Chapter 5

Other Dietary Proteins besides Gluten could Affect some Celiac Patients

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Abstract

Some patients with celiac disease do not improve even after following a gluten-free diet upon diagnosis; therefore, nutritionists and physicians might conclude that this is due to the fact that their dietary recommendations were not strictly obeyed. However, in some cases, this is because these patients suffer from refractory celiac disease; dietary treatment is not the solution for these cases. Some of the cases considered to be refractory improve if, besides gluten, other dietary proteins, such as prolamins from oats (avenins) or maize (zeins) and sometimes, caseins from bovine milk, are withdrawn. Although there are very few published papers about such cases, there are clinical and practical facts, as well as published *in vitro* and *in silico* experiments, supporting the idea that other proteins induce an immune response similar to that provoked by gluten in celiac patients. In this chapter, the clinical evidence of these special celiac disease cases is discussed, as well as the information about experimental models and their possible relationship to an immune response against dietary protein antigens other than wheat gluten.

1. Introduction

By definition, celiac disease (CD) is an immunologically mediated systemic disorder, induced by gluten (the wheat protein fraction insoluble in water) and related prolamins (alcohol-soluble protein fraction from any cereal) in genetically predisposed individuals.[1] Thus considered, prolamins from oats or maize, which could affect some celiac patients, would not be excluded from this definition, even though they are not usually considered “related prolamins”. Those that are recognized as such, rye and barley, are taxonomically closer to wheat since they belong to the same group and subgroup, while oats and maize belong to the same family and subfamily to which wheat belongs.[2] What seems far stranger is that bovine milk caseins may exacerbate CD.

The issue here is that some CD patients continue experiencing symptoms and signs typical of the disease even after removing gluten from their diet, although effective adherence to this diet fails between 9 and 58% of the cases.[3-5] Another cause which may keep the disease active may be that celiac patients ingest gluten inadvertently. This is because the "gluten-free" or "no gluten" labels which indicate a maximum level of 20 ppm[6] are not always truthful. In fact, nearly half of the cases that do not respond to a gluten-free diet are due to the ingestion of improperly labeled foods[7] and one quarter of these patients are not aware of what they consume.[3] Although some of the symptoms improve with a reduced gluten intake, if its suppression is not strict, there is damage to the intestinal mucosa.

When symptoms of CD remain despite a strict adherence to the gluten-free diet, it could be due to other non-immune causes such as exocrine pancreatic insufficiency or intestinal bacterial overgrowth. Similarly, intolerance to fructose and lactose, resulting from damage to the intestinal mucosa while CD was active, can induce some of the symptoms of the disease.[8]

However, there are real CD cases in which there is no response to the gluten-free diet. Due to this, various studies and practical experiences lead to consider the possibility that other proteins, in addition to wheat, barley and rye, increase the CD immune response. Thus, it is clear that there are aspects of CD which have not been sufficiently studied. For instance, “Non-celiac gluten hypersensitivity”[9] is a new entity, and unresponsive CD or refractory celiac disease, has only recently been characterized. Both entities are two different health problems.

Refractory CD is defined by persistent or recurrent malabsorption symptoms and the presence of intestinal villous atrophy despite having followed strict gluten-free diet for 6-12 months.[10] Although there is no epidemiological data, it is considered that 5-10% of celiac patients do not recover through the gluten-free diet (GFD).[3] Patients with Type 1 refractory CD do not respond to the gluten-free diet, but their intraepithelial lymphocytes are normal. Type 2 is characterized by the presence of abnormal intraepithelial lymphocyte clones, which do not express CD3 and CD8 T-cell receptor markers, but express instead intracellular CD3; it is associated with a poor prognosis since it could evolve into a T-cell intestinal lymphoma.[11] Thus, continuous monitoring of both the immunophenotype as well as of lymphocytes clonality is recommended in these cases.[10]

In refractory CD, especially in Type 1, we should consider the possible influence of other dietary proteins besides gluten. The question is not whether there is a closely-related taxonomy, because this presumes a high prolamin homology with those from gluten, but to what extent they share the same immunogenic peptide sequences. This is the key to CD pathogenesis.

CD is triggered in genetically predisposed individuals, due to the properties of the gluten proteins which contain 15% proline and 35% glutamine. Proline, due to its cyclic structure, prevents intestinal proteolytic enzymes from breaking the peptide bond which forms it. Thus, some peptides comprising 10 to 50 amino acid residues with high immunogenic potential still remain and are able to cross the damaged intestinal barrier into the intestinal lamina propria. The lateral amino groups of the glutamines are removed by tissue transglutaminase, producing negatively charged peptides. The new-formed charged sequences (neoepitopes) have an increased affinity for class II human leukocyte antigens (HLA), specifically HLA-DQ2 and DQ8 in antigen-presenting cells. These cells present the neoepitopes to T cells which activate and proliferate to produce pro-inflammatory cytokines and stimulate to B cells to produce antibodies against gluten and the body's own tissular transglutaminase.[12]

Thus, any dietary protein which, once digested by the gastrointestinal tract, produces peptides with sequences and/or loads similarly arranged to those of gluten peptides could exacerbate an already developed CD. This chapter discusses some CD cases as well as complementary experiments dealing with a possible reaction to dietary proteins other than those of wheat.

2. Response to Proteins Generally Regarded as Safe

The persistence of symptoms in CD despite the gluten-free diet should lead to look for other causes of malabsorption. Among these, the most common is intolerance to food proteins other than gluten.[13] It would be interesting to test whether these proteins exacerbate a CD case which was previously triggered by gluten proteins or if they were the primary instigators.

Table 1 summarizes a series of tests with antibodies, cells or cell lines from CD patients, case reports, dietary and food protein contact challenges and *in silico* studies. These, along with some clinical experiences, have in common the analysis of the effects on CD of dietary protein different to prolamins from wheat, barley and rye. Overall, in refereed publications, three types of dietary proteins have been tested, generally unrecognized, which are suspected to have some effect on this disease: prolamins from oats (avenines) and maize (zeins), as well as bovine caseins.

Protein	Test performed	Outcome	Reference
Oat Proteins	Stimulus of lysosomal and K562 cells.	Positive response of lysosomal cells and agglutination of K562 cells as indicators of cytotoxic activity.	Silano et al.[14]
	Proliferation and activation of lymphocytes from CD patients.	Proteins from three oat varieties with proliferative and stimulant capacity of mononuclear peripheral blood cells, releasing IFN-γ.	Silano et al.[15]
	Two-year follow-up of CD children cases.	The children (4/9) exhibited symptoms due to oat ingestion. Oat peptides were identified in a HLA-DQ2 context.	Arentz-Hansen et al.[20]
Maize Proteins	Case report. Recording of symptoms and markers under maize stimulus.	No response to gluten removal but positive to maize in an oral challenge (double blind test, using maize and rice). CD remission on a gluten-free and maize-free diet.	Accomando et al.[13]
	Reactivity of anti-maize proteins' antibodies.	The competitive ELISA demonstrated that CD patient antibodies were specific for maize proteins; there was no cross-reactivity.	Skerritt et al.[28]
	Reactivity of CD patients' IgA and *in silico* analysis.	Positive titers of 5/24 CD patients for IgA anti-maize prolamins. Digested peptides with potentially immunogenic sequences were identified *in silico* for recognition and binding to HLA-DQ2/DQ8.	Cabrera-Chávez et al.[33]
	T-cell response.	IFN-γ production by intestinal cell line of 1/7 CD patients after stimulation using maize prolamins.	Bergamo et al.[32]
	Rectal mucosa challenge using maize prolamins and inflammation analysis.	CD patients (6/13) developed inflammatory reaction signs (nitric oxide production and granulocyte markers).	Kristjansson et al.[27]
	In silico analysis.	Presentation of peptide sequences from different maize proteins with homology to toxic wheat peptides.	Darewickz et al.[24]
Bovine caseins	Rectal mucosa challenge using bovine caseins and inflammation analysis.	Caseins induced an inflammatory response similar to that induced by gluten in CD patients in remission (nitric oxide, myeloperoxidase and eosinophil cationic protein production).	Kristjansson et al. [43]
	IgA reactivity of 150 CD patients (ELISA).	Inmunoreactivity was of 39% to hydrolyzed caseins, considering 100% inmunoreactivity to wheat gliadins.	Berti et al.[38]
	Identification of reactive proteins by IgA from CD patients.	IgA antibodies from some CD patients (9/14) recognize bovine but not human caseins by ELISA and immune-blotting.	Cabrera-Chávez, et al.[30]

Tabla I. Studies on the possible involvement of oat and maize prolamins as well as bovine casein in CD

3. Oat Prolamins and their Controversial Effect on CD

Oats, used in various foods for CD patients, may not be so safe, even if they are not contaminated with wheat gluten. Their proteins may affect the regeneration of the intestinal mucosa in recovering patients because they are able to promote T-cell response due to their immunogenicity, as well as that of lysosomal and K562 cells, which highlights their cytotoxic properties.[14,15] Oats contain a very special prolamin which, once digested, provides a peptide structurally rich in beta twists, soluble and quite immunoreactive, which the IgA antibodies of CD children recognize with high sensitivity and specificity.[16]

In contrast, other studies show that oats are safe for CD patients. According to Kilmartin et al[17], oat prolamins are not involved in the pathogenesis of CD because they do not induce a Th1 response in intestinal biopsies from a cohort of CD patients. It has also been published that these proteins do not trigger the characteristic CD autoimmunity, that is to say, they do not induce production of anti-tissue transglutaminase antibodies.[18] To reach common ground in this dispute, several authors recognize that while many CD patients can eat oats without any symptoms, some may not be able to tolerate it.[19-21] The behavior must be taken into account when considering the introduction of this cereal in the diet of CD patients. It is a fact that barley, rye and oats contain proteins with varying degrees of homology to wheat prolamins, due to their taxonomic relationship. The immune response to wheat, barley and rye prolamins is based on T-cell response to its homologous peptides.[22-23] The homology of avenins with gliadins is lesser than that of barley and rye[24], since is not so close. This gives rise to non-immunodominant peptides in oats, which would induce a response only in some CD patients.

As for the cellular immune response, there is knowledge of at least two avenin peptides which stimulate the T-cells of CD patients within the context of antigen presentation involving the HLA-DQ2.[20,25]

Finally, the manner in which oats are prepared as breakfast cereal makes them have a lower prolamin content than analogue products based on wheat, barley and rye. This results in a lower exposure to the immunogenic peptides of oat than to those of wheat.

4. The Intriguing Maize Prolamins and their Effect on CD

Maize is widely accepted as safe wheat substitute in foodstuffs for CD patients. Thus, to evaluate the effect in CD of microbial transglutaminase (mTG) in baking, we compared regular wheat bread with gluten-free bread made with rice and maize flours. In both cases, the bread was prepared with and without mTG treatment. Prolamins were then extracted from the four bread samples and tested as antigens for IgA from CD patients using ELISA. Unexpectedly, the IgA in one of the sera showed a much higher titer against prolamins in rice and maize bread treated with mTG than for wheat prolamins. In this case, it was the serum of a young CD patient who was unresponsive to the gluten-free diet. After performing a membrane immunodetection test with the isolated prolamins, it was inferred that this patient's CD was exacerbated by deamidated maize prolamins, as it happens with wheat gliadins in the pathogenesis of CD.[26]

In a case very similar to the one described in the previous paragraph, Accomando et al.[13] describe the follow-up of a young female CD patient who did not respond to the gluten-free diet. During follow-up, the patient had decreased levels of anti-gliadin IgA, but continuing symptoms of classic CD, including damage to the intestinal mucosa. When performing a double-blind provocation test with maize and rice, intolerance to maize was observed, but not to rice. After prescribing a gluten-free and maize-free diet, the symptoms gradually disappeared as well as the damage to the intestinal mucosa.

The two previously mentioned cases involved 16 year-old adolescents with atypical CD manifestations. The male was emaciated, had developmental delay, anemia, malabsorption, but mainly neurological problems. The female patient, in turn, had recurrent fatigue and loss of consciousness due to anemia. In both cases, anti-gliadin IgA and anti-transglutaminase levels decreased with gluten-free diet, but malabsorption, diarrhea and abdominal pain persisted. Symptoms remited only when maize was excluded from the diet. On neither case there are data on the age of CD onset; it is possible that it had been developing years before diagnosis and maybe maize prolamins induced a side effect to those of wheat.

In Table 1, in addition to clinical cases involving maize, a challenge with zeins in direct contact with the rectal mucosa of 13 adult CD patients is summarized. In this study, 6/13 of the patients showed signs of inflammatory reaction, although the response was lesser than that obtained in a gluten wheat challenge.[27] In this same study, a group of healthy individuals, showed response neither to gluten nor to wheat or maize proteins. Although the study by Kristjansson et al[27] evaluated the innate response involved in CD, their results demonstrated the activation of neutrophils and eosinophils in the early stages of inflammatory reaction in CD patients.

Regarding the humoral response, several authors have argued that some CD patients show high levels of antibodies against maize antigens.[28-31] Competitive ELISA immunoassays have shown that IgA from some CD patients recognizes specific maize protein sequences and that this is not a case of simple cross-reactivity. In the stage previous to antibody production, T helper cells must be activated, so that they, in turn will stimulate B cells.

Although there is not much information on the subject, in a study where the intestinal T cells of celiac patients were stimulated, a cell line from a patient (from a cohort of seven) with CD produced gamma interferon (IFN-γ) after stimulation with maize prolamins.[32]

In order to explain maize antigen presentation in CD, there have been diverse *in vitro* and *in silico* modelling studies. From their results it is inferred that some sequences in maize prolamins are good candidates for an efficient binding to HLA-DQ2 and HLA-DQ8 molecules[33]; it is a key step in the pathogenesis of CD. It has also been shown that certain sequences in maize remain immunoreactive for IgA from CD patients after a simulated gastrointestinal digestion with pepsin and trypsin. Furthermore, once thoroughly treated with proteases, there are still zein peptide sequences capable of binding to HLA class II molecules, involved in the pathogenesis of CD.[33]

The side chains of amino acids that bind to the HLA class II receptors make contact with specific sites in the molecule. For HLA-DQ8, the amino acid residue on the antigen required for binding is glutamine (converted into glutamic acid by tissular transglutaminase) in positions P1 and P9 on the peptide. For HLA-DQ2, glutamine is required on the P4, P6 and P7 positions on the peptide.[34]

Among the products of an exhaustive zein digestion, LQQAIAASNIPLSPLLFQQSPALSLVQSLVQTIR can be found, a peptide with glutamine residues at the appropriate positions to effectively bind to HLA-DQ8.[33]

According to Köning[35], in the early stages of CD development, a broad, gluten-specific T-cell response originates and may be directed towards any of the immunogenic peptides. On the other hand, IFN-γ secretion increases expression of HLA-DQ2 in the surface of the antigen-presenting cells, making peptide presentation more efficient. Eventually T-cell response will focus on the most immunogenic and stable peptides. It is likely that, among them, one of the maize prolamin peptides could be found.

The fact that few studies have been published on the adverse effect of maize gluten requires extensive review. An important consideration would be the weight of dietary maize, especially its intake before the development of CD. In the Mexican and Central American populations maize is a primary staple food; *atole* (a porridge made with maize flour mixed with water or milk) is introduced early into the infant diet. Thus, in a recent study, a total of 5 members of a cohort of 24 celiac patients showed positive reactivity of IgA class against zeins.[33]

In the Northwest Mexican population involved in our study, maize is used as much as wheat in the current diet.[36] In this way, the zein immunogenic sequences would be introduced at the same time that the immunogenic gliadin sequences, in individuals with a genetic predisposition to CD. However, not in all cases there is an immune response against zeins because, as with avenins, they possess less immunogenic sequences than gliadins.

5. Bovine Casein and its Surprising Effect on CD

Lactose intolerance is common in CD patients, especially when the intestinal mucosa has not completely recovered when starting a gluten-free diet. However, there are cases in which, even if they have recovered well, cow's milk is not tolerated and not because of lactose intolerance. A few years ago, it was suspected that some gluten peptides could pass through the wheat-containing pasture into the cow's milk. Dekking et al.[37] demonstrated, in a very convincing experiment, that there were no immunogenic peptides in cow's milk proteins, even if they had been fed pasture containing 100% wheat. Therefore, symptoms which were triggered in CD patients after ingesting cow milk were not due to gluten protein contamination but to the bovine milk proteins themselves.

IgA in some CD patients recognizes certain alpha and beta-casein sequences but not kappa caseins from cow milk, while it does not recognize any of the human milk caseins.[30] According to Berti et al.[38], IgA immunoreactivity is considerably reduced after casein digestion. However, the *in vitro* hydrolysis conditions used are quite different of the physiological conditions for the gastrointestinal digestion. Moreover, due to the processing of milk (heat treatment), bovine caseins form aggregates, which increases their resistance to digestion.[39]

On the other hand, beta casein contains sequences homologous to those of wheat gluten.[24] For example, in the 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) which is widely recognized as an immunogenic gluten peptide for CD, the PYPQ sequence is repeated three times.[40] This sequence can be found in seven peptides of digested bovine beta casein, with pepsin and trypsin.[41] The reason why IgA from CD patients does not recognize human beta casein may be due to small differences in contrast with sequences found in bovine casein and gluten. While the digestion of wheat gluten and bovine casein yields peptides with PYPQ sequences, hydrolysis of human milk casein peptides yields sequences such as PIPQ or PVPQ.[42] Thus, the residues of

branched chain amino acids with aliphatic groups from human casein peptides, provide considerably different properties compared to the aromatic group of tyrosine from bovine beta casein peptides.

In an analogous fashion to what has been described above for maize proteins, bovine caseins may induce an inflammatory reaction in a patch test on the rectal mucosa of CD patients.[43] However, even with such evidences from humoral and cellular response, tissular anti-transglutaminase antibodies, characteristic of the CD autoimmune response, may not appear. For their generation, the presentation of the enzyme and its substrate to the gluten peptides is necessary. Evidence shows that the humoral response is the same for untreated bovine caseins than for those previously treated with transglutaminase.[29] Thus, the hypothetically immunogenic peptides of bovine caseins do not require deamination for an efficient binding to the HLA-DQ2/DQ8 molecules. Thus, with the mere presence of bovine beta casein, transglutaminase would not be presented and an immune response against it would not be launched with the consequent lack of autoimmunity.

The low proportion of CD patients with symptoms after ingesting of cow's milk is surprising in the light of the hypothesis of its immunogenic peptides. In this case, the same assumptions cannot be made regarding the scant number of reactive sequences or amount ingested according to dietary habits, as it was done for oat and maize prolamins, because milk is a widely consumed food. Perhaps there would be differences in the degree in casein digestion; some CD patients would digest it less than others, especially if they have exocrine pancreatic insufficiency.[44]

6. Non-Refractory Celiac Patients who do not respond to the Gluten-Free Diet

When there is no good response to gluten-free diet, it becomes necessary to monitor possible responses against other dietary proteins. Regardless of the mechanism by which the immune system of these patients is being activated or not, simple and relatively non-invasive tests, such as ELISA immunoassays, can be used to obtain helpful information about treatment. According to published evidence, the three types of proteins discussed in this chapter may be related to persistent symptoms in CD patients. Thus, they would be the first subjects of ELISA immunoreactivity analysis, followed by a dietary challenge.

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