

Review article: safe amounts of gluten for patients with wheat allergy or coeliac disease

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Publication data

Submitted 23 May 2005
First decision 15 June 2005
Resubmitted 16 November 2005
Accepted 16 November 2005

SUMMARY

For both wheat allergy and coeliac disease the dietary avoidance of wheat and other gluten-containing cereals is the only effective treatment. Estimation of the maximum tolerated amount of gluten for susceptible individuals would support effective management of their disease.

Literature was reviewed to evaluate whether an upper limit for gluten content in food, which would be safe for sufferers from both diseases, could be identified. When setting gluten limits for coeliac disease sufferers, the overall potential daily intake should be considered, while for wheat allergy limits should be based on single servings. For coeliac disease sufferers this limit should lie between 10 and 100 mg daily intake. For wheat allergy, lowest eliciting doses for children lie in the lower milligram range, while for adults they are most significantly higher.

Gliadins (part of the gluten proteins) not only trigger coeliac disease, but are also major allergens in wheat allergy. Therefore, measurement of gliadins with validated enzyme-linked immunosorbent assay methods provides an appropriate marker for assessing gluten and/or wheat protein contents in food. Available data suggest that a maximum gluten content for 'gluten-free' foods could be set, which protects both wheat allergy sufferers and coeliac patients.

Aliment Pharmacol Ther 23, 559–575

INTRODUCTION AND OBJECTIVES

Coeliac disease is estimated to affect approximately 1% of the population in regions such as Europe, North and South America, north Africa and the Indian sub-continent. Coeliac disease is thus an important public health issue. Reflecting this, wheat and other gluten-containing cereals feature among the substances listed in the General Standard for the Labelling of Prepackaged Foods as 'known to cause hypersensitivity'.¹ Their initial inclusion in the list, which comprised mainly foodstuffs that elicited immunoglobulin E (IgE)-mediated allergic reactions, was related to their role in the aetiology of coeliac disease. Recent data indicate that wheat allergy – both IgE- and cell-mediated – occurs more frequently, at least in some regions, than had previously been thought, and affects a high proportion of food allergy sufferers in some populations, making it a public health issue of similar magnitude as that of coeliac disease.^{2–5} There is scarce information on the overlap between coeliac disease and wheat allergy patients.

Treatment of both coeliac disease and wheat allergy relies on avoidance of wheat, rye and barley proteins. However, total avoidance is extremely difficult, if not impossible in most diets. In coeliac disease, the concept that gluten-free refers to a level of gluten that is harmless, when ingested indefinitely, rather than to total absence, is widely accepted. Indeed, with a view to protecting coeliac disease sufferers, Codex Alimentarius specified in 1981 a standard for food rendered gluten-free, i.e. wheat starch and derivatives, establishing an upper limit for total nitrogen (0.05%),⁶ equivalent to about 300–500 mg gluten/kg as measured by a method available in 1992.⁷ This nitrogen figure was derived from available clinical experience. However, the concept of an upper safe limit is still not universally accepted for food allergy, although much work has been, and is being carried out to establish experimentally minimal eliciting doses for many food allergens.⁸ These may be considered as the maximum tolerated dose for a selected sensitive section of the at-risk (i.e. allergic) population. Such data thus not only help the clinician to provide better guidance, but also when analysed at the population level, provide essential information for allergen risk management. Data on minimal eliciting doses for wheat protein have begun to be generated through challenge studies. These have revealed that children and adults show very different patterns of reactivity. The results indicate that children typically react at lower doses, and

manifest symptoms such as atopic dermatitis (AD), while adults require much greater quantities and react more frequently with anaphylaxis, accompanied by exertion in most cases. Similarly, longitudinal studies on coeliac disease sufferers have suggested that compliance with a gluten-free diet, based on the current Codex standard, ensures that the disease does not recur, and that they remain as healthy as a control population. Codex is currently reviewing this standard.⁹ There is considerable debate about new proposed limits (20 mg/kg gluten for naturally gluten-free food and 200 mg/kg gluten for food rendered gluten-free, e.g. wheat starch) and pressure to replace the 1981 standard with a more stringent and comprehensive one also covering naturally gluten-free food. Such a replacement would obviously have a major impact on coeliac disease sufferers, potentially decreasing the range of products available to them, as well as likely increasing their cost. Food manufacturers would also be affected through the need to implement more stringent measures to meet the new standard. It is thus important for all stakeholders that any change is based on sound scientific and clinical evidence.

Since 1981, considerable research has been carried out for coeliac disease sufferers with a view to determining the maximum tolerable dose of gluten. Similarly, much new data have been generated on wheat allergy. The purpose of this study is firstly to review the evidence supporting the proposed standards for gluten-free foods, and secondly to establish whether gluten-free products as defined for coeliac disease sufferers could be considered safe for wheat allergy patients. If a single limit could be set protecting both coeliac disease sufferers and wheat allergic subjects, consumer communication regarding 'gluten-free food' would be eased considerably.

Literature search was performed using the data collections of the authors, Medline search using search terms such as wheat allergy; wheat allergy and coeliac disease; coeliac disease and challenge; gluten and detection and food.

COELIAC DISEASE

Clinical manifestations and prevalence

Coeliac disease is genetically determined and is more frequent in women than in men. The disease is an autoimmune condition triggered by ingestion of gluten, the insoluble wheat, barley or rye protein

fraction.^{10, 11} Clinical data are now available suggesting that the great majority of coeliac patients tolerate oats although some concerns remain about the safety of this cereal for coeliacs,^{12–15} and oats remain currently on the Codex list of gluten-containing cereals. The abnormal immunological response is characterized by an inflammatory reaction of the small bowel leading to flattening of the mucosa. The accepted diagnostic criteria for coeliac disease, as defined by the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), are based on detection of flat mucosa on small bowel biopsy and disappearance of symptoms following a gluten-free diet.¹⁶

Coeliac disease was for many years considered a disease of the very young infant. Symptoms and signs of malabsorption become obvious within months of starting a gluten-containing diet. Chronic diarrhoea or loose stools, vomiting, a distended abdomen and failure to thrive were common presentations. Similarly, diarrhoea, weight loss and general weakness were the most common symptoms in adults.¹⁷ Today, coeliac disease presenting with a malabsorption syndrome is not the general rule.^{18, 19} Indeed when serological tests based on the detection of coeliac disease-associated autoantibody were developed as safe screening tools, it became clear that coeliac disease is a complex disorder with manifestations that are not confined to the gastrointestinal tract.^{20, 21} Isolated iron deficiency anaemia is the most common example. Other manifestations include permanent tooth enamel defects,²² epilepsy and cerebral calcification,²³ isolated liver diseases,^{24, 25} osteopenia,^{26, 27} idiopathic ataxia,²⁸ infertility,²⁹ non-Hodgkin's lymphoma³⁰ and myocardiopathy.³¹ Furthermore, coeliac disease is related to specific conditions such as selective IgA deficiency³² and Down's syndrome.³³ It is also frequently associated with other autoimmune diseases³⁴ of which type I diabetes is the most frequently studied.³⁵ In most cases the mechanisms underlying these associations remain to be determined.

Screening programmes within populations indicate that the disease is underdiagnosed. Serological testing detects otherwise undiagnosed disease in one in 100 individuals.^{36–42} Biopsy revealed a flattened mucosa in the majority of patients that tested positive.

A life-long gluten-free diet is in fact the only effective treatment for coeliac disease. Wheat-, rye- and barley-based products must be avoided. Oat-based

products, as long as they are free from contamination with other gluten-containing cereals, are tolerated by most patients. On a gluten-free diet the small bowel mucosal lesion of coeliac patients heals and the symptoms disappear.

Cellular mechanism and characterization of antigens

CD4+ T lymphocytes in the jejunal mucosa of coeliac patients react to gluten-derived peptides presented by antigen-presenting cells in the context of class 2 histocompatibility molecules. The recognition of these peptides leads to activation and release of inflammatory cytokines characterized by a Th1 pattern dominated by γ -interferon.^{43, 44} The activation of innate immunity mechanisms seems to precede the adaptive immune response.⁴⁵ The main autoantigen of coeliac disease is the enzyme tissue – transglutaminase (TG2).⁴⁶ Antitissue TG2 autoantibodies are deposited in different tissues, but their pathogenic role is still undefined.⁴⁷ TG2, among other functions, deamidates gluten protein^{48, 49} and increases the affinity between the hydrolysed peptides and human histocompatibility leucocyte antigen (HLA) class 2 DQ2 or DQ8 molecules on antigen-presenting cells. Activated CD4+ T cells not only induce a Th1 cytokine response, and downstream the secretion of metalloproteinases which are involved in mucosa remodelling, but also stimulate B lymphocytes leading to production of antigliadin and anti-TG2 antibodies. Other phenomena occur in the coeliac epithelium such as epithelial cell apoptosis⁵⁰ and lymphocyte killing of enterocytes.^{51, 52}

By directly introducing a purified gliadin fraction to the small bowel of intubated volunteer patients, Ciclitira *et al.*^{53, 54} showed that this particular class of cereal protein was able to induce mucosal flattening. Using a combination of *in vivo* and *in vitro* techniques, it was then demonstrated that all gliadins were toxic, with α -gliadins producing the most severe effects.⁵⁵ More recently gliadin epitopes able to stimulate T-cell responses have been identified. They are all HLA-DQ restricted, most DQ2-restricted, some DQ8-restricted. As there is evidence that only HLA-DQ8-positive coeliacs, and non-DQ2, react to DQ8-restricted epitopes, this could open the possibility of defining different dietary requirements according to the genotype. Although there is no evidence for establishing a hierarchy of T-cell epitopes, a large proportion of T lymphocytes isolated from the intestinal mucosa of

untreated coeliac patients recognize *in vitro* a 33 amino acid α -gliadin-derived peptide, which intraluminal and brush border enzymes are unable to fully digest, and which contains three overlapping epitope sequences (PFPQPQLPY, PQPQLPYQ and PYPQPQLPY).^{56–59} Direct administration by intubation of a peptide including these sequences into the small bowel of volunteer coeliac patients in remission leads to mucosal damage.⁶⁰ Some glutenin-derived peptides also induce *in vivo* as well as *in vitro* T-cell activation.^{61, 62}

Eliciting dose

A life-long gluten-free diet is the only known medical treatment for coeliac disease. However, the sensitivity of coeliac patients to gluten varies on an individual basis. This complicates the setting of acceptable limits for trace amounts of gluten in gluten-free foods.⁶³

It is generally accepted that the *in vivo* gluten challenge is the gold standard for the assessment of the level of gluten tolerance. Both 'acute' and prolonged *in vivo* challenge tests have been used in investigating the effects of small amounts of gluten in the diet. Table 1 summarizes published research on ingestion of small amounts of gluten and on gluten challenge studies.

Ciclitira *et al.*⁵³ performed an *in vivo* study with increasing amounts (10–1000 mg) of unfractionated gliadin. Results showed that 10 mg unfractionated gliadin did not cause mucosal damage, while minimal changes were seen after 100 mg. However, after an additional challenge with 500 mg gliadin, development of mucosal ridges and changes in the villous height/crypt depth ratio and in the amount of intraepithelial lymphocytes were significant. After 1000 mg gliadin challenge more marked abnormalities began to appear.

Catassi *et al.*⁶⁴ performed a 4-week challenge test with 20 coeliac children in order to investigate the effects of chronic ingestion of small amounts of gliadin. They were given a daily dose of either 100 mg or 500 mg of gliadin. Patients in whom the daily intake was 100 mg showed a significant increase in the intraepithelial lymphocyte count, while in the group that had ingested 500 mg gliadin, more marked histological changes were noted. A prospective randomized study is presently being performed with a larger number of patients and with doses of <100 mg, i.e. either 10 or 50 mg of purified gluten per day. Preliminary results

suggest that both 10 and 50 mg daily gluten are well tolerated, but that there is a trend for mucosal changes to occur at the 50 mg dose.⁶⁵

Over the past 40 years, industrially purified wheat starch-based flours have been widely used and accepted as part of the gluten-free diet for coeliac patients in the UK and northern Europe. These products have not been used in southern European countries because of fears of an excessively high-gluten contamination. Table 2 summarizes the clinical studies on wheat starch-based gluten-free flours in the coeliac diet. In 1988, Ejderhamn *et al.*⁶⁶ showed that a daily intake of 4–14 mg gliadin did not cause changes of villous height or intraepithelial lymphocyte counts in coeliac patients (mean age 14 years) who had been on a wheat starch-containing gluten-free diet for a mean period of 10 years. Later, it was shown that coeliac patients on a strict gluten-free diet, even based on products containing wheat starch, show mortality or morbidity rates similar to those exhibited by the general population.^{20, 67} On this particular diet, the mucosa heals and stays morphometrically normal over at least 10 years of wheat starch ingestion.

In a subsequent study of duodenal biopsy specimens from 89 adult coeliacs⁶⁸ no relationship was found between the presence or absence of villous atrophy and ingestion of a gluten-free diet as defined by Codex Alimentarius or a gluten-free diet that contained no detectable gluten. Intraepithelial lymphocyte counts were higher in subjects with an abnormal mucosa than in those with normal mucosa, but this was independent of the type of gluten-free diet.

Kaukinen *et al.*⁶⁹ investigating the small bowel mucosal condition in 52 adults and children who had been on a gluten-free diet for an average of 8 years, and 40 of whom had been on a wheat starch-based diet, found that the wheat starch-based gluten-free products, with a calculated daily gluten intake of 36 mg, did not cause aberrant upregulation of mucosal HLA-DR and the mucosal integrity was unrelated to the daily intake of wheat starch in all patients on a strict diet.

In a paper from the same group, Lohiniemi *et al.*⁷⁰ investigated whether long-term consumption of wheat starch-based gluten-free products sustained abdominal symptoms or had an adverse effect on the general well being. The daily amount of wheat starch had no effect on the Gastrointestinal Symptom Rating Scale (GSRS) and the mean GSRS score in coeliac patients did not differ from that of the control subjects. Dietary

Table 1. Small bowel biopsy findings in coeliac patients taking small amounts of gluten (adapted from Ref.⁷²)

Author	n	Age group	Consumption of gluten	Duration of the diet	Symptoms	Abnormal findings in small bowel biopsy
(A) Small amounts of gluten in diet						
Dissanayake <i>et al.</i> ⁷³	13	Adults	<0.5 g gluten daily	6–72 months	Abdominal symptoms in 1	PVA in 10
Baker <i>et al.</i> ⁷⁴	24	Adults	<2 g gluten daily	4–132 months	–	PVA in 16
Ejderhamn <i>et al.</i> ⁶⁶	11	Adolescents	4–14 mg gliadin daily†	8–14 years	Asymptomatic	No
Kumar <i>et al.</i> ⁷⁵	15	Adolescents	Occasional gluten (2.5–10 g)	Several years	Asymptomatic	PVA or SVA in 7
Mayer <i>et al.</i> ⁷⁶	14	Adolescents	0.06–2 g gluten daily	9–16 years	Symptoms in 29%	PVA or SVA in 4, villous shortening in 7, increase in IELs
Troncone <i>et al.</i> ⁷⁷	6	Adolescents	<0.5 g gluten daily	≥10 years	Asymptomatic	PVA in 1; increase in IELs in 2
(B) Gluten challenge studies						
Ciclitira <i>et al.</i> ⁵⁴	7	Adults	1.2–2.4 mg gliadin daily, 1 week†	>1 year	–	No
Ciclitira <i>et al.</i> ⁷⁸	10	Adults	1.2–2.4 mg gliadin daily, 6 weeks†	>1 year	Diarrhoea in 4	No
Montgomery <i>et al.</i> ⁷⁹	8	Adults	2.5–5 g gluten daily, median 6 months	6–27 months	–	Increase in IELs
Catassi <i>et al.</i> ⁶⁴	(1) 10	Children	(1) 100 mg	14 ± 3 months	(1) Asymptomatic	Dose-dependent decrease in VH/CrD, increase in IELs
	(2) 10	Children	(2) 500 mg gliadin daily, 4 weeks		(2) Anorexia and pale stools in 3	

† Gliadin from wheat starch-based gluten-free products.

VH/CrD, villous height and crypt depth ratio; PVA, partial villous atrophy; SVA, subtotal villous atrophy; IEL, intraepithelial lymphocyte.

Table 2. Studies on the effects of wheat starch-based gluten-free diet in coeliac disease and dermatitis herpetiformis (from Ref.⁷³)

Author	Study design	Number of patients with wheat starch diet (all patients)	Patient group	Duration of wheat starch intake	Outcome methods	Untoward effects of wheat starch-based gluten-free diet
Ciclitira <i>et al.</i> ⁵⁴	Open challenge	7 (7)	Adults	1 week	Small bowel biopsy	No
Ciclitira <i>et al.</i> ⁷⁸	Open challenge	10 (10)	Adults	6 weeks	Small bowel biopsy, Cr-EDTA excretion	Abdominal symptoms in some
Ejderhamn <i>et al.</i> ⁶⁶	Cross-sectional	11 (11)	Adolescents	10 years on average	Small bowel biopsy	No
Chartrand <i>et al.</i> ⁸⁰	Open challenge	17 (31)	Children and adults	0.5–10 months	AGA, EmA	Symptoms in 11/17
Kaukinen <i>et al.</i> ⁶⁹	Cross-sectional	40 (52)	Children and adults	8 years on average	Small bowel biopsy, AGA, EmA, ARA	No
Selby <i>et al.</i> ⁶⁸	Cross-sectional	39 (89)	Adults	0.6–29 years	Small bowel biopsy, AGA, EmA	No differences to naturally gluten-free diet
Lohiniemi <i>et al.</i> ⁷⁰	Cross-sectional	48 (53)	Adults	9–11 years	Quality of life, small bowel biopsy to 23	No
Peräaho <i>et al.</i> ⁷¹	Prospective, randomized	20 (40)	Adults	1 year	Small bowel biopsy, AGA, EmA, BMD, quality of life	No differences to naturally gluten-free diet

AGA, anti gliadin antibodies; EmA, antiendomysial antibodies; ARA, antireticulin antibodies; BMD, bone mineral density; Cr-EDTA, Cr-ethylenediaminetetraacetic acid.

compliance was noted to be good and the daily intake of gluten from wheat starch was estimated to be 36 mg on average, based on the assumption that 100 g of gluten-free wheat starch-based flour contains up to 20 mg gluten. Villous atrophy was found only in two of 23 patients that had a biopsy.

More recently, a controlled study by Peräaho *et al.*⁷¹ concluded that wheat starch-based flour is acceptable in the gluten-free diet. No differences were observed in clinical responses, small bowel mucosal morphology, intraepithelial T-cell densities, mucosal HLA-DR expression, serum endomysial, tissue TG2 or gliadin antibody levels, bone mineral densities or quality of life measurements, between patients on a diet-containing wheat starch-based flour and those of a group of coeliac patients on a gluten-free diet which did not contain wheat starch. Typically wheat starch-based flours contain 100 mg gluten/kg or less as measured by enzyme-linked immunosorbent assay (ELISA) methods. Assuming that the patient ingests 200 g of wheat starch-based flour per day, full remission is thus achieved with a daily gluten intake of approximately 20 mg.

Very recently, the gluten content of 59 naturally gluten-free and 24 wheat starch-based gluten-free products were analysed by ELISA.⁷² Both naturally gluten-free (22% of total) and wheat starch-based gluten-free products (46% of total) were found to contain gluten (up to 200 mg/kg, most below 100 mg/kg). Based on these results, a median daily flour intake of up to 300 g was calculated, giving a daily gluten intake of 30 mg; this amount was found not to affect mucosal histology.

In conclusion, available gluten challenge studies show that daily intakes of <10 mg have no effect on mucosal histology, whereas definite alterations are caused by a daily intake of 500 mg and observable alterations by 100 mg. A calculated daily intake of 30 mg seems not to harm the mucosa. Therefore at present, a safe limit could be set between 10 and 100 mg. Ongoing prolonged *in vivo* microchallenge studies will provide further assurance regarding the validity of these figures. These data indicate that wheat starch-based foods (provided they contain <100 mg gluten/kg) are safe for coeliac patients. On the contrary, data also indicate that a certain proportion of naturally gluten-free products may contain gluten.⁷² The overall potential daily intake of gluten should be considered in setting a safe limit for the claim 'gluten-free', taking into account all foods which contain gluten, whether naturally gluten-free, or rendered gluten-free.

WHEAT ALLERGY

Prevalence

Wheat is included among the foods identified by Codex Alimentarius as responsible for most of the food allergies. Wheat is also often implicated in food-dependent, exercise-induced anaphylaxis (FDEIA).

Wheat allergy appears more frequent in northern⁸¹ than in southern Europe. In France, wheat ranks as the eighth most frequent food allergen in children and the 12th in adults.⁸² It represents 20% of the entire food allergy clinical population in the study of Sicherer,² 14% in the study of Niggemann³ and 10.9% of children and 25% of adults recorded in Moneret-Vautrin *et al.*⁸³ In contrast, in one American study on food allergy in children, wheat-related food allergy was present in only 2.5% of the cases.⁸⁴ These numbers could be underestimated because they represent only the most severe cases where hospital care was necessary.

Pathogenic mechanisms

Pathogenic mechanisms include IgE-mediated and cell-mediated allergy, and are characterized by the timing of reactions during oral challenges, by skin prick tests (SPT) or prick-in-prick tests, epicutaneous tests and by specific IgE measurements and lymphocyte activation tests.

The time interval between food ingestion and the appearance of symptoms, distinguishes immediate and non-immediate reactions. The former occur within a few hours of food ingestion, and are characterized mainly by one or more of the following symptoms: urticaria and/or angio-oedema, anaphylaxis, nausea, vomiting, diarrhoea, rhinitis and bronchial obstruction. They are IgE-mediated, and are diagnosed on the basis of positive responses to prick tests, specific IgE assays and oral provocation tests.⁸⁵

Non-immediate reactions occur from several hours to 1 or 2 days after food intake, and are characterized mainly by eczematous manifestations (or exacerbation of AD) and loose stools or diarrhoea. In these patients, a T cell-mediated pathogenic mechanism has been demonstrated on the basis of positive responses to patch testing with the implicated food and to oral provocations tests.^{86–89} Roehr *et al.*⁸⁶ showed that positive atopy patch tests (APT) to wheat were correlated with positive challenge results in 94% of patients assessed. In the study by Strömberg⁸⁷ on children with

AD, APTs were correlated with positive challenge results in 90% of cases of wheat allergy and 93% of cases of rye allergy. False-negative patch test results to wheat occurred in 6% of patients and to rye in 10%. However, patch tests and oral provocation tests with food allergens in patients who have experienced non-immediate reactions are not yet fully standardized.

Clinical manifestations

Clinical manifestations of wheat allergy are similar to those of other food allergies, with symptoms on the skin, the gastrointestinal tract and the respiratory tract.² They occur in children as well as in adults but possibly with different clinical patterns. The main symptoms in children are AD, either alone or associated with respiratory symptoms and digestive problems.^{81, 90–94}

In adults, FDEIA, anaphylactic shock, angio-oedema,^{95–99} irritable bowel syndrome, eosinophilic oesophagitis and rare cases of ulcerative colitis^{100–103} are the presentations most often described.

Wheat may be introduced very early in the diet, around the fifth month after birth. However, sensitization may occur much earlier through maternal milk,¹⁰⁴ in which undegraded gliadins have been reported in the case of breast-feeding mothers not following a specific diet.¹⁰⁵ Sensitization to other foods-like milk or egg frequently occurs by the same route.

Wheat-dependent exercise-induced anaphylaxis (WDEIA) was mostly observed in adults, but also sometimes in children.^{99, 106–108} This particular presentation is difficult to predict and to diagnose as the ingested wheat quantities, as well as the exercise level necessary to induce the symptoms, are very variable.^{107, 109, 110}

Wheat allergens

Wheat food allergy has received much less attention than bakers' asthma.^{111–116} In children the IgE response appears quite heterogeneous. Studies with immunoblots have shown that IgE binding to different proteins of the salt-soluble fraction.^{81, 117} James *et al.*¹¹⁸ showed that a 15 kDa protein belonging to the α -amylase inhibitor family was recognized by serum IgE from five wheat allergic children. CM3 protein from the tetrameric inhibitor of wheat α -amylases

could also be an important allergen for AD patients acting as sensitizing allergen both by ingestion and by inhalation.¹¹⁹

Morita *et al.*¹²⁰ reported the presence of IgE directed against wheat γ -gliadins in four patients with FDEIAN. An epitope recognized by IgE from four wheat-related AD patients has been identified as a pentapeptide with QQQPP structure belonging to the repetitive domain of the low-molecular weight (LMW) glutenin subunits.¹²¹ In a recent study, the IgE fraction from the serum of adults and children with cereal-induced food and respiratory allergy reacted with the salt-soluble protein fraction from wheat, oat and barley,¹²² suggesting that glutenins were involved. In both pathologies a similar protein pattern was recognized suggesting that the allergens implicated could be the same.

In a more recent study, Battais *et al.*¹²³ studied 28 patients, children and adults with wheat allergy confirmed by double-blind, placebo-controlled food challenge (DBPCFC). Sixty percent of sera were shown to have specific IgE antibodies against α - and β -gliadins and LMW weight glutenin subunits and 28% against lipid transfer proteins (LTP). IgE antibodies against the albumin/globulin protein fraction were recorded in 72% of patients. In a more extensive study with 60 patients, the same authors showed that different antigenic profiles could be detected in food allergy to wheat, according to age and symptoms. α -, β -, γ -gliadins and albumins/globulins appeared to be more important allergens for children with AD with or without asthma, while ω -gliadins were major allergens for adults with FDEIA and/or anaphylaxis (100%) or urticaria (55%). B-type LMW glutenin subunits also featured in anaphylaxis cases in adults. Only 23% of patients with AD and 8% of those with AD and asthma reacted to ω -gliadins.¹²⁴ Another recent study¹²⁵ showed that ω -gliadins were also the major allergens in children with wheat-induced anaphylaxis. Differences therefore exist between children and adults in the pattern of response to major wheat allergens and in disease outcome.

A Finnish team published several articles on characterization of wheat food allergens recognized by IgEs from AD and FDEIAN patients. Varjonen *et al.*,^{126, 127} in a study of AD in children suspected of wheat-dependent food allergy, reported wheat protein-specific IgE recognizing 26, 38 and 69 kDa proteins. In a more recent study, they showed that gliadins could be important allergens for this category of patients.⁹⁴ In 1997, they published data on five patients exhibiting

FDEIAN after ingestion of cereals.¹⁰⁹ Prick tests were performed with a salt-soluble protein suspension, and immunoblots with the ethanol-soluble fraction of different cereals using patients' sera containing specific IgE. The five patients were positive in the prick tests and possessed IgE antibodies directed against proteins of wheat, barley, spelt and oats, but not against those of other cereals. Immunoblots revealed that different proteins from the wheat gliadin fraction were recognized by IgE antibodies, suggesting a role for those allergens in the pathogenesis of FDEIAN after wheat ingestion.

A follow-up study was performed on 18 patients with the same symptoms.¹¹⁰ All 18 patients exhibited urticaria after positive prick tests with wheat flour. Symptoms appeared only when the patients ate food-containing wheat after physical exercise. IgEs from a pool of patients' sera recognized two wheat proteins, an ω -5 gliadin and an α -gliadin. In ELISA, the 18 patients' sera were positive to the ω -5 gliadin and 13 to the α -gliadin. *In vivo* reactivity of the ω -5 gliadin was confirmed by prick tests in 15 patients while α -gliadin gave positive prick tests for only five of these patients. Therefore, for FDEIAN patients, ω -5 gliadin is a major allergen and α -gliadin only a minor one.

In a study by Palosuo *et al.*,¹²⁸ 16 (84%) of 19 children with immediate hypersensitivity to wheat – diagnosed on the basis of positive responses to oral challenges – had IgE antibodies against purified ω -5 gliadin in ELISA tests. The same group^{99, 119} showed that all patients with WDEIA reacted positively to ω -5 gliadin in SPT and had detectable IgE antibodies to it by ELISA.

The ω -5 gliadin induced release of histamine from the basophils of patients with WDEIA but not from those of controls. The authors also presented data suggesting that tissue TG2 in the intestinal mucosa of these patients could be activated during exercise.¹²⁹ Gliadin peptides released by pepsin digestion would react with the enzyme and build large protein complexes that could boost the IgE response. This hypothesis, if substantiated would make an interesting parallel between the mechanisms involved in coeliac disease and in FDEIAN to wheat. Finally, the manufacture of wheat isolates including deamidated gliadins could create neoallergens, as has been shown in one case of allergy restricted to wheat isolates,¹³⁰ in the absence of reactivity to unmodified wheat itself.

In vitro cross-reactivity between different cereals has been demonstrated in several studies. Varjonen *et al.*¹²⁶ performed an IgE immunoblot with serum samples from 40 adults with allergic reactions after ingestion or inhalation of cereals: 35 patients with AD, four with urticaria and one with rhinitis. Sixteen simultaneously staining bands were observed in wheat, rye and barley extracts, suggesting cross-reactivity among these cereals. In the study by Armentia *et al.*,¹²² IgE immunodetection using serum pools from patients sensitized to cereals by inhalation (adults with baker's asthma) or ingestion (adults and children) displayed very similar patterns of IgE-binding using salt extracts from wheat, barley, and rye. Protein bands of around 11–16 kDa, probably allergenic members of the α -amylase inhibitor family, were immunodetected in wheat, barley and rye. Moreover, when examining the sera from 23 adults with WDEIA, Palosuo *et al.*¹³¹ observed cross-reactivity between ω -5 gliadin-specific IgE antibodies and rye γ -70 and γ -35 secalins, and barley γ -3 hordein. These findings suggest that rye and barley may also elicit symptoms in patients with WDEIA.

Thresholds of clinical reactivity (minimum eliciting doses)

Diagnosis of wheat allergy, in common with other food allergies, and in contrast with coeliac disease, relies on the observation of clinical signs, and their timing in response to food challenge. Minimum eliciting doses are thus quite difficult to establish unequivocally. Oral provocation tests with wheat have been performed in several studies.^{2, 81, 86–89, 92, 117, 122, 127, 128, 132–135} Wheat-specific IgE concentrations do not seem to be such useful predictors of the provocation test outcome as to reduce the use of the latter tests, in particular compared with milk-, egg- and peanut-specific IgE concentrations.^{3, 132, 134, 136}

Jones *et al.*,¹¹⁷ reported that up to 10 g of wheat flour (about 1.2 g of protein) are needed to induce symptoms in children suspected of wheat allergy on the basis of positive prick tests. Wheat allergy was only confirmed in 31% of sensitized children and 24% of them were also allergic to other gluten-containing cereals (oats, barley, rye). Children respond to lower amounts than adults with 62% of children reacting to <8–10 g of wheat flour.^{2, 81, 127} However, most of the studies cited assessed children with AD, who had not suffered severe immediate reactions.^{2, 81, 86, 87, 92, 122, 127, 128} In some

studies,^{127, 128} challenges began with the application of a drop of wheat porridge to intact skin and the upper lip, and positive responses to this method were reported.¹²⁷ In a more recent study based on a large population (326 children and 164 adults) screened for food allergy, Moneret-Vautrin *et al.*,⁸³ reported 38 children and 41 adults with wheat allergy. Clear differences were observed between wheat-allergic adults and children in their reaction to controlled oral challenge with wheat protein. One gram or less of wheat protein provoked reactions in 40% of the children while 80% reacted to <2 g. Thus, only 20% of children needed more than 2 g of wheat protein to provoke a reaction. However, 6% (two of 32) of the children reacted to <10 mg of protein. In the adult allergic population, the entire panel required at least 1 g of wheat protein and half required more than 6 g to induce an allergic response (D. A. Moneret-Vautrin, personal communication). D. Hill (personal communication) performed wheat challenges with 62 children aged 7–103 months and also observed four patients (3%) who reacted to 2 mg of wheat protein.

Wheat-dependent exercise-induced anaphylaxis appears to be triggered by large amounts of allergen.¹³⁷ In a study by Hanakawa *et al.*,⁹⁶ which assessed one patient with WDEIA, generalized urticaria was induced during exercise following the ingestion of 64 g (but not 45 g) of bread.

Patients with T cell-mediated hypersensitivity to wheat required about 10 g of wheat protein to produce positive responses to provocation tests.^{86–89} Altogether these studies strongly suggest that eliciting doses of wheat protein for wheat allergic patients are higher than for coeliac patients.

However, with few exceptions,^{83, 122} patients with a convincing history of anaphylaxis to isolated wheat ingestion have not been challenged.^{2, 117, 118, 128, 132, 135}

C. Ortolani (personal communication) also challenged about 30 subjects with histories of immediate reactions to wheat ingestion. Results were consistent with published findings by other authors, the rate of positive responses being about 50%, and the amount of wheat protein, which produced such responses ranging from 100 mg to 25 g.

Wheat-allergic patients are treated the same way as coeliac disease patients – with a gluten-free diet. Most of these patients tolerate naturally gluten-free cereals, i.e. maize and rice and also oats, even though cross-allergy between wheat and these cereals has been described.

MEASUREMENT OF GLUTEN IN GLUTEN-FREE FOODS

Background

There is no general agreement yet on the analytical method to be used to measure gluten in ingredients and food products to check compliance with maximum limits for gluten-free food. The proposed Codex Alimentarius limits for gluten,⁹ i.e. 20 mg/kg for naturally gluten-free food and 200 mg/kg for food rendered gluten-free (e.g. wheat starch), are still under discussion. However, a prerequisite for setting such limits is the availability of a reliable method, which can measure gluten traces from about 10 to more than 200 mg/kg in a variety of foods and ingredients.

In the course of the last few years, important progress towards a standardized method has been achieved. For several reasons, it is very difficult to develop an acceptable test: (i) gluten refers to a complex mixture of proteins found in several different cereals with natural fluctuations in their protein profile depending on the variety, year, geographic origin, etc. (ii) Derivatives of cereals such as wheat starch do not have the same protein profile as the native cereal. (iii) To obtain at least comparable results among different laboratories, an internationally recognized gluten reference standard should be defined. Ideally, an analytical test should be able to detect all epitopes involved in either coeliac disease or wheat allergy and the test should give the same response to gluten which has undergone various process steps, e.g. very strong heat treatments or protein hydrolysis, as even very small peptide sequences are known to be reactive in coeliac disease.

The development and validation of methods to determine gluten in gluten-free food has been a research subject for more than 20 years. Both the extraction process for quantitative recovery of gluten from the foodstuff and reliable measurement of the extracted gluten are very difficult issues, due to the particular protein solubility properties of the polypeptides that constitute gluten and their specific repetitive primary sequence. Several methods have been developed along the years mainly based on immunological tests,¹³⁸ mass spectrometry (MS)¹³⁹ and the polymerase chain reaction (PCR)¹⁴⁰ and several commercial ELISA kits have been put on the market. However, until recently, none of these methods was considered as universally acceptable.

Although ELISA tests are widely used in analytical laboratories in the food industry and in the compliance branches of food authorities, results lacked reproducibility because of the use of different reference standards, extraction protocols and antibodies.

Gluten, prolamins and gliadins

Prolamins constitute about 50% of gluten which is defined as the water-insoluble protein fraction of flour from wheat,¹⁴¹ barley, rye, oats and their crossbred varieties. The prolamins of wheat, barley, rye and oats are called gliadins, hordeins, secalins and avenins respectively. The gluten fraction isolated from wheat flours exhibits an enormous complexity due to the broad genetic polymorphism of several classes of these proteins.

As gliadins are the proteins mainly involved in the pathophysiology of coeliac disease and they are also recognized to be major allergens in wheat allergy, they represent good markers to measure gluten traces both with respect to wheat allergy and coeliac disease. Quantification of gliadin levels could therefore provide an appropriate tool for defining an acceptable limit for gluten-free food by Codex Alimentarius and national regulatory authorities. Obviously, for a quantitative method, an accepted analytical standard substance needs to be available. Due to the complexity of the gluten fractions, the definition of a reference material has proved difficult.

However, under the leadership of the Prolamin Working Group, a new gliadin reference material has been prepared using gluten extracted from the most common European wheat varieties. Recently, it has been further characterized by the EU Commission's Joint Research Center (JRC) at the Institute for Reference Materials and Measurements (IRMM), Geel, Belgium with a view to certification as reference material CRM IRMM-480, 'Gliadin from European Wheat'.

Determination of gluten traces by measurement of gliadins

There have been several attempts to design methods based on the measurement of a gliadin fraction or a gliadin peptide in the past. Most have been antibody-based, because of the specificity of these tools, which can measure components even in complex

matrices. In addition, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was used to detect gluten and gliadins.^{139, 142} Although different immunological techniques are available, ELISA continues to be the preferred format,¹³⁸ mostly because of the ease of use and the availability of commercial test kits.

Several commercially available test kits are based on a monoclonal antibody raised against an ω -gliadin from the Australian wheat variety Timgalen originally developed by Skerritt and Smith.¹⁴³ The method has been collaboratively tested and approved as an official method by the American Association of Official Analytical Chemists,¹⁴⁴ but only for gluten levels above 160 mg/kg. This method allows the extraction from and detection of gluten in highly heat-processed products, which was only possible to a limited extent with the methods described in literature so far, e.g. based on α -gliadin¹⁴⁵ or an α -gliadin peptide.¹⁴⁶

With the classic extraction solvent for gliadins, 60% aqueous ethanol, mainly the heat-stable ω -gliadin fraction, which does not contain -SH-groups, is extracted from cooked foods, while most of the remaining gliadin fractions, α , β and γ , stay as insoluble aggregated material and cannot be solubilized.¹⁴⁷ To specifically extract ω -gliadins, 40% ethanol was recommended.¹⁴³ However, wide fluctuations in results have been observed when comparing the test kits based on ω -gliadin antibodies and those based on antibodies directed to other gliadin fractions or peptides, both due to fluctuation in the ω -gliadin content of wheat-based ingredients and to differences in the standard materials used.

A few years ago, a method, which makes use of a new monoclonal antibody, called R5, was developed by Valdés *et al.*¹⁴⁸ R5 recognizes the epitope QQFPF, a pentapeptide present twice in α -gliadins and 11 times in γ -type gliadins.¹⁴⁹ Several commercially available test kits are now on the market making use of the procedure and the antibodies developed. A patented quantitative extraction procedure for gluten involving guanidinium hydrochloride and 2-mercaptoethanol was introduced to improve the extraction of gluten from heat-processed samples¹⁵⁰ and the gliadin reference material developed by the Prolamin Working Group was introduced as standard. The test allows equivalent recognition of wheat, barley and rye gluten protein.¹⁵¹ Valdés *et al.*¹⁴⁸ also demonstrated by Western blotting the reactivity of R5 towards enzymatically or heat-modified wheat prolamins. In the frame of the

Prolamin Working Group, Immer *et al.*¹⁵² performed a collaborative study using the R5 ELISA with 20 laboratories and concluded that the method is able to detect gliadin in the range of 2–5 mg/kg food. In addition, the results correlate well with those obtained by the MS method.¹⁵³

A controversial issue might be whether it is justified to calculate the gluten content of wheat starch as 'gliadins $\times$ 2', as strong differences in gliadin to gluten ratios exist in starch. This is due to the fact that in wheat starch gliadin and gluten proteins make only a minor part of the nitrogen-containing compounds besides the more abundant starch granule proteins, phosphatidyl choline and albumin-globulin. The contents of these compounds differ from starch to starch.¹⁵⁴

Last but not least, if the ratio of gliadins to glutenins varies considerably and HMW glutenins are also involved in the pathophysiology of coeliac disease,⁶² the validity of immunoassays recognizing gliadins but not glutenins could be challenged.

The sandwich R5 ELISA of Valdés *et al.*¹⁴⁸ has been proposed as an official Codex method. At the Codex Committee Meeting on Methods of Analysis and Sampling, Budapest, 2004, the method was endorsed as a type IV method.¹⁵⁵ This means, if further validation data of this method are submitted, the method might be accepted as an official Codex method at the next meeting.

Measurement of hydrolysed gliadins

Prolamins in foods or ingredients can be partially hydrolysed as result of processing (residual prolamins in wheat starch hydrolysates such as maltodextrins, prolamins in malt and beer, etc.), but might still be toxic for subjects suffering from coeliac disease. The ELISA tests based on ω -gliadin or R5 antibodies, which are commercially available, hardly recognize prolamins peptides in this type of food ingredients. A competitive ELISA was developed by Ferre *et al.*,¹⁵⁶ which might be a new tool to also detect hydrolysed gliadins.

CONCLUSION

Recent epidemiological surveys reveal that both coeliac disease and wheat allergy are more frequent than indicated by earlier data. Prevalence of coeliac disease in many countries is indeed estimated as high as 1%.

Both conditions therefore represent significant public health problems in large parts of the world.

Thorough studies of coeliac disease patients, conducted over many years, have demonstrated that diets containing the small, but measurable amounts of gluten found in current 'gluten-free', including 'naturally gluten-free' products lead to healing of the intestinal mucosa. These studies have therefore narrowed the range within which the maximum tolerated daily intake lies, i.e. to a figure higher than 10 mg, but lower than 100 mg/day. Based on a consideration of the diet of coeliac patients, current data indicate that wheat starch-based food is safe, provided it contains <100 mg gluten/kg. Furthermore, there is no evidence to support a definition of naturally gluten-free requiring no detectable gluten (i.e. a 'zero tolerance').

The clinical features of wheat allergy are broadly similar to those of other food allergies, but it possesses some unique characteristics. It tends to be more frequent, but less severe in infants and children, with AD the most prominent symptom. The minimum doses of wheat protein to elicit reactions in the more sensitive children lie in the lower milligram range, but the allergy is often outgrown in later childhood. In contrast, in adults, wheat allergy is more persistent, and manifestations are often associated with physical exertion (FDEIA). However, the lowest doses necessary to provoke reactions are also much larger in adults (often >1 g protein), and thus are unlikely to be ingested inadvertently or to be present as residual allergen from cross-contact. Wheat also induces cell-mediated immune reactions, but the minimum provoking doses are even higher than for IgE antibody-mediated responses (approximately 10 g or more).

Gliadins trigger coeliac disease, but they are also major allergens of food allergy to wheat, capable of provoking IgE-mediated reactions. Measurement of gliadins therefore constitutes a good estimate of the amount of gluten and of wheat proteins in products containing wheat. In recent years, more reliable and sensitive assays to determine the presence of gliadin in

foods have been developed and commercialized. These are either based on the detection of the heat-stable ω -gliadins or on the detection of a 'toxic' motif in gliadin (the so-called R5 antibody assay) and thus have overcome many of the drawbacks of the earlier methods. Although natural variability of gliadin fractions in gluten-containing cereals, denaturation and cross-links because of strong heat treatments contribute a certain measurement uncertainty, the new assays based on the R5 antibody seem to be sufficiently reliable and have been proposed as international reference methods. However, they still remain to be adopted by Codex Alimentarius.

Taken as a whole, the evidence obtained through recent studies indicates that the maximum tolerated daily dose of gluten for coeliac disease patients would also constitute a base for a safe upper limit per serving for the vast majority of sufferers from wheat allergy. It provides the basis for calculating the maximum permissible gluten content for 'gluten-free' foods, based on a thorough analysis of the dietary patterns of both these patient groups. New assays for gliadin provide promising tools for manufacturers and regulatory bodies to verify compliance of products with such a limit.

TRANSPARENCY STATEMENT

This work was commissioned by the Food Allergy Task Force of the European branch of the International Life Sciences Institute (ILSI Europe). Industry members of this task force are Ajinomoto Europe, Barilla G&R F.lli, Bayer CropScience Bioscience, Coca-Cola European Union Group, Danisco, Groupe Danone, H.J. Heinz, Kraft Foods R & D Inc., MasterFoods, Monsanto Europe-Africa, Nestlé, PepsiCo International, Royal Numico, Tate & Lyle Speciality Sweeteners, Unilever, Wild Flavors, Berlin. For further information about ILSI Europe, call +32 2 771 00 14 or e-mail info@ilsi-europe.be. The opinions expressed herein are those of the authors and do not necessarily represent the views of ILSI or ILSI Europe.

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