapa en la línea celular CACO-2 al estimularlas con digeridos de zeínas y gliadinas. La respuesta inmune se evaluó estimulando biopsias del duodeno de pacientes celiacos con los péptidos sintéticos Z34-mer y G33-mer, productos inmunogénicos de la digestión de α-zeína y α-gliadina respectivamente. Ambos péptidos deamidados fueron utilizados para estimular linfocitos T de sangre periférica extraídos de dos pacientes con EC y tres controles sanos retados *in vivo* con gluten después de una dieta sin gluten de al menos un mes. Las zeínas aumentaron la permeabilidad de la monocapa celular y la liberación de la proteína zonulina-1 (ZO-1). Simultáneamente, la respuesta pro-inflamatoria fue evidenciada por el aumento de los marcadores moleculares como interleucina 8 (IL-8), ciclooxigenasa 2 (COX2) y la proteína fosforilada p38 cinasa (pp38 MAPK). Z34-mer aumentó la producción de interferón gamma (IFN-γ) en 3 de 5 biopsias de pacientes celiacos de forma similar al G33-mer. Además, en las células T de uno de dos pacientes, Z34-mer indujo una mayor producción de IFN-γ respecto a los controles y similar al G33-mer. En conclusión, las zeínas son capaces de inducir una respuesta inmune celular similar a la producida por gliadinas, en algunos pacientes celiacos.

Palabras Clave: Enfermedad celiaca, prolaminas del maíz, zeínas, respuesta inmune celular.

ABSTRACT

Celiac disease (CD) is an immunologically mediated systemic disorder in predisposed individuals induced by wheat gliadin, mainly after deamidated by intestinal tissue transglutaminase (TG). In rare cases, corn induces symptoms so it is hypothesized that the zeins, although different gliadins but deamidables by TG, are able to induce a similar cell response in some celiac patients. Our goal was to demonstrate that the zeins induce a cellular immune response and the pro-inflammatory reaction that precedes it. Pro-inflammatory response and monolayer permeability was measured in the cell line CACO-2 in response to digested zeins and gliadins. The immune response was evaluated by stimulating duodenal biopsies of celiac patients with the synthetic peptides Z34-mer and G33-mer, immunogenic products of digestion of α-zein and α-gliadin respectively. Both deamidated peptides were used to stimulate T cells from peripheral blood extracted from two CD patients and three healthy individuals challenged *in vivo* with gluten after a gluten-free diet for at least one month. Zeins increased permeability of the cell monolayer and the zonulin release of the protein-1 (ZO-1). Simultaneously, the pro-inflammatory response was evidenced by increase of molecular markers such as interleukin 8 (IL-8), cyclooxygenase 2 (COX2) and phosphorylated p38 protein kinase (MAPK pp38). Z34-mer increased production of interferon gamma (IFN-γ) in 3/5 biopsies of celiac patients similarly to G33-mer. Furthermore, Z34-mer induced higher production of IFN-γ in T cells from 1/2 patients compared with controls and similar to G33-mer. In conclusion, zeins are capable of inducing similar cell immune response to that produced by gliadin in some celiac patients.

Keywords: Celiac disease, maize prolamins, zein, cellular immune response.

SINOPSIS

La enfermedad celiaca (EC) es un desorden sistémico mediado inmunológicamente en el cual intervienen la predisposición genética, la ingestión de gluten y la inflamación en la mucosa intestinal. Debido a esta última, se facilita la entrada de los péptidos de gliadinas, un tipo de proteínas presentes en el gluten de trigo, a través del epitelio intestinal. El padecimiento se consideraba exclusivamente intestinal debido a que entre sus síntomas figuran la diarrea, la distención y dolor abdominales, así como mala absorción. Actualmente se reconoce que la EC puede manifestarse con síntomas extra-intestinales como la dermatitis herpetiforme, anemia, osteoporosis, crecimiento deficiente y retardo de la pubertad.

La prevalencia de EC se estima en 1:200 a 1:100[1, 2], en países caucásicos de Europa y en Estados Unidos. En nuestro país se estima que 1:170[3] personas padecen esta enfermedad, cifra que puede estar subestimada debido a la falta de información tanto en la población general como de los profesionales de la salud. De cualquier forma, de acuerdo al estimado, se trata de un problema de salud que requiere atención.

El único tratamiento para las personas que padecen EC es la dieta sin gluten, evitando alimentos que contienen trigo, cebada o centeno. Sin embargo, en algunos pacientes no hay respuesta a dicho tratamiento, no hay alivio en los síntomas, aumentando así el riesgo de padecimientos neoplásicos como linfomas, con pronóstico poco favorable. Una causa de la EC no responsiva puede ser la presencia de péptidos en los alimentos de la dieta sin gluten, que sean reconocidos por las células del sistema inmune y desencadenen la respuesta característica de este padecimiento. Entre estos alimentos figuran la avena, el arroz y el maíz; de los cuales, solo se ha encontrado respuesta celular a aveninas de dos variedades de avena[4]. El maíz es un alimento importante en nuestra cultura y es una de las principales opciones para sustituir trigo a pesar de sus limitaciones tecnológicas para la panificación. Debido a que se encuentra alejado

taxonómicamente del trigo, se clasifica como un alimento sin gluten; sin embargo, hay evidencias de que su consumo no es seguro para algunos pacientes celiacos[5,6].

En el 2006 se publicó el caso de una paciente con EC que no respondía al tratamiento sin gluten, curiosamente sus alimentos se acompañaban de un tipo de atole de maíz[5]. No fue hasta que se excluyó de la dieta al maíz además del trigo, que los síntomas remitieron. Poco tiempo después, nuestro grupo de trabajo trató el caso de un paciente celiaco cuyo padecimiento parecía agravarse a pesar de la dieta sin gluten. En el suero de este paciente, había anticuerpos IgA específicos a las prolaminas del pan de maíz tratado con transglutaminasa, en un nivel incluso más alto que a las prolaminas de pan de trigo[6]. En ambos casos se demostró que las prolaminas del maíz, llamadas zeínas, pueden ser responsables de la falla en el tratamiento de dieta sin gluten y solamente al restringir el maíz además del trigo, se logró aliviar los síntomas.

Cabrera-Chávez y colaboradores[7] mediante análisis de secuenciación masas/masas encontraron péptidos derivados de la α-zeína reconocidos por IgA de algunos pacientes celiacos. Entre estos péptidos figura el Z34-mer, un péptido de 34 aminoácidos resistente a la digestión con residuos de glutamina deamidables por la TG que facilitaría su unión a las moléculas HLA-DQ8. Las propiedades inmunogénicas de éste péptido pudieran ser comparables a las del péptido G33mer, un potente estimulador células T *in vitro*.

Para evaluar la respuesta celular a los péptidos de gliadinas en pacientes celiacos, se utilizan tanto células obtenidas de biopsias de la mucosa intestinal, como las biopsias mismas, y células aisladas de sangre periférica. Así mismo, se utilizan líneas de células epiteliales derivadas de tumores cancerígenos del colon (CACO-2), como modelo de membrana intestinal para estudiar efectos sobre la permeabilidad. Estas herramientas analíticas han sido utilizadas para detectar respuesta celular a las aveninas y otras moléculas de ingredientes alimentarios, por lo que también se pueden aplicar en la evaluación de la respuesta celular a las prolaminas del maíz.

Con los antecedentes antes descritos, nos planteamos la siguiente hipótesis: las prolaminas del maíz, aunque poseen secuencias antigénicas diferentes a las del trigo pero también son deamidables por la transglutaminasa tisular, son capaces de inducir una respuesta celular similar al gluten en algunos pacientes celiacos.

Para contrastar la hipótesis, revisamos exhaustivamente la información que asociara EC y proteínas de maíz. Hicimos además la búsqueda de secuencias proteicas y comparación con las del trigo, para inferir sobre la formación de epítopes con la posibilidad de inducir respuesta celular en pacientes celiacos. Así mismo, seleccionamos herramientas analíticas utilizadas para evaluar la respuesta celular ante estímulos con prolaminas del gluten, para reproducirlas con las del maíz. Una vez identificadas las técnicas analíticas y los péptidos inmunogénicos a utilizar, estudiamos si las zeínas pueden inducir una respuesta inmune celular y pro-inflamatoria análoga a las gliadinas, en cultivo primario de celiacos y en la línea celular CACO-2, respectivamente. Finalmente estudiamos si la respuesta inmune celular de las zeínas es análoga a la de gliadinas, en células T de sangre periférica después de un reto *in vivo* con gluten. El desarrollo de los estudios realizados para cumplir los objetivos y contrastar la hipótesis, se describe en tres capítulos que junto a la presente sinopsis, conforman el trabajo de tesis.

Capítulo I: Propiedades de las Prolaminas del Trigo y del Maíz y su Interacción con las Células Responsables de las Respuestas Inmunes Innata y Adaptativa: una Revisión.

Este primer capítulo, está conformado por un artículo de revisión y el resumen de un trabajo en cartel.

El artículo de revisión se titula: Maize prolamins could induce a gluten-like cellular immune response in some celiac disease patients. Nutrients 2013; 5: 4174–4183. Aquí se plantea que en algunos pacientes celiacos, como un evento poco común, los péptidos derivados de las prolaminas del maíz pueden inducir una respuesta inmune por

mecanismos similares o alternativos a los que utilizan los péptidos del gluten. Esto es avalado por algunos resultados en trabajos de experimentación en los cuales las prolaminas del maíz y las del trigo tienen varias características compartidas. Dado que los péptidos del gluten inducen una respuesta inmune en la mucosa intestinal ya sea *in vivo* o *in vitro*, los péptidos del maíz se pueden evaluar en la misma forma para determinar su capacidad de inducir alguna respuesta inmune celular similar. Hipotéticamente, las prolaminas del maíz podrían ser perjudiciales para un muy limitado grupo de pacientes celiacos, especialmente para aquellos que no responden al tratamiento y, de ser confirmado, deberían seguir una dieta sin maíz ni trigo. Con esta revisión, hicimos una selección adecuada de las herramientas analíticas para cumplir el objetivo de comparar los trabajos realizados por otros autores sobre la respuesta celular de las prolaminas del maíz con las del trigo, en pacientes celiacos.

El resumen del trabajo: Gluten-like cellular response of maize prolamins in intestinal biopsies and CACO-2 cells. The FASEB Journal 2014; 28 [1] Supp. 916.6, presentado en el Congreso Internacional de Biología Experimental (Experimental Biology) en San Diego, California, EUA, describe brevemente la viabilidad del uso de biopsias del bulbo duodenal de pacientes celiacos para la evaluación de la respuesta inmune celular y la línea comercial de células CACO-2, para evaluar el efecto en la inflamación y la permeabilidad de la membrana del epitelio intestinal a causa de las prolaminas de maíz o de trigo.

Los péptidos inmunogénicos G33-mer de α-gliadinas y Z34-mer de α-zeínas, se utilizaron para evaluar la activación celular en cultivos de biopsias obtenidas del intestino de pacientes con EC u otro tipo de inflamación intestinal. El péptido inmunogénico G33-mer de α-gliadinas está bien tipificado desde hace años, mientras que el Z34-mer fue obtenido previamente al secuenciar productos de digestión de zeínas inmunodetectados por IgA de pacientes celiacos[7]. Por estudios *in silico* este péptido presentó afinidad a las moléculas HLA-DQ8 de células T en EC[8]. Adicionalmente, se evaluó el cambio en la permeabilidad de la monocapa de células CACO-2 después de estimularlas con péptidos de gliadinas y/o zeínas digeridas con pepsina y tripsina. El

informe patológico indicó que de 10 pacientes, 7 presentaron con inflamación, 2 atrofia y 1 no mostró daño en la mucosa duodenal.

La producción de interferón gama (IFN-γ) e interleucina 2 (IL-2) aumentaron respecto al control por efecto del péptido G33-mer en biopsias de mucosa atrofiada, mientras que el péptido Z34-mer indujo un aumento menor en las biopsias de mucosa intestinal atrofiada o inflamada con respecto al control. Los péptidos de zeínas digeridas y el G33-mer indujeron una mayor permeabilidad de la monocapa de células CACO-2 en comparación con el efecto inducido por los péptidos de gliadinas digeridas y el Z34-mer. Con estos resultados preliminares se infiere que las zeínas podrían tener un efecto en la disrupción de la mucosa intestinal de una forma similar a las gliadinas. Sin embargo, esto no necesariamente se da por la inducción de un daño inicial que desencadene una respuesta celular característica de EC.

Capítulo II: Las Prolaminas del Maíz y del Trigo Afectan Diferencialmente a las Células Intestinales en Biopsias de Celiacos y CACO-2.

El capítulo está conformado por un artículo original y una carta al editor. El artículo es: Prolamins of maize and wheat differentially affect intestinal cells both in biopsies of celiac patients and CACO-2 cell line. Food and Agricultural Immunology 2016; 27(2): 259-272. La carta es: The age-related immunoreactivity to gluten peptides in celiac disease. Gastroenterology 2016 (Aceptado, a publicarse en el número de marzo).

En este capítulo comparamos el efecto de los péptidos inmunogénicos en pacientes celiacos con aquel en personas sanas. Además evaluamos la permeabilidad y la respuesta pro-inflamatoria en la monocapa de células CACO-2 por efecto de las fracciones de los péptidos digeridos de maíz equivalentes en tamaño a los péptidos inmunogénicos de maíz y trigo.

En este estudio se comparó el efecto de las zeínas del maíz en la respuesta inmune celular con el de las gliadinas del trigo, para verificar el efecto en pacientes celiacos.

Como indicador de la respuesta inmune adaptativa, se evaluó la activación *in vitro* de la mucosa duodenal de pacientes celiacos con péptidos inmunoreactivos de zeínas y gliadinas. La respuesta pro-inflamatoria y la permeabilidad en células CACO-2 ocasionada por las fracciones de los péptidos de zeínas y gliadinas digeridas, se evaluaron como indicadores de la respuesta inmune innata. Las zeínas aumentaron la producción de IFN-γ en 3 de 5 biopsias de pacientes celiacos, de una forma similar a como lo hicieron las gliadinas en todos los 5 pacientes. El IFN-γ es una citocina de importancia central en la respuesta celular de la EC ya que es producida por los linfocitos T efectores. Éste, induce los efectos característicos de la mucosa intestinal en la EC como son la atrofia de las vellosidades, la hiperplasia de las criptas y la linfocitosis intraepitelial.

Dos de las fracciones de zeínas digeridas (de 3–5 y de 1–3 kDa) estimularon de manera análoga a las de gliadinas, cuatro indicadores de inflamación. El primero, la producción de IL-8, una de las principales quimiocinas producidas durante los procesos pro-inflamatorios. El segundo indicador, la fosforilación de la proteína p38 MAPK, la cual participa en la cascada de señales que controlan la respuesta celular como la inflamación y la apoptosis a diferentes procesos de estrés[9]. El tercer indicador, es la producción de ciclooxigenasa 2 (COX2), la cual en presencia de daño oxidativo convierte el ácido araquidónico en prostaglandinas que juegan un papel crucial en la inflamación[10]. Por último, la liberación de la proteína ZO-1, la cual forma parte del complejo proteico que integra las uniones estrechas y su desensamble del complejo es concomitante a la liberación de zonulina. Esta última es importante porque está demostrado que su liberación se induce en las células CACO-2, en respuesta al gluten[11].

Particularmente, la fracción de zeínas digeridas con un rango de tamaños de 3 a 5 kDa, causaron el aumento en la permeabilidad en la monocapa de células CACO-2 aunque en menor proporción que la fracción de gliadina del mismo rango de tamaño.
Los péptidos de zeínas demostraron ser inmunogénicos para algunos pacientes celiacos y también ser capaces de inducir una respuesta innata similar, pero en menor grado, que los péptidos de gliadinas.

En la carta al editor se discute que, contrario a lo publicado por Hardy y colaboradores[12], las células T de niños y adultos con EC no reconocen de forma similar los péptidos del gluten. Estos autores concluyen que el diagnóstico y tratamiento basado en información generada por el uso de péptidos inmunogénicos en adultos, podría ser empleado también para niños con EC. Sin embargo, es difícil establecer que los péptidos del gluten inducen respuestas similares en células T de niños y adultos con EC si la edad media de los niños y los métodos empleados difieren.

Además, en nuestro argumento se destaca que debido a la inmadurez del sistema inmune, las células T de niños con EC pueden reconocer péptidos del gluten deamidados o no[13]. También es posible detectar inmunoglobulinas IgA contra gliadinas deamidadas principalmente en niños mayores de 8 años, mientras que en niños menores de 3 años hay reconocimiento de péptidos nativos[14]. Finalmente, con la realización de esta carta procuramos ser coherentes con la justificación de dos aspectos importantes en los métodos empleados para nuestra investigación: i) en el primer estudio realizado con biopsias de pacientes celiacos, se utilizaron péptidos nativos ya que los participantes fueron principalmente niños y ii) en nuestro último ensayo, que se expone en el siguiente capítulo, se emplearon péptidos deamidados para estimular células T de sangre periférica de adultos.

Capítulo III: Efecto de las Prolaminas del Maíz en Células Mononucleares de Sangre Periférica de Pacientes Celiacos.

Este capítulo consiste en un artículo original: Effect of maize prolamins on peripheral blood mononuclear cells from celiac disease patients. Fue preparado para la revista Molecular Nutrition & Food Research en la modalidad “Food & Function article”.

El objetivo en este trabajo fue comparar la respuesta en células mononucleadas de sangre periférica de pacientes celiacos a prolaminas de maíz o de trigo. Dos pacientes celiacos y tres no celiacos adultos en dieta sin gluten por un mes como mínimo, consumieron gluten durante tres días. Seis días después, se les extrajo sangre venosa y

aislaron células mononucleadas (entre ellas células T) para estimularlas con gliadinas o zeínas. Para el estímulo *in vitro* se emplearon los péptidos inmunogénicos G33-mer y Z34-mer de gliadinas y zeinas respectivamente, así como las fracciones de péptidos digeridos de gliadinas y zeínas de 3 a 5 kDa todos ellos deamidados por la enzima TG. Estos últimos, como se muestra previamente en nuestro segundo artículo, habían mostrado inducir efectos en la respuesta pro-inflamatoria y permeabilidad en células CACO-2. La respuesta celular fue evidenciada por el aumento en la producción y liberación de IFN-γ en el sobrenadante del medio de cultivo celular.

No se observó aumento considerable en la producción de IFN-γ en uno de los pacientes ni en los individuos control debido al reto *in vivo* con gluten. Sin embargo, en las células T de uno de los pacientes celiacos se indujeron niveles de IFN-γ altos al estimularlos con los péptidos de zeína, principalmente el Z34-mer. Dicho aumento fue similar al inducido por el péptido inmunogénico G33-mer y significativamente mayor al de células no estimuladas ($p<0.05$).

La estimulación *in vitro* de las células T con el péptido Z34-mer fue comparable a la estimulación generada con el péptido de la α-gliadinas G33-mer. Éste, es considerado el más inmunogénico por contener una epítope dominante que induce liberación óptima de IFN-γ en las células T sensibles al gluten[15]. La secuencia peptídica del Z34-mer difiere mucho de la del G33-mer, sin embargo también es resistente a la digestión y contiene residuos de glutamina deamidables por la enzima transglutaminasa. Esto le permite presentar epítopes capaces de estimular una respuesta celular que necesita ser evaluada más detalladamente en el futuro. Inicialmente se propuso que la deamidación del Z34-mer generaría residuos de carga negativa afines a residuos de carga positiva de la molécula HLA-DQ8 en las células presentadoras de antígeno, a su vez, estos complejos péptido/HLA presentados a las células T iniciarían la respuesta inmune anormal característica de la EC[8]. Sin embargo, ninguno de los pacientes en los cuales se indujo respuesta celular posee el haplotipo HLA-DQ8, lo cual indica que la respuesta celular fue independiente del haplotipo.

Conclusión

En algunos pacientes celiacos las prolaminas del maíz pueden inducir, de forma análoga a las del trigo, una respuesta inmune celular en biopsias de intestino delgado y en células T circulantes. Además, al igual que los péptidos del trigo, los péptidos del maíz inducen una respuesta pro-inflamatoria y de disrupción de membrana en un modelo celular *in vitro*. También, el péptido Z34-mer derivado de las α-zeínas, fue capaz de inducir una respuesta similar al péptido inmunogénico G33-mer de las α-gliadinas a pesar de sus diferencias antigénicas. Este conjunto de resultados nos permitieron contrastar la hipótesis de que las prolaminas del maíz son capaces de inducir una respuesta inmune celular de forma similar al gluten en algunos pacientes celiacos a pesar de presentar secuencias antigénicas diferentes a las de trigo. Sin embargo, en nuestros resultados no estudiamos la deamidadación de los péptidos por la transglutaminasa tisular en el intestino delgado ni comparamos su efecto en la respuesta inmune celular contra péptidos nativos.

CAPÍTULO I

Revisión de las Propiedades de las Prolaminas del Trigo y del Maíz y su Interacción con las Células Responsables de las Respuestas Inmunes Innata y Adaptativa

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Review

Maize Prolamins Could Induce a Gluten-Like Cellular Immune Response in Some Celiac Disease Patients

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Abstract: Celiac disease (CD) is an autoimmune-mediated enteropathy triggered by dietary gluten in genetically prone individuals. The current treatment for CD is a strict lifelong gluten-free diet. However, in some CD patients following a strict gluten-free diet, the symptoms do not remit. These cases may be refractory CD or due to gluten contamination; however, the lack of response could be related to other dietary ingredients, such as maize, which is one of the most common alternatives to wheat used in the gluten-free diet. In some CD patients, as a rare event, peptides from maize prolamins could induce a celiac-like immune response by similar or alternative pathogenic mechanisms to those used by wheat gluten peptides. This is supported by several shared features between wheat and maize prolamins and by some experimental results. Given that gluten peptides induce an immune response of the intestinal mucosa both *in vivo* and *in vitro*, peptides from maize prolamins could also be tested to determine whether they also induce a cellular immune response. Hypothetically, maize prolamins could be harmful for a very limited subgroup of CD patients, especially those that are non-responsive, and if it is confirmed, they should follow, in addition to a gluten-free, a maize-free diet.

Keywords: celiac disease; cellular immune response; maize prolamins; zeins

1. Introduction

Celiac disease (CD) is an immune-mediated enteropathy triggered by dietary wheat, rye and barley gluten (water-insoluble proteins) in genetically predisposed individuals [1]. Characteristic features of CD involve crypt hyperplasia, intra-epithelial lymphocytosis and villus atrophy of the intestinal mucosa. These injuries affect intestinal function and nutrient absorption, which can cause a variety of gastrointestinal and extra-intestinal symptoms [2].

Intestinal mucosa damage in CD patients begins with an innate response that leads to a cellular immune response [3]. First, prolamin peptides from gluten, which are resistant to human digestion, interact with a chemokine receptor, inducing zonulin release and a subsequent tight junction disassembly [4]. Then, the damaged barrier allows the arrival of gliadin peptides to the lamina propria, where tissue transglutaminase (tTG) deamidates specific glutamine residues to confer an overall negative charge. These peptides are bound to the human leucocyte antigen (HLA) DQ2 or DQ8 molecules, in antigen presenting cells, which present them to T-cells to develop the full immune response required for CD [5]. In addition to gluten peptides, self tTG is presented to T-cells, which triggers an auto-immune response. Therefore, CD is considered an autoimmune disease.

CD symptoms disappear in the majority of patients after dietary gluten withdrawal; however, in some patients, the symptoms are still present even after they adopt a strict gluten-free diet [6]. This is due to either refractory CD or to the presence of gluten as a contaminant or as a non-declared additive in foods [7]. Additionally, the lack of response to dietary gluten withdrawal in a very limited subgroup of patients, could be due to other dietary proteins present in the gluten-free diet, such as those from maize, which is a common alternative ingredient used in gluten-free diets.

It has been demonstrated that zeins, the maize prolamins, are able to induce an inflammatory response through contact with the mucosa in some CD patients [8]. Furthermore, IgA antibodies from some CD patients can recognize zeins [9], even after lime and/or enzymatic treatments [10]. Perhaps, in active CD, peptides derived from zeins could exacerbate the immune response in the intestinal mucosa, because they have sequence characteristics and/or electronegative residues that resemble gluten peptides.

2. Supporting Experimental Results

Table 1 summarizes the similarities between maize prolamin peptides and wheat celiac-toxic gluten peptides that are involved in the pathogenesis of celiac disease. These results support the hypothesis that peptides from zeins that are resistant to human digestion are able to induce a celiac-like immune response in some CD patients by a similar mechanism to that triggered by wheat gluten peptides.

2.1. Incomplete Protein Digestion

Pepsin and trypsin, the main peptidases of the intestinal tract, cannot completely digest wheat gluten, because they are unable to cut its 15% proline-containing polypeptides [11,12]. The result is the release of peptides larger than nine amino acids, which are capable of eliciting innate and adaptive immune responses [13]. The proline content of zeins is also high (9%) and, although zeins contain bonds that pepsin can cut, they also contain cysteine residues with disulfide bonds that obstruct

digestion by pepsin [14]. All together, the ability of trypsin to digest zeins is low due to their low number of cleavage sites, low solubility [15] and secondary conformation [16].

Table 1. Similarities between maize prolamin peptides and wheat celiac-toxic gluten peptides that are involved in the pathogenesis of celiac disease (CD). NO: nitric oxide; NOS: nitric oxide synthase; HLA-DQ2 or DQ8: human leucocyte antigen molecules; IFN-γ: interferon gamma.

Step in CD Pathogenesis	Characteristics of Celiac-Toxic Peptides from Wheat Gluten	Characteristics of Maize Prolamins That Could be Inducers for CD
Incomplete protein digestion	Gastrointestinal peptidases do not digest the proline-rich wheat gluten polypeptides completely, which releases peptides larger than nine amino acids [11,12].	Digestion of zeins is poor due to relatively high concentrations of glutamine, proline and cysteine residues [14–16].
Innate immune response	Increased levels of NO were produced by challenged granulocytes and NOS expression was increased in enterocytes from CD patients' small intestine biopsies [17,18].	Proteins from maize caused granulocyte activation in a rectal challenge in six out of 13 CD patients tested [8].
Adaptive immune response: deamidation of peptides by tTG	Gluten peptides deamidated by tTG in the lamina propria contain negative charges [19–21].	Maize prolamins deamidated by TG *in vitro* were better recognized than native ones by IgA from some CD patients' sera [22].
Adaptive response: increased affinity of HLA-DQ2/DQ8 on antigen presenting cells to bind peptides	HLA-DQ2 prefers negatively charged amino acids from gluten peptides at the p4, p6 or p7 positions in the peptide, while HLA-DQ8 prefers them at positions p1 or p9 [20].	Peptides from digested maize prolamins have glutamine at positions p1 and p9 that can be deamidated by tTG and bind to HLA-DQ8 [23,24]. Other peptides can be bound by HLA-DQ2 [10].
Adaptive response: processing and presentation of peptides	After processing, the deamidated gluten peptides are presented to T-cells. Then, B-cells are induced to proliferate and produce antibodies [25].	T-cells from the intestine of one out of seven CD patients stimulated by maize prolamins and teff produced low IFN-γ as compared to wheat, but higher than control and other non-wheat grains [26]. Additionally, IgA antibodies against maize prolamins were detected in several CD patients [10,27].
Adaptive response: role of antibodies against dietary prolamins	Roles of tTG-specific antibodies induced by gluten in CD patients could be: inhibiting epithelial cell differentiation and inducing their proliferation, increasing epithelial and blood vessel permeability and affecting angiogenesis [28].	Although the levels of antibodies against gluten decrease in some CD patients following a gluten-free diet, antibodies against maize prolamins remained high until both gluten and maize were avoided [29,30].
Adaptive response: activation of T-cells	Activated T-cells drive the inflammatory response that leads to the development of the characteristic celiac lesions and the symptoms [31]. T-cells induce damage mostly by IFN-γ production [32].	Neither the intestinal lesions nor the CD symptoms were alleviated with a gluten-free diet when maize was still eaten [29].

2.2. The Inflammatory Process

Nitric oxide (NO) production is involved in the innate inflammatory response mediated by macrophages in CD, and it has been detected in cultured gluten-challenged small intestine biopsies [17]. Additionally, there is an elevated expression of mRNA encoding the major inducible isoform of NO synthase II (iNOS) in untreated CD patients [18]. After rectal wheat gluten challenge in CD patients, granulocyte activation precedes NO production. Furthermore, some patients have been found to display signs of a similar inflammatory reaction after challenge with maize prolamins [8].

2.3. Deamidation of the Peptides

Gluten peptides are transported across the epithelial barrier to the lamina propria, where tTG changes the glutamine residues to glutamic acid. Antigen-presenting cells then process these negatively charged peptides and increase their affinity for the major histocompatibility complex (MHC) class II molecules, HLA-DQ2 and HLA-DQ8. These immunogenic peptide fragments can stimulate HLA-DQ2- and HLA-DQ8-restricted T-cells and trigger an adaptive response in the lamina propria [19–21]. Maize prolamins likely are also deamidated by tTG, because IgA from CD patients was more immunoreactive against maize prolamins extracted from maize bread, treated with microbial transglutaminase, than against maize prolamins from untreated bread [22].

2.4. Affinity of HLA/DQ8 Molecules to Bind Peptides

Adaptive responses to gluten initiate when dendritic cells phagocytose gliadin peptides and present them to undifferentiated T helper cells, whose activation is crucial for the development of CD. Peptide deamidation by tTG increases the affinity of HLA-DQ2/DQ8 for these peptides. HLA-DQ2 has an affinity for negatively charged amino acids at the p4, p6 or p7 positions in the peptide, while HLA-DQ8 has an affinity for those residues at positions p1 and p9 [23]. The primary amino acid sequences of maize zeins can fit into these HLA binding sites once they are deamidated. Through *in silico* analysis, Darewicz *et al*. [24] identified a high degree of homology between two zein peptides and the celiac-toxic peptides from prolamins found in wheat, barley and rye (gliadins, hordeins and secalins, respectively). Moreover, we have identified a peptide sequence (α-zein 58–91) that is resistant to complete digestion and which has characteristics that would allow it to bind to HLA-DQ8 [10]. In addition to this peptide, Table 2 provides the sequence of a 33-mer (α2-gliadin 56–88) peptide that is a potent T-cell stimulator [19].

Table 2. Theoretical peptide sequences that bind to HLA-DQ2/DQ8 molecules. After deamidation by tTG [33], glutamine residues (underlined) became glutamic acid, which is an electronegative residue that binds to p4 and 6 in HLA-DQ2 and p1 and 9 in HLA-DQ8.

Food	Peptide	Sequence	Affinity	Reference
Wheat	α-Gliadin	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	HLA-DQ2	[19]
Wheat	α Gliadin	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	HLA-DQ8	[19]
Maize	α-Zein	LQQAIAASNIPLSPLLFQQSPALSLVQSLVQTIR	HLA-DQ8	[10]

2.5. Processing and Presentation of Peptides

Peptides of gliadin are deamidated by tTG, phagocytosed, processed and transported to the cell surface in dendritic cells via MHC class II molecules. Subsequently, the peptides are presented to infiltrated T helper cells that recognize deamidated peptides and trigger the proliferation of specific B-cells and the production of IgA anti-gliadin and anti-transglutaminase antibodies [25]. Some celiac patients contain B-cells that produce anti-maize prolamin IgA antibodies that do not cross-react with anti-wheat prolamins [10,27].

2.6. Role of Antibodies

After the DQ2-/DQ8-dependent activation of CD4+ T-cells, B-cells are stimulated and produce auto-antibodies. These auto-antibodies in the intestinal lumen could be involved in disease pathogenesis in various ways. For instance, they could be involved in inhibiting epithelial cell differentiation, augmenting epithelial cell proliferation, increasing epithelial and blood vessel permeability and affecting angiogenesis [28]. In some CD patients on a gluten-free diet, including maize-based foods, the anti-gliadin and anti-tTG antibody titers diminished, but the symptoms persisted [29,30]. Total symptom remission in these cases was achieved only with a gluten- and maize-free diet [30]. It is possible that partial production of anti-tTG antibodies, in addition to anti-zein antibodies, continued to affect the intestinal mucosa when dietary maize was present.

2.7. Activation of T-Cells

The activation of gliadin-reactive CD4+ T-cells results in the production of cytokines that drive an inflammatory response, which leads to the development of the characteristic CD lesions and symptoms [31]. Gluten-specific T-cells induce tissue damage mostly by the production of interferon (IFN)-γ [32]. There is some evidence of T-cells being simulated by maize prolamins: intestinal T-cells cultured from CD patients were challenged with maize prolamins *in vitro*, and T-cells from one out of seven samples produced IFN-γ as a result of T-cell stimulation [26]. Although this patient response was not specific, maize and teff peptides produced higher levels of IFN-γ (145.6 and 154.4 pg/mL, respectively) than the negative control (10.9 pg/mL) and others "non-toxic" grains (≈110 pg/mL).

Dietary gluten withdrawal has been demonstrated to induce mucosal recovery and the disappearance of CD symptoms. Nevertheless, some patients on gluten-free diet have forms of CD that do not respond to this diet. This could be due to a higher sensibility of these patients to "gluten-free" foods that still contain some traces of gluten [34] or to the presence of other cereal prolamins, such as those in maize in a very limited subgroup of CD patients.

3. Potential Links between Zeins and CD

Based on the similarities between wheat and maize prolamins discussed above, we can infer that the innate and adaptive responses to zeins would be similar to the response against gliadins in CD patients. Nevertheless, it is necessary to identify whether zeins contain immunodominant and minor epitopes similar to those found in gliadins after proteolysis. Some authors have found that there is no effect on T-cell activation or pro-inflammatory cytokine secretion when CD patient biopsies were treated with

whole pepsin-trypsin digested prolamins from maize [26,35]. Therefore, there is a need to evaluate the effect of isolated immunogenic peptides from maize prolamins, which can be obtained by *in silico* analysis [10].

The evaluation of the response of immune cells to gliadins includes the increased expression of surface receptors and the production of different cytokines for both tissue and immune cells. Some of these receptors include HLA-DR (human leucocyte antigen), CD54 or ICAM-1 (intercellular adhesion molecule), CD3 (in mature T-cells), CD25 (interleukin-2 receptor) and CD69 (in activated T-cells and natural killer cells) [36,37]. Cytokines that would be produced include interferon gamma, interleukins (IL) 2 and 15 and zonulin [13,38–40]. To evaluate the immune response, an analysis of the protein expression of these markers can be performed after CD patient biopsies are challenged with zein peptides. These *ex vivo* digested-peptide challenge analyses are considered useful tools to evaluate the safety of non-gluten prolamins in a gluten-free diet [26,40].

There is evidence that after a short gluten challenge in treated CD patients, gluten-specific T-cells are present in peripheral blood [41–44]. After this *in vivo* challenge, peripheral blood mononuclear cells can be isolated and activated with gluten peptides for quantitative detection of pro-inflammatory cytokines and direct detection of HLA-DQ2 tetramer specific for gliadins. For both cytokine measurements and the detection of an immune response, these techniques would be very useful in the evaluation of the effect of maize prolamins on the immune response in CD patients.

4. Conclusions

Although reaction to maize prolamins in CD patients appears to be a rare event, the confirmation that they play a role in the pathogenesis of CD will be useful information for the follow-up of some non-responsive celiac patients. It is estimated that approximately 10% to 18% of these cases are refractory CD, which represents a more severe CD, with a clear malignity and a less favorable prognosis [7]. Therefore, it is important to assess these clinical cases, because uncontrolled CD can lead to several malabsorption problems, osteoporosis and other autoimmune diseases [45].

Maize is one of the most commonly consumed grains in the gluten-free diet. Despite the low content of zeins in maize-containing foods compared with that of gliadins in wheat-containing foods, maize could be responsible for persistent mucosal damage in a very limited subgroup of CD patients. If our hypothesis is proven, zeins could be classified as harmful for some CD patients, especially those showing a poor response to a gluten-free diet.

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Conflicts of Interest

The authors declare no conflict of interest.

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Gluten-like cellular response of maize prolamins in intestinal biopsies and Caco-2 cells (916.6)

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Abstract

Celiac disease (CD) is an autoimmune-mediated enteropathy triggered by wheat gliadins in genetically prone individuals. Some celiac patients are affected by maize zeins, possibly by a gliadin-like immune response. The immunogenic peptides of gliadin (G33mer) and zein (Z34mer) were used to evaluate activation of cultured intestinal biopsies of patients with different gastrointestinal diseases. Additionally, changes in the permeability of a monolayer of Caco-2 cells challenged with peptides of digested gliadin (GdDig) and zeins (ZeDig), were evaluated. Pathological report indicated inflamed duodenal mucosa (7), mucosal atrophy (2) and normal mucosa (1) in 10 patient biopsies. Interferon-gamma (IFN-γ) and interleucin (IL-2) specifically increased in atrophic mucosa by effect of G33mer, while the Z34mer induce a smaller increase in cases of atrophic and inflamed mucosa compared to control. ZeDig peptides and G33mer induced a higher permeability of the monolayer of Caco-2 cells, as compared to that induced by GdDig or Z34mer. Zeins could have an effect on disrupted mucosa similar to gliadins but not necessarily by induction of the initial damage.

CAPÍTULO II

Las Prolaminas del Maíz y del Trigo Afectan Diferencialmente a las Células Intestinales en Biopsias de Celiacos y CACO-2

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Prolamins of maize and wheat differentially affect intestinal cells both in biopsies of celiac patients and CACO-2 cell line

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ABSTRACT

We study the effect of maize zeins on cellular immune response as compared to that of wheat gliadins for exacerbating celiac disease due to a hypothetical similar response in some patients. *In vitro* activation of celiac duodenal mucosa with gliadin or zein immunoreactive peptides and pro-inflammatory response of CACO-2 cells to digested gliadin or zein fractions were evaluated as indicators of adaptive and innate response, respectively. In 3/5 biopsies, zein increased production of IFN-γ, whereas gliadin has done it in all the patient biopsies. In CACO-2 cells, two zein fractions (3–5 and 1–3 kDa), similar to gliadin fractions, stimulated the production of IL-8, p38 MAPK, COX2, and release of ZO-1 as compared to medium alone. The 3–5 kDa zein fraction increased permeability in cell monolayers, although less than gliadin. Zein peptides are immunogenic for some patients and induce a similar innate response, but to a lesser extent than gliadin peptides.

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celiac disease; cell immune response; maize prolamins; zeins

1. Introduction

Celiac disease (CD) is an autoimmune-mediated enteropathy in genetically predisposed individuals exposed to dietary wheat gluten (Ludvigsson et al., 2012). The poor digestion of gluten proteins by intestinal proteases originates several toxic and immunogenic peptides. Toxic peptides are capable of stimulating the innate immune response that precedes the abnormal adaptive immune response in CD. The internalization of toxic peptides intra or inter-enterocytes can trigger a series of events that lead to membrane disruption and cell apoptosis (Di Sabatino & Corazza, 2009). Then, the immunogenic peptides enter the lamina propria and are deamidated by the tissue transglutaminase (tTG), captured by dendritic cells and presented to gluten-sensitive Th1 cells in the context of human leukocyte antigen (HLA)-DQ2/8 (Green & Cellier, 2007).

A gluten-free diet is the only treatment for CD, where in addition to wheat, barley and rye must be avoided. One of the more common wheat substitutes is maize as a principal ingredient for different gluten-free foodstuffs. However, some unexpected responses to

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maize proteins have been found in some CD patients after oral challenge (Accomando, Albino, Montaperto, Amato, & Corsello, 2006); as humoral IgA immunoreactivity (Cabrera-Chávez, Rouzaud-Sánchez, Sotelo-Cruz, & Calderón de la Barca, 2008); and other *in vivo* and *in vitro* findings previously reviewed (Ortiz-Sánchez, Cabrera-Chávez, & Calderón de la Barca, 2013). Hypothetically, maize prolamins could be harmful for a very small group of CD patients, especially those that are nonresponsive, and if it is confirmed, they should follow a gluten-free diet that also excludes maize foodstuffs. The principal information about maize in CD comes from casual results by using its proteins as "negative" controls in studies of CD immune response. There are no experiments intended to directly prove the immune response to maize proteins in CD patients.

Because of the wide use of maize in gluten-free foods, it is important to study the effect of its prolamins (called zeins) on innate and adaptive immune response as compared to those elicited by wheat gliadins for exacerbating CD. To achieve this objective, duodenal bulb biopsies of CD patients were challenged with immunogenic zein and gliadin peptides. Also, CACO-2 cells were stimulated with fractionated pepsin–trypsin (PT)-digested gliadins and zeins in order to elucidate their toxic effect, by measuring their ability to affect the permeability and induce pro-inflammatory markers.

2. Materials and methods

2.1. *Patients*

Biopsies were taken from duodenal bulb of each five patients undergoing upper endoscopy at the Hospital Infantil del Estado de Sonora (HIES) in Hermosillo, Mexico. The study was approved by the ethical committee of the HIES and all samples were taken under a parent's informed written consent. Whole blood was also extracted from each patient for genomic DNA extraction using the QIAamp DNA Blood Mini Kit (QIAGEN, USA) and genotyping of HLA-DQ2/DQ8 was done by real-time Polimerase Chain Reaction (Step One Plus, Applied Biosystems) using specific primers (Olerup, Aldener, & Fogdell, 1993). Serum anti-gliadin IgG, anti-gliadin IgA and anti-tTG IgA antibodies were analyzed by a direct enzyme-linked immunosorbent assay (ELISA), as previously reported (Cabrera-Chávez, Rouzaud-Sánchez, Sotelo-Cruz, & Calderón de la Barca, 2009). Briefly, microplates were coated with 5 μg/mL gliadins or guinea pig liver transglutaminase (Sigma-Aldrich, St Louis, MO) in coating buffer overnight at 4°C. The plates were blocked for 1 h with 3% gelatin and then incubated for 4 h at 25°C with human serum samples in incubation buffer. After washings, plates were incubated for 1 h with horse radish peroxidase-conjugated anti-human IgA antibodies (DAKO, Carpinteria, CA), at 25°C. Peroxidase activity was developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma-Aldrich). Absorbance at 450 nm was read (Microplate reader, Bio-Rad, Hercules, CA).

2.2. *Peptide preparation*

The immunogenic peptides α-gliadin 33-mer (LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF; Molecular Weight (MW) = 3914.51 Da) (Shan et al., 2002), α-zein 34-mer (LQQAIAASNIPLSPLLFQQSPALSLVQSLVQTIR; MW = 3646.32 Da) (Cabrera-Chávez et al., 2012), and the toxic peptide α-gliadin p31–49 (LGQQQPFPPQQPYPQPQPF;

MW = 2222.51 Da) (Maiuri et al., 2003; Matysiak-Budnik et al., 2003), were supplied by United Biosystems (USA) with purities of 97.54%, 95.66% and 95.18%, respectively. The immunogenic peptides were used in the experiments at 1 mg/mL and the toxic peptide at 50 μg/mL, as it has been seen in previous work (Capozzi et al., 2013; Maiuri et al., 2003; Picarelli et al., 2010).

2.3. *Duodenal biopsies, tissue culture and cytokine assays*

Five duodenal bulb fragments were collected from each patient. Two fragments were used for routine histology and three were placed on culture plates and cultured in Dulbecco's Modified Eagle's Medium (D-MEM) containing 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL gentamicin (Gibco, USA) at 37°C in a 5% CO_2 atmosphere. One fragment was used for α-gliadin 33-mer challenge, other for α-zein 34-mer challenge, both peptides at a concentration of 1 mg/mL, and the third fragment left without challenge. After 24 h, supernatants were collected and frozen at −70°C prior to cytokine evaluation.

ELISA kits were used for IFN-γ (Mabtech) and IL-15 (eBioscience) detection according to manufacturers' recommendations. ZO-1 release in biopsies was determined by ELISA as above for anti-gliadin antibodies detection. Microplates were coated with 100 μL of cell supernatant diluted 1 : 5, after blocking, were incubated for 2 h with rabbit polyclonal anti-human ZO-1 antibodies (Abcam, Cambridge, UK) and detected by incubation for 1 h with goat anti-rabbit IgG-HRP (DAKO, Carpinteria, CA).

2.4. *Cell line culture*

CACO-2 cells [HTB-37™ American Type Culture Collection] were cultured in 9.5 cm^2 surface wells containing D-MEM with 10% FCS, 4 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL gentamicin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmosphere. The culture medium was replaced three times a week. Subculture was performed at 80% of confluence. All experiments were performed between passages 65 and 78. Monolayers (passages 22–35) were grown on 0.33 cm^2 permeable collagen-coated polytetrafluorethylene filters with 0.4 μm pore size (Corning, Lowell, MA) for 14–21 days until confluency, polarized and differentiated state.

2.5. *Gliadin and zein peptic–tryptic digestion*

Gliadins from wheat and zeins from maize (Sigma Chem Co, St Louis, MO, USA) were subjected to PT digestion, as previously described (Cabrera-Chávez et al., 2008) with some modifications. Briefly, gliadins or zeins were exposed to pepsin from porcine gastric mucosa (3200–4500 units/mg protein, Sigma-Aldrich, USA) at 37°C, pH 2.0 for 4 h, followed by trypsin from porcine pancreas (1000–1500 Benzoyl-L-arginine ethyl ester units/mg solid, Sigma-Aldrich, USA) at 37°C, pH 7.8 for 4 h. After reaction, the incubation was stopped at 80°C for 45 min and then sequentially fractionated in Millipore membranes with four different Molecular Weight Cut-Offs (MWCO: 30, 5, 3 and 1 kDa). Table 1 presents the molecular weights of digested gliadin or zein fractions. Protein fractions were filtered through 0.22 μm pore filter and $NaHCO_3$ was added to a

Table 1. Molecular weight range of protein fractions of PT-digested gliadins and zeins.

Prolamins	Fraction	Protein size (kDa)
Gliadins or zeins	1	≥30
	2	5–30
	3	3–5
	4	1–3

Note: kDa, kilodaltons.

final concentration of 44 mM. Protein concentration was determined by absorbance at 280 nm using a Nano-Drop 2000 UV-Vis Spectrophotometer (ThermoScientific, USA) and used at a concentration of 1 mg/mL for cell stimulation.

2.6. *Cytokine determination*

CACO-2 cells were stimulated separately with fractions 1, 3 and 4 of digested gliadins and zeins, with p31–49 or left untreated for 24 h at 37°C in 5% CO_2 atmosphere. After treatment, cell supernatants were collected and frozen at −70°C until analysis. Production of the pro-inflammatory cytokines IL-6 and IL-8 was measured in cell supernatants by ELISA and quantified using the provided reference standard curve (Affymetrix, eBioscience, San Diego, CA, USA). The experiments were performed twice in duplicate assays.

2.7. *Cell lysis and pro-inflammatory markers by ELISA*

After the former stimulation, CACO-2 cells were placed on ice, washed once in phosphate buffer saline (PBS) and detached with trypsin– ethylenediaminotetraacetic acid (EDTA) for 10 min at 37°C. Whole cell extracts were prepared as described before (Capozzi et al., 2013). Briefly, cells were re-suspended in lysis buffer (20 mM Hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF and 1 mM Na_3VO_4). DNA was fragmented by brief sonication and released proteins were recovered after centrifugation of lysates at 12,000 rpm for 15 min at 4°C.

For ELISA, microplates were coated with 100 µL of cell lysate (1:10) and washed, as previously described at the former ELISAs for ZO-1. The coated microplates were incubated for 2 h with rabbit monoclonal anti-phospho-p38 mitogen-activated protein kinases (MAPK) (Thr180/Tyr182) from Cell Signaling Technology, or with rabbit monoclonal anti-ciclooxigenase 2 (COX2) from Abcam (1:1000 dilution). Detection was made by incubation for 1 h with goat anti-rabbit IgG-HRP (DAKO, Carpinteria, CA) and further chromogenic convertion of TMB.

2.8. *Cell monolayer polarization and permeability*

After culture medium replacing with PBS at room temperature, the trans-epithelial electric resistance (TEER) was measured using a silver chloride adjustable electrode (Millicell ERS; Millipore Co., Bedford, MA) adapted to a Fluke multimeter (Everett, WA, USA). TEER values were expressed in ohms (Ω)/cm^2.

The permeability of the CACO-2 cell monolayer was evaluated according to Fiorentino, Levine, Sztein, and Fasano (2014) with some modifications. Bovine serum albumin (BSA)

was dissolved in P buffer (10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 10 mM glucose, 3 mM $CaCl_2$, 145 mM NaCl) or P/EDTA buffer (10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 10 mM glucose, 145 mM NaCl, 2 mM EDTA). The apical surface of CACO-2 cell monolayers was stimulated with fractions 3 and 4 of PT-digested gliadin, zein or α-gliadin peptide p31–43 in cell culture medium and incubated at 37°C for 6 h, and then apical an basolateral sides were washed with PBS. In order to measure the paracellular flux, the apical and basolateral cell culture media was replaced with P buffer containing 10 mg/mL BSA or P buffer alone, respectively. P/EDTA buffer containing BSA (10 mg/mL) and P/EDTA buffer were used as positive controls. After incubation for 4 h, BSA in the basolateral media was measured at 280 nm.

2.9. *Statistic analysis*

Cytokine concentrations, as well as absorbance values from ZO-1 release in biopsy supernatants and activation of pro-inflammatory proteins in CACO-2 culture supernatants were calculated from duplicates of two experiments by ELISA. Mean values were compared by ANOVA and statistic significance by the Tukey's test using the statistical software NCSS, version 2001.

3. Results and discussion

The clinical characteristics of participants, all of them CD patients whose biopsies were studied, are presented in Table 2. Patients represent a variety of clinical manifestations with typical and atypical symptoms, alleles of genetic predisposition and positive indexes of antibodies for CD. Ages ranged from 0.9 to 18 years and histology shows villus atrophy. All of the patients presented at least two alleles that encode for HLA-DQ2 or HLA-DQ8 molecules.

3.1. *Prolamin-induced cytokine responses in biopsies*

IFN-γ was measured in the culture supernatants of biopsies following 24 h of culture with immunogenic peptides from gliadins or zeins. Three of the patient biopsies produced an

Table 2. Characteristics of CD patients.

Patient	Age (in years)	Diagnostic	Haplotype or alleles	Positive index for antibodies[a]	Biopsy	Symptoms
1	0.9	CD	DQA1*501, DQB1*0301	ND	Villus atrophy	Seizures and GERD
2	1.5	CD	DQA1*501, DQA1*302	Anti-Gd IgG; anti-tTG IgA	Villus atrophy	Diarrhea, bloating and vomit
3	10	CD	DQB1*0201, DQB1*0301	Anti-Gd IgG; anti-Gd IgA; anti-tTG IgA	Villus atrophy	GERD, abdominal pain and diarrhea
4	18	CD	HLA-DQ2, DQB1*301	Anti-Gd IgG; anti-tTG IgA	Villus atrophy	Gastritis
5	1	CD	DQA1*0501, DQA1*0302	Anti-Gd IgG; anti-Gd IgA	Villus atrophy	Chronic constipation seizures

Notes: ND, not done; DQA1, alpha-chain DQ alleles; DQB1, beta-chain DQ alleles; IgG, G isotype immunoglobulin; IgA, A isotype immunoglobulin; Gd, gliadins; GERD, gastroesophageal reflux disease.
[a]Antibodies were analyzed before biopsies were taken.

IFN-γ increase when stimulated with the 34-mer zein peptide, two of them similar to it of the 33-mer gliadin peptide ($p = 0.05$). A 14-month-old patient who carries the HLA-DQ2 haplotype and suffers from gastritis and chronic diarrhea has not presented CD antibodies or mucosal damage, thus considered non-celiac patient. Nevertheless, its biopsy increased IFN-γ levels when stimulated with gliadins, but the levels were lower than those of biopsies of CD patients (data not shown).

The former results confirm the theoretical immunogenicity of the 34-residue peptide of maize, a product of the PT-digested alpha-zein A20, with glutamine residues situated in favored positions to bind effectively to the HLA-DQ8 molecules (Cabrera-Chávez et al., 2012; Calderón de la Barca & Cabrera-Chávez, 2014). As expected, all the five biopsies produced significant higher ($p = 0.05$) IFN-γ levels in response to gliadins than their respective negative controls (Figure 1(A)). Gluten-reactive CD4+ T cells of CD patients recognize the gliadin immunogenic peptide 33-mer in the HLA-DQ2/8 context and elicit a Th1 type

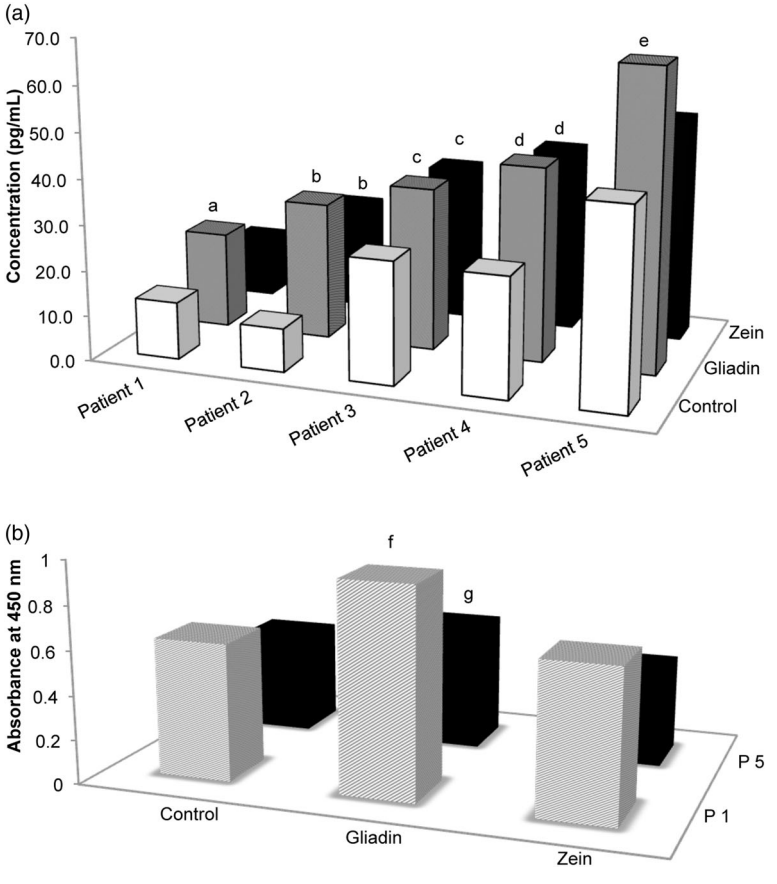

Figure 1. Zein peptides induce increased levels of IFN-γ but no ZO-1 in intestinal biopsies. (A) Patient biopsies stimulated with either gliadin (α-gliadin 33-mer), zein (α-zein 34-mer) or unstimulated were analyzed for IFN-γ production in supernatant by ELISA. (B) Patient biopsies either stimulated with gliadin (α-gliadin 33-mer), zein (α-zein 34-mer) or unstimulated were analyzed for ZO-1 liberation to the supernatant by ELISA. Statistic analysis was made within each patient: a to g $p = 0.05$ versus control. Results are expressed as mean ± SD of duplicate analysis in two different experiments. P1 and P5: patient 1 and patient 5, respectively.

cytokine response, mainly with the production of IFN-γ (Nilsen et al., 1998). This cytokine induces damage on the intestinal mucosa through the activation and release of enzymes such as matrix metalloproteinases in lamina propria mononuclear cells and fibroblasts (Ciccocioppo, Di Sabatino, & Corazza, 2005).

The previous results are consistent with Brottveit et al. (2013), who observed that IFN-γ increased after a short-term gluten challenge in celiac and non-celiac gluten sensitivity patient biopsies. Nevertheless, the induction of pro-inflammatory cytokines by gliadin 33-mer in macrophages does not include IFN-γ (Thomas, Sapone, Fasano, & Vogel, 2006). Hence, dendritic cells or gluten-reactive CD4+ T cells can be mainly responsible for production and secretion of INF-γ, suggesting an adaptive immune response. Although the alpha-gliadin 33-mer is considered to have a higher affinity to the HLA-DQ2 molecule (Qiao et al., 2004), our results suggest that both gliadin and zein peptides elicited an adaptive response regardless of DQ2 or DQ8 haplotype.

No significant ($p = 0.05$) differences were detected in the production of IL-15 from biopsies stimulated with the 34-mer zein (50.13 ± 13.92 pg/mL) or 33-mer gliadin peptides (56.33 ± 5.66 pg/mL) as compared to negative controls (60.81 ± 2.75 pg/mL). IL-15 is the main innate response cytokine overexpressed in untreated CD and can be induced not only by gliadin "toxic" peptides such as p31–42 and p31–49 (Maiuri et al., 2003; Mention et al., 2003) but also immunogenic peptides as the 33-mer, with effect on membrane integrity and permeability (Thomas et al., 2006). Nevertheless, even gliadin 33-mer could not elicit IL-15 in patient biopsies. Our results agree with those of Mention et al. (2003) who found out very low IL-15 secretion in CD intestinal biopsies of active or treated CD patients after 24 h incubation with digested gliadin peptides.

IL-15 is mostly delivered at the enterocytes surface (Meresse et al., 2004), and it can also be secreted in untreated CD patient enterocytes (Di Sabatino et al., 2006). Therefore, the lack of cellular response for producing secreted IL-15 after gliadin 33-mer treatment can be attributable to that all of the five studied patients are currently in gluten-free treatment. Finally, there is a need to evaluate IL-15 expression in biopsy specimens by immunohistochemistry assays, in order to clarify if both assayed maize and wheat peptides have an exclusive immunogenic effect and do not bind to the receptors for cytotoxic peptides in enterocytes.

ZO-1 release was higher in two of the five patients (P1 and P5) biopsies that were stimulated with the 33-mer gliadin peptide ($p = 0.05$), but there was no ZO-1 liberation under the 34-mer zein stimulus (Figure 1(B)). ZO-1 is part of the tigh-junctions (TJ) protein complex, released after membrane disruption (Fasano, 2008). In the same way that IL-15, ZO-1 is also an innate response indicator and it was expected to remain the same after challenged with the immunogenic gliadin peptide or the zein peptide. Nevertheless, the ZO-1 releasing found could be related to a high concentration of IFN-γ, a cytokine which can disrupt the intestinal barrier integrity in murine intestinal cell lines (Zufferey, Erhart, Saurer, & Mueller, 2009) and induce internalization of transmembrane proteins, with the release of ZO-1, a cytosolic plaque TJ protein (Bruewer et al., 2005).

3.2. *Cytokines production by zein PT-prolamin fractions in CACO-2 cells*

Due to most of the immunogenic and toxic peptides are under 5 kDa (Ciccocioppo et al., 2005); we use fractions 3 and 4 of zeins or gliadins for cytokine assays and fraction 1 was

used as an irrelevant peptide fraction. To analyze the innate response to the zein peptides, the production of pro-inflammatory cytokines interleukins 6 and 8 (IL-6 and IL-8) was measured in challenged CACO-2 cells. Fractions 3 and 4 of PT-digested zeins stimulated production of IL-8 in a similar way as gliadin fractions 3 and 4 did (Figure 2(A)). Gliadin peptides induced releasing of IL-8 from enterocytes that leads to the recruitment of neutrophils in the lamina propria (Fasano, 2011). An increased level of IL-8 in CACO-2 cells stimulated with PT-digested gliadin was also observed by Capozzi et al. (2013). Zein peptides considered within fractions 3 and 4 (1–5 kDa) can stimulate the production of IL-8, an important pro-inflammatory cytokine induced by oxidative stress in intestinal cells.

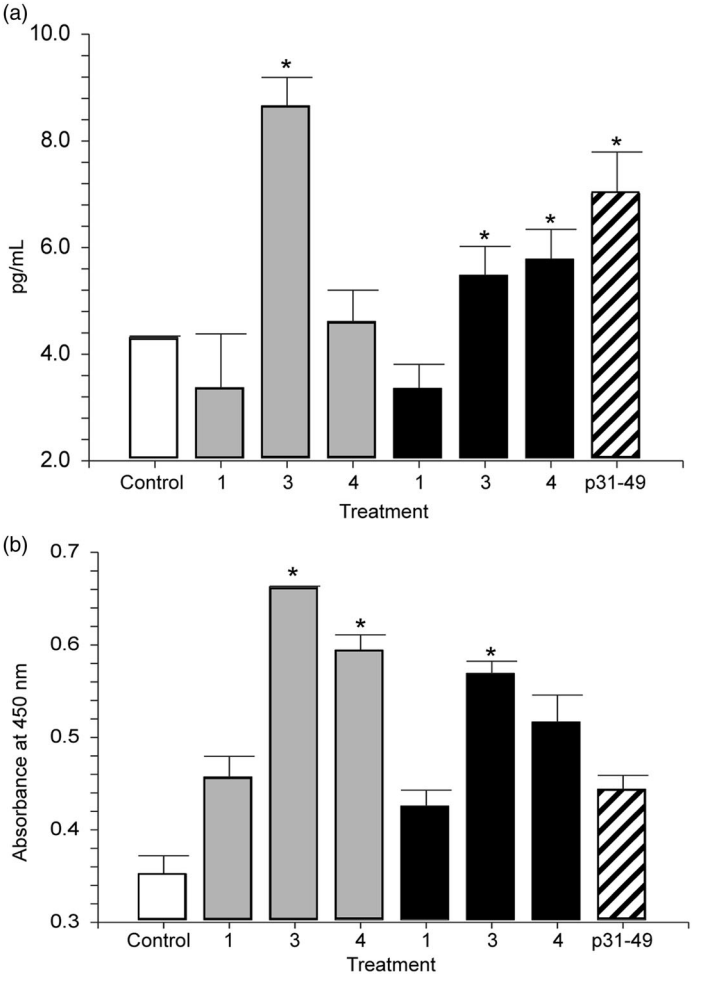

Figure 2. Chemotactic and release of ZO-1 is increased by zein in the same way of gliadin peptides in CACO-2 cells. (A) CACO-2 cells were stimulated with gliadin fractions 1, 3 or 4 (gray columns), zein fractions 1, 3 or 4 (black columns) or toxic peptide *α*-gliadin p31–43 (striped column) for 24 h and were analyzed for IL-8, statistical analysis: $^*p = 0.05$ versus unstimulated (control) cells (white column). (B) Liberation of ZO-1 to supernatant by ELISA in the same stimulated CACO-2 cells. Statistical analysis: $^*p = 0.05$ versus unstimulated (control) cells (white column). Results are expressed as mean ± SD of duplicate analysis in two different experiments.

Production of IL-6 was not detected for any of the zein nor gliadin PT-digested fractions as well as for the positive control p31–49. A similar result of IL-8 production but not IL-6 was obtained by Fiorentino et al. (2014) after stimulation of CACO-2 cells with *Shigella* antigens. However, Capozzi et al. (2013), observed a small increase in IL-6 in comparison to the IL-8 increase after stimulation of CACO-2 cells with PT-gliadin. Perhaps, we did not detect IL-6 due to the use of PT-digested gliadin fractions, a different control peptide (p31–49 instead of p31–43) or to different sensitivity of the analysis tools. Furthermore, in cells of an isolated medium with a lack of cytokines like IL-1 and tumor necrosis factor, the chemotactic functions in the gliadin-stimulated enterocytes given by IL-8 could be prioritized.

3.3. *Zein PT-prolamin fractions in CACO-2 cells activate MAPK inducing a pro-inflammatory response*

We analyzed phosphorylation of the p38 group of the MAPK (p38 MAPK), by challenging intestinal CACO-2 cells with our fractions in comparison to the gliadin peptide p31–49, an inducer of innate response in CD mucosa (Hüe et al., 2004). We found out that the pro-inflammatory response elicited by fractions 3 and 4 of PT-digested zeins were similar to those of the PT-digested gliadin fractions and to the p31–49 for increasing p38 MAPK, as shown in Figure 3(A).

The p38 MAPK participates in a signaling cascade controlling cellular responses, like inflammation and cell apoptosis, to different cellular stresses (Zarubin & Han, 2005). According to Luciani et al. (2010), gliadin peptide p31–42 is internalized in human intestinal cell lines and induces oxidative stress by the generation of reactive oxygen species. In addition to p31–42, gliadin peptide p31–49 can induce a pro-oxidative environment that induces activation of stress-sensitive signaling pathways. Interestingly, in CACO-2 cells, inhibition of the p38 and NF-$\kappa\beta$ signaling pathways results in the reduction in pro-inflammatory cytokines (Garat & Arend, 2003). On epithelial cells, stress induced by gliadin peptides activates p38 MAPK in order to regulate the actin dynamics during adverse environmental conditions (Guay et al., 1997). Therefore, in the same way as gliadin, maize peptides can activate signaling pathways that induce to enterocyte oxidative stress and a consequent damage to the membrane.

The oxidative stress on CACO-2 cells stimulated with PT-digested zeins also increased the COX2 levels. Figure 3(B) shows that COX2 amounts after stimuli with fractions 3 of PT-digested gliadins, 4 of PT-digested zeins or p31–49 are not different from negative control (unstimulated). However, COX2 levels of fractions 3 and 4 PT-digested zeins were similar to the corresponding fractions of gliadins ($p = 0.05$). It seems that zein peptides induced an oxidative imbalance as well as gliadins. COX2 response is to oxidative damage and converts arachidonic acid to prostaglandins that plays a crucial role in inflammation, although in enterocytes is mainly due to the way of the NF-$\kappa\beta$ activation (Ferretti, Bacchetti, Masciangelo, & Saturni, 2012). However, it has been reported that in mononuclear cells of lamina propria, COX2 is activated through the p38 MAPK pathway when cells were stimulated with the gliadin peptide p31–43 (Maiuri et al., 2003). Increased levels of phosphorylated p38 MAPK and COX2 were also observed by Capozzi et al. (2013) when CACO-2 cells were stimulated with PT-digested gliadins. For all the above, our results suggest an evidence that zein peptides induce an pro-inflammatory

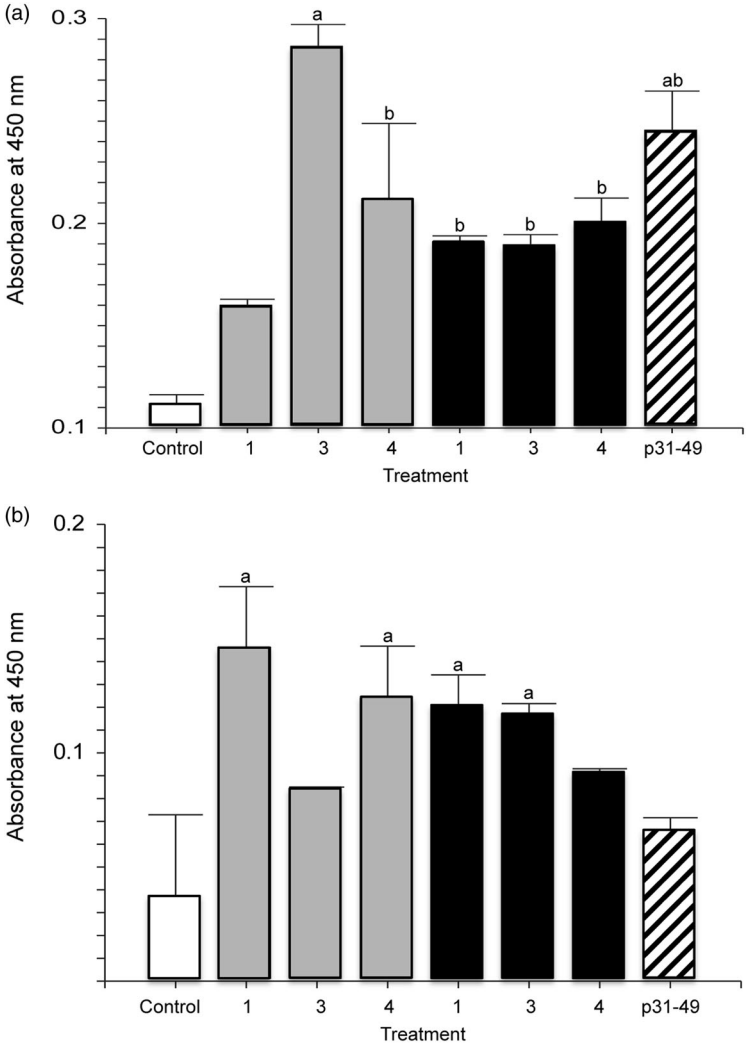

Figure 3. Induction of oxidative response by zein peptides in CACO-2 cells via activation of p38 MAPK and COX2. (A) CACO-2 cells were stimulated with gliadin fractions 1, 3 or 4 (gray columns), zein fractions 1, 3 or 4 (black columns) or toxic peptide *α*-gliadin p31–43 (striped column) for 3 h and were analyzed for pp38 in cell lysate by ELISA. Statistic analysis: $^{ab}p = 0.05$ versus unstimulated (control) cells (white column). (B) Production of COX2 in cell lysates by ELISA in the same stimulated CACO-2 cells. Statistic analysis: $^{a}p = 0.05$ versus unstimulated (control) cells (white column). Results are expressed as mean ± SD of duplicate samples of two experiments.

effect in CACO-2 cells and this mechanism is regulated by the p38 MAPK, which is also involved in damage on cellular integrity.

3.4. *ZO-1 liberation by zein PT-prolamin fractions in CACO-2 cells*

Similar to IL-8, fractions 3 and 4 of PT-digested zein stimulate production of ZO-1 the same way gliadin fractions 3 and 4 do (Figure 2(B)). ZO-1 is an important component of the TJ protein complex. The release of zonulin from the enterocytes is concomitant to the

disengagement of ZO-1 from the TJ complex between cells and its consequent disassembly leads to increased membrane permeability. Zonulin increases in culture media of CACO-2 cells when exposed to gliadin peptides (Orlando, Linsalata, Notarnicola, Tutino, & Russo, 2014), additionally, cell apoptosis induced by gliadin (Giovannini et al., 2000) can also increase ZO-1 levels in the culture media and this represents a double marker of membrane permeability and cell destruction simultaneously. Our results show, indirectly by the increase in ZO-1, that maize prolamins, as well as gliadin "toxic" peptide p31–49, can induce liberation of zonulin in CACO-2 cells and affecting membrane integrity.

3.5. *Permeation effects of zein PT-prolamin fractions in CACO-2 cells*

After 18 days, CACO-2 cells had covered the insert membrane and reached constant TEER values. As positive control for well-polarized cells in the inserts, TEER was measured in untreated CACO-2 cells or stimulated with p31–49. TEER values were of 1.18 and 0.38 Ω/cm^2, respectively. Membrane permeation was measured by albumin concentration in the basolateral zone of insert well of the CACO-2 cell layer incubated with fraction 3 of PT-digested zeins or p31–49 peptide (0.107 mg/mL for both). Although the two albumin concentrations were lower than that for fraction 3 of PT-digested gliadins (0.301 mg/mL), no other fraction of gliadins nor zeins induced disruption of the cell monolayer (Table 3). Albumin concentration of basolateral zone of cells with full permeation buffer P/EDTA was 0.636 mg/mL. Gliadin peptide concentrations of 1 mg/mL were also used by Orlando et al. (2014) to alter cellular permeability in CACO-2 for 6 h. Interestingly, in our study, the use of unmarked BSA proved to be a reliable and economic method for testing cell membrane permeability.

4. Conclusions

The zein 34-mer (α-zein 58–91) peptide possesses immunogenic capacity in some patients' biopsies through the production of IFN-γ, a key cytokine in the adaptive response in CD. From the pro-inflammatory and the permeation effects of the fractions of PT-digested zeins on the intestine cell line CACO-2, we inferred that there are toxic peptides in zeins (1–5 kDa). These results suggest that there are innate and adaptive responses to zeins similar, but to a lesser extent than, to gliadins.

Although reaction to maize prolamins in CD patients appears to be a rare event, the confirmation that they play a role in the pathogenesis of CD will be useful information for the follow-up of some non-responsive celiac patients. It is estimated that

Table 3. Permeability effect of PT-digested zeins and gliadins in CACO-2 cells monolayers, albumin corresponds to basolateral concentration after 4 h.

Challenge	Albumin (µg/mL)
Blank	76
α-Gliadin 31–49	107
Gliadin fraction 3	301
Gliadin fraction 4	82
Zein fraction 3	107
Zein fraction 4	23

approximately 10% to 18% of these cases are refractory CD, which represents a more severe CD, with a clear malignity and a less favorable prognosis (Rubio-Tapia & Murray, 2010). Despite the low content of zeins in maize-containing foods compared with that of gliadins in wheat-containing foods, maize could be responsible for persistent mucosal damage in a very limited subgroup of CD patients (Ortiz-Sánchez et al., 2013). However, before considering maize as non-safe for patients who do not respond to a gluten-free diet, an oral challenge needs to be assessed.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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CORRESPONDENCE

Readers may submit letters to the editor concerning articles that appeared in *Gastroenterology* within one month of publication. Detailed guidelines regarding the content are included in the Instructions to Authors.

Age-Related Immunoreactivity to Gluten Peptides in Celiac Disease

Dear Editor:

In the November 2015 issue of *Gastroenterology*, Hardy et al[1] present a comprehensive study comparing T-cell response to gluten peptides between children and adults with celiac disease (CD). The article highlights the hierarchy of immunogenicity of α- and ω-gliadin peptides in both groups. Remarkably, the authors conclude that T cells from children and adults with CD recognize similar gluten peptides, and therefore, "peptide based diagnostics and therapeutics for adults may also be used for children."

We do not completely agree with Hardy et al[1] about CD children, because of the immaturity and limited memory response of the immune system of younger children. Although deamidation of gliadin peptides by tissue transglutaminase (tTG) strongly enhances the CD specific T-cell response, clones from young CD patients seem to recognize a diversity of gluten peptides whether deamidated or not, as demonstrated by Vader et al.[2] The study included 26 patients of 4 years of age, 7 of them <2 years, showing an inverse relationship between age and peptides recognized, while immune recall plays an important role in older children.[2]

In addition, there are studies involving CD children younger than 18 months, with elevated antigliadin immunoglobulin (Ig)A antibodies but low or no tTG IgA antibodies, in contrast to CD children >18 months old.[3] We have also studied age-related IgA reactivity to dietary proteins.[4] Serum IgA reactivity of ≤2-year-old (n = 6) and 3-year-old (n = 3) patients to tTG-treated gliadins was similar to the reactivity against untreated gliadins, whereas in older patients (>8 years; n = 5), where reactivity with deamidated gliadins increased by 33% in respect to the untreated ones. Results agree with those of Vader et al[2] because IgA antibodies are indirect markers of activated gluten-specific T cells.

A mayor discrepancy between the studies by Hardy et al[1] and Vader et al[2] or other studies of IgA reactivity, may well be related to the patients' age. In the Hardy et al[1] study, there were 51 CD patients, all but one >4 years of age, whose T cells were tested only against one peptide.

Prior studies have indeed found differences in immune response between CD adults and children >3 years old. A similar T-cell reactivity to deamidated but not to native gliadin epitopes between adults and children was described in recent onset CD (mean age, 8.3 years).[5] However, none of the patients recognized the immunodominant epitopes of the α-gliadin 33-mer (57-89), whereas one-half of the patients in the Hardy et al[1] study did. Moreover, α-gliadin peptides that activate adult T cells did not uniformly induce a response in stimulated T cells shortly after diagnosis or in untreated CD children (mean age, 7.1 years),[6] supporting the concept of heterogeneity of T-cell responses between children and adults in the study of Vader et al.[2]

In contrast, Hardy et al[1] argue that differences in respect to Vader et al[2] could be related to methodology. For instance, they analyzed an immunogenic gluten peptide described by Vader et al[2] finding a poor response in patients' T cells, possibly owing to a missing glutamine residue at the amino terminus of the peptide sequence. Therefore, the peptide was suggested to be less susceptible to deamidation by tTG in the glutamine residue at the third position to generation of a more potent T-cell epitope.[7]

It is difficult to establish whether or not there are differences in T-cell responses to gluten peptides between children and adults with CD if the studies differ in children age and methodology. Because gluten challenge should be discouraged in children <5 years of age according to the ESPGHAN criteria,[8] a more reliable comparison may be achieved with novel technology based on T-cell responses that are detectable in the blood without a gluten challenge.[6] Meanwhile, it could be convenient to use a wider variety of peptides and tests for the diagnosis of CD in children than in adults.

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CAPÍTULO III

Efecto de las Prolaminas del Maíz en Células Mononucleares de Sangre Periférica de Pacientes Celiacos

Effect of maize prolamins on peripheral blood mononuclear cells from celiac disease patients

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Abbreviations: CD, celiac disease; PBMC, peripheral blood mononuclear cells; IFN-γ, gamma interferon; ELISA, enzyme linked immunosorbent assay; kDa, kilodaltons; tTG, tissue tranglutaminase; PHA, phytohaemaglutinin A. IgA, isotype A immunoglobulins; IgG, isotype G immunoglobulins.

Keywords: Celiac disease, T-cell response, maize prolamins, zeins.

ABSTRACT

Scope: Celiac disease (CD) is an enteropathy induced by wheat gliadins and in some rare cases by maize zeins due to a similar immune response. The aim of this study was to compare the cellular response in CD peripheral blood mononuclear cells (PBMC) after a gluten challenge to maize and wheat prolamins.

Methods and results: *In vitro* gliadin and zein challenges were made in isolated PBMC's from two treated CD patients and three non-CD individuals after an *in vivo* gluten challenge by assessing gamma-interferon (IFN-γ) release in culture medium supernatants. PBMC's were stimulated with gliadin or zein immunogenic peptides and prolamins digested fractions (3-5 kDa), which have shown to induce cellular response in CD patient's biopsies. There was no higher production of IFN-γ after challenge for one of the CD patients neither to the non-CD individuals. Nevertheless, PBMC's from one of the CD patients produced higher IFN-γ than controls both after stimulation with zein or gliadin peptides.

Conclusion: In one of CD patient PBMC's, there was a cellular response to zein immunogenic peptide and digested fractions that response in a similar way than gliadin immunogenic peptides after a challenge.

1 Introduction

CD is an immunologically mediated systemic disorder developed in genetically predisposal 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 known treatment to alleviate symptoms. One of the most important alternative cereals used for the gluten-free bakery products is maize; additionally, it has been used as a negative control in different studies on CD. By chance, in some of the studies it has demonstrated adverse effects[2, 3] generating doubts about its use for dietary treatment of refractory CD patients. It could be due to similarities between maize (zein) and wheat (gliadin) prolamins both with a high percentage of glutamine 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]. This cytokine promotes tissue inflammation and lacks of autocrine effect on the other cells like monocytes; therefore, it is used for the assessments of cell response to different gluten peptides by *in vitro* assays[5, 6, 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]. These T-cells can be isolated and stimulated *in vitro* which generates a reliable tool to evaluate the reactivity of other dietary peptides different of those of wheat gluten[6]. The aim of this study was to

evaluate the T-cell response to zein deamidated peptides in CD patients as compared to non-CD individuals after gluten-free diet followed by a three-day gluten challenge.

2 Materials and Methods

2.1. Patients

Patients underwent gluten-free diet for one month, after which, they were subjected to a three days challenge with at least 50 g/day of gluten. 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]. Twelve mL of blood was used for the isolation of peripheral blood mononuclear cells (PBMC's) 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 was expressed as an index value and it was calculated based on the mean of absorbance values of control individuals as reported before[9], index values of 1.0 and above were considered as positive.

2.2. Peptide preparation

The immunogenic peptides α-gliadin 33-mer (LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF; MW = 3914.51 Da), referred to as G33-mer, and α-zein 34-mer (LQQAIAASNIPLSPLLFQQSPALSLVQSLVQTIR; MW = 3646.32 Da), 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 $CaCl_2$ 2 mM for 60 min at 37°C and then placed on ice. Separation of TG was performed using a 30 kDa Amicon Ultra-0.5 mL columns and peptides were recovered into sterile water.

2.3. Cell culture and cytokine assays

Isolated PBMC's were cultured at a final concentration of $2X10^5$ cells/mL on culture plates 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 37ºC in a 5% CO_2 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 a concentration of 25 μg/mL. After 20 h, supernatants were collected and frozen at -70ºC prior to IFN-γ determination. ELISA kits (Mabtech, Sweden) were used for detection

according to manufacturers' recommendations.

2.4. Statistical analysis

Experiments were performed by triplicate and results are given as mean values and compared by 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.

3 Results and Discussion

Characteristics of three control individuals and two celiac patients are described in table 1. Both celiac patients described as patient 1 and 2, reported extra-intestinal and intestinal symptoms, suggestive for CD that were alleviated after gluten-free diet. All the control individuals showed low levels of anti-Gd IgG, anti-Gd IgA and anti-tTG IgA antibodies. Celiac patient 1 presented positive indexes for anti-Gd IgG and anti-tTG IgA antibodies, while patient 2 had for anti-Gd IgG, anti-Gd IgA and anti-tTG IgA antibodies (table 1). Interestingly, only patient 2 had a positive index for anti-zein IgA antibodies, as it was previously found in some CD patients by Cabrera-Chávez et al[9].

Production of IFN-γ in PBMCs of control individuals was not obtained 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). In both CD patients, α-gliadin immunogenic peptide (G33-mer) increased the release of IFN-γ in PBMC's respect to controls ($p<0.005$) at days 0 and 6. Additionally, IFN-γ

release on patient 1 was higher at day 6 compared to day 0 ($p<0.05$) and this effect was not observed on patient 2. Peripheral blood effector T-cells reactive to gliadin 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 gliadin 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.

Interestingly, increased release of IFN-γ after stimulation with the immunogenic peptide of α-zeins (Z34-mer) was observed at day 0, mostly on patient 2 ($p<0.0005$) but stimulation diminishes at day 6 although it still higher than controls and patient 1 ($p<0.05$). Regarding to patient 1, IFN-γ levels increased in a lesser extent than patient 2 but greater than controls at day 0 ($p<0.05$), however at day 6, levels decrease in a manner comparable to controls (figure 1). 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]. High serum IgA anti-zein was also detected on patient 2 (table 1), and this is a marker of humoral response that is preceded by a specific recognition of zein antigens as a latent threat to the immune system. Cell response to Z34-mer is independent to the gluten challenge and the higher response

could be explained by the fact that maize is a common food constituent of the gluten-free diet and patients could be were highly exposed to large quantities of this protein.

An increase of IFN-γ release was also observed by the PT-digested fraction of zeins on patient 2 and this was greater than patient 1 and controls ($p<0.005$) on day 0 and 6 (figure 1). PT-digested fraction of gliadins did not induce response in neither of the two patients respect to controls at day 0 as expected due to treatment. Nevertheless, at day 6 after gluten challenge, an increase in IFN-γ release respect to day 0 was only significant on patient 2 ($p<0.05$) and this was greater respect to controls and patient 1 ($p<0.05$). 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. Nevertheless, stimuli with gliadin peptides was clearly observed when α-gliadin peptide G33-mer was used which 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 proposed to have affinity to the HLA-DQ8 tetramer[3]. Z34-mer has a very different peptide sequence to G33-mer, nevertheless, shares prolamin features like resistance to digestion and the possibility to be deamidated on its glutamine residues to

enhance a cellular response. Isolation of PBMCs 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.

Concluding remarks

In PBMCs of a celiac patient a cellular response to maize zeins was induced and this response was similar to that induced by wheat gliadins although independent of the gluten challenge. In vitro stimulation of PBMCs 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.

Author contributions:

AMC contributed with facilitation of resources, data analysis, writing and edition of this work, JPOS contributed with the design and realization of this work.

Conflict of interest statement

The authors declared no conflict of interest.

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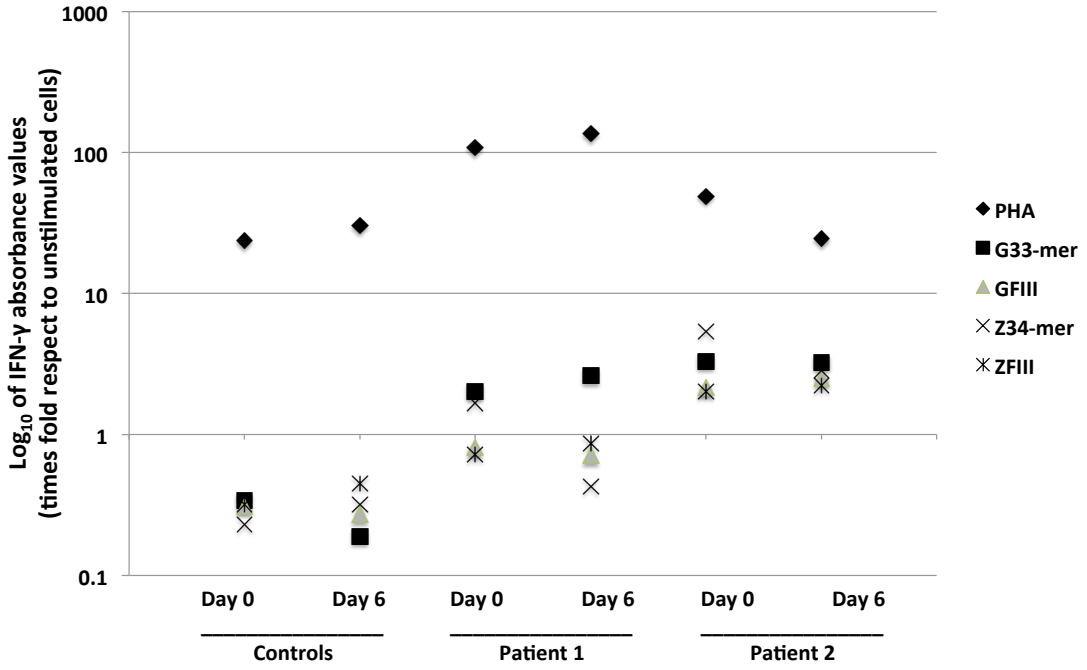

Figure 1

Release of the Th1 response related cytokine IFN-g in control individuals and CD patients. Absorbance values of IFN-γ are plotted based on $\log_{10}$ and represents the fold increase of cytokine in PBMC's without stimulation. All cytokine values of controls individuals were averaged and presented as a single value. CD patients were plotted individually as patient 1 and 2. G33-mer at patient 1 in day 0 vs. day 6 ($p<0.05$); GFIII at patient 2, day 0 vs. day 6 ($p<0.05$); Z34-mer at patient 2 vs. control and patient 1 ($p<0.005$); G33-mer in both patients vs. controls ($p<0.005$); ZFIII at patient 2 vs. controls and patient 1 ($p<0.005$). **IFN-γ**, interferon gamma; **PHA**, phytohemagglutinin A; **G33-mer**, α-alpha-gliadin 33-mer; **GFIII**, gliadin digested fraction; **Z34-mer**, alpha-zein 34-mer; **ZFIII**, zein digested fraction.

Table 1. Characteristics of control individuals and celiac disease patients

Subject	Age (years)	Haplotype or alleles	Index of antibodies				Symptoms
			IgG anti-Gd	IgA anti-Gd	IgA anti-TG	IgA anti-Zn	
Control 1	30	DQA1*501, DQA1*0301	0.899	0.773	0.744	0.768	None
Control 2	30	DQA1*501, DQB1*302/3	0.695	0.451	0.356	0.353	None
Control 3	27	DQA1*0301	0.796	0.728	0.668	0.619	None
Patient 1	31	HLA-DQ2	1.316	0.748	1.046	0.938	Migraine, fatigue and bloating
Patient 2	46	DQA1*0501	1.217	1.238	1.280	**1.68**	Anemia, constipation, bloating and arthralgia

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.

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