

# In vivo gluten challenge in celiac disease

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**HJ Ellis, PJ Ciclitira.** In vivo gluten challenge in celiac disease. *Can J Gastroenterol* 2001;15(4):243-247. In vivo gluten challenge has been used since the early 1950s to study the role of cereal fractions in celiac disease. While early studies relied on crude indicators of celiac toxicity, the advent of jejunal biopsy and sophisticated immunohistochemical techniques has allowed accurate studies to be performed.

Studies to determine the nature of the cereal component that is toxic to patients with celiac disease have concentrated on wheat because of its nutritional importance. A number of *in vitro* studies indicated the presence of one or more celiac-activating epitopes with the N-terminus of the A-gliadin molecule. In vivo challenge with three synthetic peptides subsequently indicated the toxicity of a peptide corresponding to amino acids 31 to 49 of A-gliadin.

In vivo gluten challenge is the gold standard for the assessment of celiac toxicity; however, jejunal biopsy is a relatively invasive procedure, thus, other methods have been investigated. Direct infusion of the rectum with gluten has been shown to result in an increase in mucosal intraepithelial lymphocytes, occurring only in celiac patients. This method has been used to study the celiac toxicity of gliadin subfractions. The *in vitro* technique of small intestinal biopsy organ culture is also a useful tool and appears to give the same results as *in vivo* challenge.

The importance of tiny amounts of gliadin in the diet, such as that which occurs in wheat starch, has been studied by *in vivo* challenge; this technique has clarified the position of oats in the gluten-free diet. Several studies suggest that this cereal may be included in the diet of most adult celiac patients.

Studies of the transport of gliadin across the enterocyte following ingestion or challenge suggest that gliadin may be metabolized by a different pathway in celiac disease. This could result in an abnormal presentation to the immune system, triggering a pathogenic rather than a tolerogenic response.

**Key Words:** Celiac disease; Gliadin; Gluten; Gluten challenge

## Test *in vivo* au gluten dans la maladie cœliaque

**RÉSUMÉ :** Le test *in vivo* au gluten est utilisé depuis le début des années 1950 pour étudier le rôle des fractions de céréales dans la maladie cœliaque. Alors que des études antérieures se fiaient aux indicateurs bruts de toxicité cœliaque, l'avènement de la biopsie jéjunale et de techniques immuno-histo-chimiques sophistiquées ont permis la mise au point d'exa- mens plus précis.

Les examens permettant de déterminer la nature des composantes céréalières qui sont toxiques pour les patients atteints de maladie cœliaque se sont attardés au blé en raison de son importance nutritionnelle. Un nombre d'études *in vitro* ont indiqué la présence d'un épitope cœlio-activateur ou plus avec la terminaison N de la molécule A-gliadine. Le test *in vivo* au moyen de trois peptides synthétiques a par la suite précisé la toxicité d'un peptide correspondant aux acides aminés 31 à 49 de la gliadine.

Le test de gluten *in vivo* est la norme pour l'évaluation de la toxicité cœliaque. Par contre, la biopsie jéjunale est une intervention relativement effractive. On a donc essayé de faire appel à d'autres méthodes. La perfusion rectale directe de gluten a fait augmenter les lymphocytes intraépithéliaux muqueux, ne survenant que chez les patients cœliaques. Cette méthode a été utilisée pour étudier la toxicité cœliaque des sous-fractions de la gliadine. La technique *in vitro* de mise en culture d'une biopsie du grêle est également un outil utile et semble donner les mêmes résultats que le test *in vivo*.

L'importance de petites quantités de gliadine dans l'alimentation, par exemple dans l'amidon de blé, a fait l'objet d'études au moyen de tests *in vivo* et cette technique a clarifié la position de l'avoine dans une alimentation sans gluten. Plusieurs études suggèrent que cette céréale puisse être incluse dans l'alimentation de la plupart des patients cœliaque adultes.

Des études sur le transport de la gliadine entre les entérocytes après l'ingestion ou après un test suggèrent que la gliadine puisse être métabolisée selon une voie différente dans la maladie cœliaque. Cela pourrait entraîner des anomalies du système immunitaire et déclencher une réponse pathogène plutôt que toléro-gène.

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## DEFINITION

Celiac disease is a chronic inflammatory response of the small intestinal mucosa in genetically susceptible individuals following the ingestion of gluten. Wheat, rye and barley have the disease-activating component. Early studies also implicated oats (1), although the celiac toxicity of this cereal is now in considerable doubt (2). Gluten ingestion by sufferers results in an abnormal flat jejunal mucosa that returns to a normal villous appearance when treated with a gluten-free diet.

To characterize the cereal fraction that exacerbates the condition, studies have concentrated on wheat because of its nutritional importance as a staple. Wheat grains can be divided into three main fractions – the outer husk or bran, the germ from which the plants grow, and the flour or endosperm that normally comprises 72% of the grain by weight. In an early challenge study, these three components were mixed separately in water and fed to an eight-year-old child. The child suffered from vomiting, diarrhea and collapse mainly due to the endosperm or flour fraction (1). The study was extended by examining the different fractions of wheat flour, which include water soluble starch and protein. The protein fraction comprises 8% to 14% by weight, depending on whether it is a weak/soft or hard/strong flour, such as that used for bread making. It became apparent that it was the protein fraction that caused the problem, and within this, it was the alcohol-soluble gliadins that were implicated (3). These early challenge studies relied on the measurement of increased fecal fat secondary to malabsorption as an indicator of celiac toxicity. This is a very imprecise measurement that has since been superseded by jejunal biopsy with morphological measurements (4).

## CEREAL CHEMISTRY

Wheat protein fractions can be subdivided. The albumins and globulins and starch granule proteins represent a small percentage of the whole. The glutenins and gliadins each form approximately 40% of the protein. The glutenins are proteins of 60 kDa to polymers of several millions. These provide wheat with its characteristic baking qualities by entrapping carbon dioxide, allowing dough to prove. The glutenins were thought to be insoluble in alcohol and most aqueous solutions, although more recent work has shown that the reduction of interchain bonds results in alcohol-soluble proteins. Proteins that are soluble in 40% to 90% alcohol without the need to break interchain bonds are termed 'cereal prolamins' – and specifically the gliadins in wheat. It is the prolamins of wheat, rye and barley that are known to cause celiac disease. Whether the other major group of wheat proteins, the glutenins, cause the immunological reaction leading to celiac disease is not known. The difficulty in obtaining pure soluble fractions has hampered investigation. The wheat prolamins or gliadins can be divided into subfractions previously termed alpha, beta, gamma, omega according to their relative electrophoretic mobility or, more recently, alpha, beta and omega types

according to their amino acid sequences. These proteins are unusual because they are comprised of 10% to 15% proline and 35% glutamine. The chemistry of these cereal proteins has been fully reviewed by Shewry et al (5).

## TOXICITY TESTING OF PROLAMINS

In vivo gluten challenge is the gold standard for testing the toxicity of gluten proteins. The advent of the small intestinal biopsy capsule and the discovery that individuals with active celiac disease had a characteristic lesion of the gut (4), which had previously been dismissed as postmortem autolysis, allowed objective testing of the toxicity of these peptides, by morphological measurement.

**Testing gliadin subfractions:** Early in vitro celiac small intestinal organ culture indicated the celiac toxicity of electrophoretically pure alpha, beta, gamma and omega fractions of wheat gliadin (6). To extend these findings in vivo by using treated patient volunteers, a Quinton multiple jejunal biopsy capsule was placed, under x-ray control, in the proximal small intestine. To this was taped an infusion catheter that allowed test fractions to be infused into the duodenum. Jejunal biopsies taken over several hours showed that 10 mg of gliadin produced no change, 100 mg produced minor changes, 500 mg produced moderate changes and 1 g produced a marked change in the mucosa. The initial changes occurred 2 h after infusion and were maximal between 4 and 6 h after infusion, after which they gradually improved. Then 1 g each of electrophoretically pure alpha, beta, gamma and omega gliadins was used, all of which proved to be celiac-activating (7). The morphometric parameters assessed were the dissecting microscopic appearance of the biopsies, changes in the villous height to crypt depth ratio, changes in enterocyte surface cell height, increases in intraepithelial lymphocyte count, altered expression of intracellular adhesion molecule in the subepithelial lamina propria and expression of histocompatibility leukocyte antigen class II by crypt enterocytes (8,9).

**Challenge studies to characterize the celiac-activating peptide:** The advent of amino acid sequencing technology and the availability of synthetic peptides allowed the study of small peptides by various methods. Complete sequences of certain alpha-type gliadins were published, greatly aiding this work (10). Whereas in vitro organ culture studies implicated N-terminal peptides of A-gliadin in the pathogenesis of celiac disease (11,12), comparison of published sequences from databases suggested homology between a C-terminal region of A-gliadin and the coat protein of human adenovirus 12 (13). A single gluten-sensitive T-cell clone from the peripheral blood of a patient with celiac disease was reactive to a synthetic N-terminal A-gliadin peptide corresponding to amino acids 31 to 49 (14). To assess the celiac toxicity of peptides implicated in the above-mentioned studies, we turned to in vivo challenge.

We synthesized three gliadin peptides, peptide A corresponding to amino acids 31 to 49 of A-gliadin, peptide B corresponding to amino acids 202 to 220 and peptide C corresponding to amino acids 3 to 21. Peptides A and C corre-

sponded to peptides implicated in organ culture studies (12) and peptide B to the adenovirus 12 peptide (13).

Four celiac patient volunteers were studied, all of whom had been receiving a gluten-free diet for at least six months and had responded well. Patients were given an initial 1 g gliadin challenge to prime the mucosal immune system and, as a positive control, to show that these individuals would show a morphological response to gliadin within the proposed time frame of the experiments. Then, with at least one week between challenges, the three synthetic peptides (100 mg) were tested in the four subjects. The histology of biopsies taken at time zero were compared with those taken 6 h after commencing the challenge (15).

In all four patients challenged with peptide A (amino acids 31 to 49), there was a decrease in the villous height to crypt depth ratio, a decrease in the enterocyte surface cell height and an increase in intraepithelial lymphocytes. With peptide C corresponding to amino acids 3 to 21 of A-gliadin there was no change in any of the morphometric parameters assessed. In one patient there were some minimal changes in the mucosa with peptide B (amino acids 202 to 220 of A-gliadin). An earlier challenge study using 100 mg of an almost identical peptide showed some histological change; however, it was not significant (16), thus the toxicity of the 'adenovirus peptide' is unknown.

In subsequent experiments, the celiac biopsies from the current challenge study at time zero were compared with those taken 2 to 4 h after the infusion of the three peptides A, B and C for expression of mRNA encoding proinflammatory cytokines. There was evidence of mRNA for the proinflammatory cytokines interferon-gamma and interleukin-2 within 2 h of receiving the challenge with both unfractionated gliadin and peptide A (amino acids 31 to 49 of A-gliadin) but not with peptide C, although a minimal increase in proinflammatory cytokines was observed 2 h after the infusion of peptide B in the one patient who had shown minimal mucosal changes (17).

**Do the results of in vitro organ culture studies correspond to those obtained in vivo?:** Early organ culture experiments showed the toxicity of alpha, beta, gamma and omega type gliadins (6), which was confirmed in vivo (7).

The in vitro organ culture system was used to assess peptides A, B and C (18). It is common to measure only enterocyte surface cell height when using this technique. As expected, it was found that a peptic tryptic digest of gluten, which served as a positive control, caused a fall in enterocyte surface cell height in jejunal biopsies from treated and untreated celiac patients. This also occurred with peptide A but not with peptides B or C, or ovalbumin, which served as a negative control (17). Thus, it appears that in vitro organ culture may give a very good indication of gliadin toxicity in vivo. Assessment of enterocyte cell height after organ culture is difficult because the biopsy is often in a poor state after culture. The method is, therefore, not without its detractors. It would, therefore, be very useful if there was a simpler test. A recent report by Piccarelli and colleagues (19) showed that small intestinal biopsies cultured in vitro

produced antiendomysial antibody when in the presence of gliadin, but not in its absence. This offered the exciting possibility of a test for peptide toxicity. However, we have shown that production of endomysial antibody (EMA) in vitro is dependent on the length of time the patient has been on a gluten-free diet, rather than the presence or absence of gluten (20). Organ culture experiments have revealed that the substitution of certain amino acids, but not others, within peptide A (15), led to the abolition of toxicity (21). This result needs confirmation in vivo; however, it is clearly not possible to test a large series of peptides by in vivo challenge, which requires large amounts of the peptide and is relatively invasive.

**Is there a more accessible site for in vivo gluten challenge for toxicity testing?:** It has long been suspected that the entire gastrointestinal tract in celiac patients is hypersensitive to gluten. The rectal mucosa appears to offer a relatively simple and safe site for gluten challenge. Celiac disease patients responded to rectally infused Frazers Fraction III (22) with a rise in mucosal intraepithelial lymphocyte count (23); controls did not respond. Loft et al (23) suggested that this might make a useful screening test for untreated CD. Ensari and colleagues (24) used this technique to confirm the toxicity of highly purified omega-gliadin fractions. More recently, it was shown that oral submucosal injection of gluten in celiac patients led to a significant rise in lamina propria lymphocytes (25). This route may offer a convenient method for diagnostic gluten challenge in the future.

#### GLUTEN CHALLENGE TO ASSESS THE SUITABILITY OF FOODSTUFFS FOR CELIAC PATIENTS

Feeding studies with pre- and postchallenge biopsies have attempted to show the effect of protracted ingestion of small quantities of gluten such as that that may be encountered in the average gluten-free diet. For this type of quantitative assessment there is no alternative route. Even so, Kumar et al (26) showed the difficulty in interpreting such studies. They showed that adults and children consuming 5 g/day of gliadin took between one week and 10 months to show histological relapse. A four-week challenge with as little as 100 mg/day of gliadin caused a significant increase in intraepithelial lymphocyte count in children who had previously been on a gluten-free diet (27). In a six-week challenge study in which patients ate six slices of gluten-free bread estimated to contain 3.6 mg/day of gliadin, we were unable to show any significant morphological changes in their biopsies, although some patients reported diarrhea (28). It is now proposed that foods labelled as gluten-free for consumption by patients with celiac disease should contain no more than 10 mg gliadin/100 g, although some patient groups are pressing for lower levels.

Recent challenge studies have suggested that consumption of reasonable quantities of oats (50 g/day) over a long period of time (six months) may not adversely affect the

majority of gluten-sensitive adults (2,29). The long held belief that this particular cereal is harmful to celiac patients may have arisen due to inaccurate methods of assessment of toxicity, such as fecal fats (1) or xylose excretion (30).

### IN VIVO GLUTEN CHALLENGE IN THE STUDY OF PATHOGENIC MECHANISMS

Small bowel biopsies from patients on gluten-free and gluten-containing diets and normal or unrestricted diets have been assessed by electron microscopy to define the transport pathways of gliadin across the enterocyte. Differences in the handling and compartmentalization of gliadin may have implications for disease pathogenesis (31-33).

Friis et al (34) used an immunofluorescent method with anti-gliadin polyclonal antibodies to show that the enterocytes of healthy controls and treated celiac patients have different staining patterns just 20 min after the start of an infusion of gliadin. The enterocytes of healthy controls are characterized by a diffuse, homogeneous staining pattern, whereas the enterocytes of celiac patients who have received gluten show a much more intense granular staining pattern in the apical region, and a conspicuous fluorescence in the intercellular space.

This difference in staining patterns might mean a different distribution of gliadin in the enterocytes of healthy controls and celiac patients. In healthy controls (diffuse pattern), gliadin appears to be in the cytoplasm of the enterocytes, but in patients with celiac disease there is a granular pattern that appears to be in an endocytic compartment that resembles major histocompatibility complex (MHC) class II-positive vacuoles containing gliadin as described by Zimmer et al (31).

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Tissue transglutaminase (tTG), a cytoplasmic enzyme for which gliadin is an excellent substrate, has recently been shown to be the antigen for EMAs (35). It has been suggested that tTG complexes with gliadin and the neoepitopes trigger the immune response that causes coeliac disease (36). Gliadin-specific T cells may provide help for tTG specific B cells, leading to antibody production, as a bystander event (37). We suggest that the abnormal compartmentalization of gliadin in the enterocytes may be the predisposing factor for binding to tTG. Thus, tTG may also be presented to the immune system in an abnormal way, which would produce antibodies (38). Withdrawal of gliadin from the diet would eliminate the abnormal presentation of tTG, by either of the above-mentioned routes, to the immune system. This would explain the high sensitivity of endomysial antibodies for coeliac disease.

### CONCLUSIONS

It is possible that in healthy controls gliadin may be metabolized through an endogenous, tolerogenic pathway, whereas in coeliac disease it may be metabolized through an exogenous MHC class II pathway. This results in abnormal presentation to the immune system, which triggers an immune response, resulting in enteropathy (38). To investigate this, the study of the absorption of gliadin, and other dietary peptides in the enterocytes of coeliac patients and healthy controls is crucial. Careful challenge studies, both in vitro and in vivo, followed by electron microscopic assessment, may help to validate this model. This could shed new light on the pathogenesis of other conditions such as inflammatory bowel disease, where damage is caused by an unknown antigen being misrepresented to the immune system.

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