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Coeliac disease: A review of the causative agents and their possible mechanisms of action

Review Article

H. J. Cornell

Royal Melbourne Institute of Technology, Melbourne, Australia

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Summary. This review outlines the main theories for the aetiology of coeliac disease and presents in more detail the work carried out in an attempt to define the nature of the toxins in wheat gluten. This includes the results of work with synthetic peptides and a discussion of the various assays used.

Evidence is presented for an enzyme deficiency in coeliac disease which leads to abnormally high concentrations of certain peptides in the small bowel. These peptides can bring about damage by direct toxic action and by immunological mechanisms.

Investigations of activity of synthetic peptides based on the structure of A-gliadin appear to be making good progress and point to certain regions of that molecule as being responsible for toxicity. Certain key sequences of amino acids appear to be of fundamental importance to these studies.

Keywords: Amino acids – Wheat – Gliadin – Coeliac disease – Gluten-sensitive enteropathy – Peptides

1. Clinical aspects

Coeliac disease (CD) is also known as gluten-sensitive enteropathy and non-tropical sprue. The symptoms of this disease were first described by Samuel Gee (1888), who reported poor growth, abnormal stools and abdominal distension as common symptoms in children.

Shiner (1959) showed that the disease could be detected in infancy by performing a biopsy of the duodenum or jejunum and examining the specimen histologically. CD is characterised by a flat appearance of the mucosa in these regions, with villous atrophy and hypertrophy of the crypts. Inflammatory cell infiltration is observed in the lamina propria and surface epithelium and changes in the basement membrane are often seen. Biopsy of the small bowel remains the cornerstone of diagnosis. These aspects and the clinical

presentation in CD have been discussed comprehensively by Cooke and Holmes (1984).

Dicke (1950) first showed that ingestion of wheat flour produced symptoms of CD. Then Anderson et al. (1952) and Van de Kamer et al. (1953) firmly established that the main protein of wheat flour, the gluten complex, was responsible for this toxicity. Foods containing rye, barley and possibly oats also contain toxic proteins and therefore must be excluded from the diet in order for the patient to go into remission. Some adults respond only slowly to a gluten-free diet but in children, Lifshitz and Fagundes-Neto (1983) showed that this response is rapid and morphological improvement of the small bowel is substantial in a matter of weeks, thus helping to confirm the diagnosis. It is important to diagnose the disease in the early years of life because Egan-Mitchell et al. (1978) found a high tolerance to gluten in some adult patients and also because of the finding by Harris et al. (1976) of an increased predisposition to malignant bowel lymphoma of patients on a normal diet. For more precise diagnosis, it has been common practice to challenge the patient on the gluten-free diet once more with gluten and if relapse occurs within two years of reintroduction of gluten, it can be concluded that the diagnosis was correct (McNeish et al., 1979). However it should be remembered that some adults with coeliac disease will take longer than this to show evidence of a mucosal lesion, while others will show no improvement after a long time on a gluten-free diet. These problems again reinforce the need for early diagnosis, where the results are more straightforward and the patient benefits from a lifelong dietary treatment (Sheldon, 1969).

2. Aetiology

The aetiology of CD remains a mystery and reflects the problem of not having a true animal model of the disease. However, the use of foetal rat (De Ritis et al., 1979) and foetal chick (Mothes et al., 1985) models, together with assays based on cultures of atrophic human mucosa, has enabled rapid progress to be made (Townley et al., 1973; Falchuk et al., 1974a). Combined with the use of synthetic peptides, this approach promises to throw further light on the basic aetiology of CD and will be discussed later in this review.

Progress in our understanding of the aetiology of CD up until 1987 has been reviewed comprehensively by Davidson and Bridges (1987). Hence only an outline of the aetiology is necessary for this paper, which concentrates instead on defining the nature of the toxins in gluten. Four theories of the aetiology of CD have been proposed, viz:

(i) The enzymopathic hypothesis

This hypothesis proposes that there is defective mucosal digestion of gluten proteins in patients with CD, leading to high concentrations of certain peptides in the small bowel which bring about damage to the tissue. Compelling evidence for this hypothesis was produced by Frazer and co-workers

(1959) who showed that the toxicity of a peptic-tryptic digest of gluten was abolished by pre-incubation with an extract of hog intestinal mucosa.

Further evidence came from Cornell and Townley (1973a) working with fraction 9 of a peptic-tryptic-pancreatinic digest of wheat gliadin, who showed that this fraction was the only one of ten fractions, obtained by ion-exchange chromatography of the digest, to be incompletely digested by small intestinal mucosa from children with coeliac disease in remission. More complete digestion of all fractions was observed with mucosa from normal children.

Cornell and Townley (1974) also showed that fraction 9 was toxic *in vivo* to patients with coeliac disease, whereas the other fractions of the digest were nontoxic, thus giving greater credence to the enzymopathic hypothesis.

Although there is evidence for defective digestion of gluten and gliadin peptides in CD, no specific peptidase has been implicated. Lindberg et al. (1968) and Dolly and Fottrell (1969a), as well as other workers, have failed to detect any defective hydrolysis of dipeptides with small intestinal mucosa from coeliac patients in remission. This could be due to a number of reasons, e.g. the mucosa is replete with peptidases and other hydrolases, many of which may be present in the various subcellular organelles and thus escape detection; the substrates used may not have been relevant to the enzyme sought.

Peters et al. (1978a,b) used subcellular fractionation techniques to study enzyme activities in the enterocyte and certain enterocyte organelles. Although they found a decrease in β-glucosidase activity of the enterocyte brush border after good morphological recovery of patients on gluten-free diets, no other deficiencies were noted except in non-responsive patients. Andria et al. (1980) and Sjostrom et al. (1981) found an enterocyte brush border amino peptidase to be lowered in patients with CD in remission. However, none of these findings can be linked with the digestibility of a toxic peptide in the gliadin or gluten.

With regard to the type of defective hydrolase activity, it appears probable that the enzyme is a peptide hydrolase. Although early work by Phelan et al. (1977) suggested that a carbohydrase could reduce *in vivo* toxicity of gliadin, it was ruled unlikely that this was a major factor on the grounds that several toxic preparations of other workers, e.g. Bernardin et al. (1976), Jos et al. (1982) contained virtually no carbohydrate. More recently, synthetic peptides free from carbohydrate, have been shown to be toxic *in vitro* (Kocna et al., 1991; Troncone 1992) and *in vivo* (Mantzaris and Jewell, 1991; Sturgess et al., 1994).

It would be very enlightening to carry out digestion studies of toxic synthetic peptides using subcellular fractions of the mucosa. However, the major problem with this type of work is that one is never sure whether the enzyme being studied is depleted as a result of incomplete recovery of the tissue, (despite the indication from morphology and disaccharidase levels) or whether the data represent a genuine depletion.

Friis et al. (1992) instilled a peptic-tryptic digest of gliadin at the ligament of Treitz in patients with coeliac disease in remission and followed the uptake using immunofluorescence. They observed an intense, but transient, reaction

in biopsy specimens from the coeliac patients but not from the controls. The processing of gliadin peptides by the enterocyte in studies such as these is of fundamental importance.

Peters and Bjarnason (1984) make the point that transglutaminase, which is increased in biopsy specimens from patients with CD, may be involved in gluten binding to membrane components and could thus provide an alternative explanation to defective digestion.

(ii) The immunological hypothesis

This hypothesis is centred around the binding of gluten to the intestinal mucosa which then becomes a target for immunological reactions (Strober, 1976). So far, there is very little evidence that an abnormal immunological response to gluten is the basic cause of CD. Practically all of the evidence obtained has to do with mechanisms of pathogenesis. When gluten is withdrawn from the diet of patients with coeliac disease, most of the immunological phenomena normalise, together with the risk of malignancy (Holmes et al., 1989).

Electron microscopy showed that the first changes to small intestinal tissue occurred in the basement membrane about 2 hours after gluten challenge and became severe after 96 hours (Shmerling and Shiner, 1970). These changes probably represent both type III (Arthus) and type IV (cell-mediated) immunological responses. In similar studies, Booth et al. (1977) detected gross damage to the enterocytes by 24 hours and suggested that antibody-dependent cell-mediated reactions and direct cytotoxicity may be occurring.

Antigliadin antibodies of the IgA class are raised in serum of patients with CD but diminish after treatment with a gluten-free diet. Although IgG class antibodies are not of such consequence in diagnosis of CD as are IgA class antibodies, the assay of both together yields good sensitivity (96–100%) and specificity (96–97%) (Bürgin-Wolff et al., 1989; Gonzi et al., 1991). Antigliadin antibodies are produced at the site of tissue damage after gluten challenge (Falchuk and Strober, 1974) and could be a mediator of mechanisms of toxicity, perhaps through complement activation (Doe et al., 1973). However, levels of such antibodies return to normal when patients are placed on a gluten-free diet (Kenrick and Walker-Smith, 1970). Skerritt et al. (1991) showed that both adult and child coeliacs had elevated levels of serum antibodies (IgA, IgG) to the coeliac-toxic cereals (bread wheat, durum wheat, rye and barley) but low levels to oats, maize and rice. Again, these higher levels were diminished when patients were placed on a gluten-free diet.

Antireticulin antibodies are found in the plasma of almost all children with active CD (Seah et al., 1978). Deposition of IgA-containing substances on the basement membrane and reticulin of jejunal mucosa occurred when children with CD were challenged with gluten (Shiner and Ballard, 1972). More re-

cently, antiendomysial antibodies were found by Ferreira et al. (1992) in sera of all patients investigated with untreated CD and sub-total villous atrophy, indicating that this test has great value in screening for CD.

Ezeoke et al. (1974), have shown that serum from patients with CD can arm normal lymphocytes to become "cytotoxic effector cells". Thus there is the possibility of certain lymphocytes being "programmed" to attack the intestinal mucosa (Scott and Losowsky, 1976).

Further support comes from the observation by Simpson et al. (1983), that specimens of jejunal mucosa from patients with CD in remission were damaged to a greater extent when lymphocytes from patients with CD were added to the culture medium. A T-lymphocyte mediated response to gliadin is believed to be the reason for the mucosal damage (Ferguson and Murray, 1971), but no single process can account for the complex features of the acute disease (Marsh, 1981). Stimulation of proliferation of peripheral blood lymphocytes from patients with CD by crude fractions of gluten digests was found by Sikora et al. (1976) to be specific and valuable as a screening test for CD. However it was later shown by Cunningham-Rundles et al. (1978) that lymphocytes from normal donors, particularly those with HLA-B8 histocompatibility antigen, were also stimulated by a peptic-tryptic digest of wheat gluten. Furthermore, Frew et al. (1980) have shown that wheat gliadin preparations produced a proliferative response in lymphocytes from normal donors irrespective of their HLA class.

Despite the likelihood that these responses may not be related to the basic cause of CD, many recent studies have reaffirmed that cell-mediated mechanisms play a vital role in the pathogenesis. Studies have now extended to the role of mediators such as interleukins and interferons. Mowat (1989) has demonstrated the importance of γ-interferon in graft-versus-host reactions in the small intestine of mice. Anti-γ-interferon treated mice did not develop villous atrophy and severe crypt hyperplasia seen in the untreated hosts. Cornell et al. (1994) have shown that γ-interferon production in cultures of blood from adults with coeliac disease, in response to gliadin-derived peptides, was related to toxicity. However the active peptides also produced a response in blood from some normal individuals. The altered expression of MHC Class II molecules on the surface of epithelial cells is suggestive of ongoing cell-mediated reactions. Mothes et al. (1995) found that tryptic digested gliadin is able to stimulate the expression of these molecules on the surface of enterocytes. This stimulation occurred in the absence of lymphocytes, but needed the presence of γ-interferon (normally produced by the lymphocytes) in the culture medium.

Mäki et al. (1991) have identified and purified proteins that specifically bind to serum IgA from patients with CD. They have postulated that an autoimmune mechanism operates to damage tissue and that these proteins act as self-antigens. It thus seems that several mechanisms of pathogenesis are involved, none of which has been shown clearly to be the primary cause of the disease.

(iii) The membrane glycoprotein defect ("lectin") hypothesis

This hypothesis, proposed by Weiser and Douglas (1976), is that glycoproteins on intestinal epithelial cells of patients with CD may be abnormal and bind in a lectin-like way to components of gluten, producing damage to the cells. Douglas (1976) prepared a crude toxic fraction known as glyc-gli from gluten by acetic acid/ethanol/water extraction and found that it bound to mucosal homogenates of small intestine from patients with CD.

Lorenzsonn and Olsen (1982) found that some plant lectins such as wheat germ agglutinin or concanavalin A are able to damage rat intestinal epithelial cells. It appears that the preparation of Douglas contained wheat germ agglutinin, as does commercial dry gluten, and in all likelihood is the basis for the lectin-like activity of crude gluten preparations. However, quite apart from such cytotoxicity, Douglas (1976) found that glyc-gli showed increased lectin-like binding to preparations of intestinal cells from patients with CD compared with preparations from normals. Without the benefit of data with specific cell types, it is difficult to assess the value of these observations. Further studies by Kottgen et al. (1983) and by Rocca et al. (1983) seem to suggest that the pattern of damage to cells by lectin-like components does not agree with what is observed *in vivo* in CD.

(iv) The mucosal permeability defect hypothesis

Bjarnason and Peters (1983) first postulated that there may be a primary defect in the permeability of the intestinal mucosa in CD, thus increasing the chance of lysosomal disruption (causing the release of damaging proteases) and producing various immunological reactions arising from increased amounts of gliadin peptides. However, there is some doubt about the abnormal permeability being a primary defect as Hamilton et al. (1982) found that normal permeability was restored by treatment with a gluten-free diet.

3. Genetics

CD is transmitted in non-Mendelian manner and no single HLA gene marker has yet been found which is specific for the disease. McDonald et al. (1965) carried out family studies and suggested that CD might be inherited through a dominant gene of low penetrance. Similar studies by Rolles et al. (1974) also showed that first-degree relatives of the coeliac child have a very high incidence of the disease (5.5%). The incidence amongst the parents and siblings was somewhat similar (6.6% and 4.8% respectively) and 20% of the families contained another coeliac. The concordance rate of CD in monozygotic twins has been shown to be around 75% (Polanco et al., 1981).

Genes that map within the HLA D region appear to be of major importance in an individual's susceptibility to CD. The disease is strongly associated with class I HLA antigen B8 (Falchuk et al., 1972) and with class II HLA antigens DR3 and DR7 (De Marchi et al., 1979). However,

more recent evidence shows an even greater degree of association of the disease with DQw2, another class II antigen (Corazza et al., 1985; Sachs et al., 1986).

While CD is undoubtedly associated with certain HLA gene products, other factors must be involved to account for the fact that DQw2 is found in many individuals who do not develop CD. These factors may be different genetic factors, e.g. those coding for a specific peptidase or an abnormal immune response, or they may be environmental, such as dietary factors, viral infection, etc.

4. The nature of the toxins in wheat gluten

(i) Work with whole proteins

The composition of the causative agents is an extremely important aspect of research on CD which has not been investigated to the same extent as the pathogenetic mechanisms. Since Dicke (1950) first showed that wheat flour was responsible for the symptoms of CD, 45 years have passed without us having a precise knowledge of that component which is the most injurious one to those with the disease. Nevertheless, considerable progress has been made.

A significant discovery was that Van de Kamer and Weijers (1955) established that the gliadin group of wheat proteins was more toxic than the gluten in group. Gliadin is a complex mixture of proteins, associated with small amounts of carbohydrates and lipids. Wrigley and Shepherd (1973) found 46 components in gliadin from one variety of flour. Different varieties of wheat yield gliadins which vary in molecular size, isoelectric point and electrophoretic mobility, the latter being used to define the four major groups, α-, β-, γ- and ω-gliadins (Woychik et al., 1961).

Ion exchange chromatography has also been employed to separate gliadin proteins. Kendall et al. (1972) used carboxymethyl cellulose to prepare crude gliadin fractions for *in vivo* tests of toxicity and found one fraction to be more toxic than the others. However, this toxic fraction was not characterised by electrophoresis. Since then it has been demonstrated by Ciclitira et al. (1984) that all four major electrophoretic fractions of gliadin are toxic to patients with CD. These workers instilled fractions prepared by ion-exchange chromatography directly into the small bowel and followed this by examination of biopsy specimens.

Bernardin et al. (1967) made a very significant advance with their development of a method for the isolation of α-gliadins using a method based on aggregation. A fraction produced, called A-gliadin-to distinguish it from electrophoretic fractions – was shown to be toxic by direct instillation (Hekkens et al., 1970). Kasarda (1978), working with wheat cultivars (nullisomic 6A wheats) in which α-gliadin was at very low levels, found that these wheats were still toxic to patients with CD. This is what could have been expected, had the results of Ciclitira et al. (1984) been known at the time.

Although the different gliadins are separable by electrophoresis, sequencing studies by Kasarda et al. (1984) suggested that α- and β-gliadins

form a single multigene family. These studies also paved the way for important studies of sections of the A-gliadin molecule.

Amino acid sequences in peptides derived from A-gliadin by cyanogen bromide cleavage and chymotryptic digestion (De Ritis et al., 1988) showed that certain common sequences were present in peptides found to be toxic *in vitro*. These sequences, PSQQ and QQQP, were absent from the non-toxic peptides, suggesting that these motifs represented problems for digestion by coeliac mucosa or were epitopes for immunological reactions leading to damage of the mucosa.

(ii) Work with protein digests

Bronstein et al. (1966) have shown that low molecular weight digests of gliadin prepared by successive digestion using pepsin, trypsin and pancreatin (PTP digest) are toxic to patients with CD. The peptides contained are of similar size to those presented to the duodenum *in vivo*. It is important to focus on the smallest peptides which are toxic and furthermore such peptides are more soluble in aqueous solutions than is whole gliadin. Enzymic digests offer greater opportunities for separation of more chemically distinct species than the latter, the major electrophoretic fractions of which are all toxic (Ciclitira et al., 1984).

Cornell and Townley (1973a) found that the ten fractions obtained by ion exchange chromatography of the PTP digest were quite different in amino acid composition. Furthermore, fraction 9 was only partially digested by homogenates of coeliac mucosa from children with CD in remission, whereas the other fractions were almost completely digested. All fractions were virtually completely digested by homogenates of mucosa from children without this disorder. Fraction 9 is a mixture of peptides of average molecular weight 1,500 daltons and represents about 5% of the gliadin.

Further work with fraction 9 showed that:

(a) Undigested material from incubation of fraction 9 with remission coeliac small intestinal mucosa retained appreciable toxicity to lysosomes (Cornell and Townley, 1973b) and to foetal rat intestinal mucosa (Cornell et al., 1988).
(b) Fraction 9 prevents morphological improvement to atrophic small intestinal mucosa in organ culture, unlike the other fractions of the digest (Townley et al., 1973).
(c) Titres of circulating antibodies in patients with active CD were highest to fraction 9, compared with the other fractions (Cornell, 1974).
(d) Fraction 9, but not the other pooled fractions, produced malabsorption of xylose in children with CD in remission (Cornell and Townley, 1974).
(e) Fraction 9 caused damage to a wide range of cells in culture, including normal human embryonic intestinal cells, whereas the other major fractions did not (Hudson et al., 1976).

Fraction 9 was subjected to reversed-phase HPLC on C_{18} columns and the sub-fractions analysed (Cornell et al., 1992). The principal peptide

was found to be a dodecapeptide corresponding to residues 75–86 of A-gliadin (RPQQPYPQPQPQ), which contains the QQPY motif. This peptide is active *in vitro* (see section 6). Other sub-fractions rich in serine were also identified and shown to have activity to lysosomes.

Wieser et al. (1984) used peptic-tryptic digestion of gliadin for their studies. Their peptide B3142, isolated from peptic-tryptic digests by HPLC, proved to be active *in vitro*. This peptide was cleaved by α-chymotrypsin into two peptides, both of which were active *in vitro* (Wieser et al., 1986). Their sequences corresponded to residues 3–24 (CT-1) and 25–55 (CT-2) of A-gliadin. Peptide CT-1 contains the motifs PSQQ and QQQP (overlapping) whilst CT-2 contains QQQP, QQPY and PSQQ overlapping with another QQPY.

5. The nature of residual peptides from coeliac mucosal digestion

Cornell and Townley (1973a) digested fraction 9 with small intestinal mucosa from children with coeliac disease in remission and isolated undigested peptides from the digest by ultrafiltration, electrophoresis and extraction. The amounts of these peptides were considerably higher than those obtained from digestion of fraction 9 with normal mucosa and they contained glutamine/glutamic acid, proline and serine as the major amino acids.

Further fractionation of fraction 9 on QAE Sephadex A-25 yielded a further five sub-fractions of which the first two (9-1 and 9-2) were shown to be the most difficult to digest and found to be toxic *in vivo* (Cornell and Maxwell, 1982).

Further characterisation of the peptides remaining from coeliac mucosal digestion of the more cytotoxic sub-fraction 9-2 was carried out by chromatography on S.P. Sephadex – C25, Ultrogel AcA 202 and C_{18} columns (Cornell, 1988). Peptides were screened for activity by their action on rat liver lysosomes as this assay utilises only small quantities of peptides. The results of amino acid analysis found glutamine/glutamic acid, proline, serine and tyrosine as the major amino acids in the peptides, which seemed to be of average molecular weight 700 daltons. It was postulated that these peptides might contain the motifs PSQQ and QQQP, found to be present in active peptides by De Ritis et al. (1988) and YPQPQ, a peptide with opioid activity in coeliac patients (Graf et al., 1987). Another motif, QQPY, which occurs three times in the A-gliadin structure, was also considered. QQPY also occurs in rye and barley prolamins.

6. Work with synthetic peptides

A major break-through in attempts to determine the nature of the causative agent in CD was the work with synthetic peptides. One of the early papers using this approach was that of Kocna et al. (1991) where peptides within the A-gliadin sequence 8–19, which contains the PSQQ and QQQP motifs, were

tested in the foetal chick assay of Mothes et al. (1985). Substitutions of amino acids in these sequences indicated that the QQQP motif was the more important factor in toxicity. However, Cornell and Mothes (1993) showed that the peptide 13–18, containing both these motifs, was inactive in this assay. Thus flanking amino acids play a vital role in the conferring of activity.

The peptide 75–86 of A-gliadin, corresponding to the principal peptide in Cornell's fraction 9, contains the QQPY motif (also found at residues 40–43 and 52–55 of A-gliadin) and was found to be active (Cornell and Mothes, 1993). It contains the pentapeptide YPQPQ, which was shown by Graf et al. (1987) to have activity in an LIF assay. The relationship between this latter activity and toxicity is not clear, but it is interesting that, like fraction 9 and its two sub-fractions 9-1 and 9-2, peptide 75–86 caused the production of high amounts of γ-interferon in cultures of blood from adult coeliacs and some normal adults (Cornell et al., 1994). Fractions and peptides which were not active in the chick assay did not produce significant amounts of γ-interferon. Experiments of this type are helping to establish the relationship between toxicity and immunological activity of synthetic peptides.

The high activity of fraction 9 in the foetal chick assay and the analysis of undigested residues from mucosal digestion suggested that a serine-containing peptide was also present. Amino acid analysis indicated that a peptide containing glutamine/glutamic acid, proline, serine, asparagine, valine and leucine was present, especially like those near the N-terminus of A-gliadin, but a little larger than the active peptide 8–19 of Kocna et al. (1991). Cornell and Mothes (1995) confirmed the activity of this latter peptide and also showed that synthetic peptides 9–19 and 11–19 were active in the foetal chick assay, with the activity increasing from the dodecapeptide to the undecapeptide to the nonapeptide. The latter contains the QQQP motif combined with the PSQQ motif and has the sequence QNPSQQQPQ. It is contained within the active peptides CT-1 (3–24) of Wieser et al. (1986) and XT (1–30) of De Ritis et al. (1988). The activities of the octapeptides 12–19 and 11–18 were less than that of the nonapeptide 11–19. Interestingly, Sturgess et al. (1994) found that the larger peptide 3–21 was not active in an intraduodenal challenge, which is surprising in view of the results above on peptide 3–24.

The PSQQ motif is also found between residues 50–53 and 213–216 of A-gliadin. Although Kocna et al. (1991) found activity in peptide 45–56, Troncone (1992) reported low activity in peptide 44–55 and Cornell and Mothes (1993) reported negligible activity in peptide 46–57. As all peptides contain the PSQQ and QQPY motifs (overlapping) we must assume that the motifs by themselves are not responsible for toxicity but that the flanking amino acids have an overriding influence. One aspect of this may be the N-terminal amino acid; N-terminal glutamine may be more important than N-terminal proline, reviving an old hypothesis by Messer et al. (1964) who believed that certain N-glutaminyl peptides were responsible for toxicity.

The PSQQ residues 213–216 forms part of an N-glutaminyl peptide 208–219, found by Kocna et al. (1991) to be active *in vitro*. Cornell and Mothes (1993) showed that peptide 213–227 was of low activity *in vitro*, whilst

Mantzaris and Jewell (1991) showed that 206–217 was active *in vivo*. However, the N-terminal leucine and then the glycine could be readily split off *in vivo* by aminopeptidases exposing the small bowel to the active peptide 208–217. Hence it is important, in studies of this type, to determine the smallest peptide that is toxic and to make comparisons of the levels of toxicity.

The other interesting area of A-gliadin is the one corresponding to the active peptide XT (31–55), derived from native gliadin (De Ritis et al., 1988). Working with the peptide 31–55, Troncone (1992) has suggested that residues 31–43 comprise the most active part. It contains the QQQP motif as well as the QQPY, whereas the 44–55 section contains the motifs PSQQ combined with QQPY as PSQQPY. Peptide 31–43, of sequence LGQQQPFPPQQPY, inhibits the increase in enterocyte height of atrophic coeliac mucosa in organ culture, unlike peptide 44–55. Ellis et al. (1992), have suggested that an antibody WC2, which recognises coeliac-toxic cereals, binds in the region of residue 36, a proline residue, where there may be an antigenic β-reverse turn.

Peptide 31–43 is contained within the peptide 31–49 which Sturgess et al. (1994) found to be toxic *in vivo*. These results are understandable. However, they found only minor histological changes in one of the four coeliac patients with peptide 202–220, which contains the peptide 206–217, found to be active *in vivo* by Mantzaris and Jewell (1991). This puzzling result suggests a variable response in coeliac patients and confirms the need for studies with more closely related peptides.

The approach of using synthetic peptides based on the A-gliadin structure has so far given some very interesting and meaningful results. Further work in this area using *in vitro* techniques based on organ culture, followed up by instillation *in vivo* promises to yield information on the most active fragments of A-gliadin and the reasons for this activity.

7. Assays for activity of peptides

(i) Organ culture assays

Assays of *in vitro* activity vary from those designed to test direct cytotoxicity or immunological activity through to those which seem to depend upon several pathogenetic mechanisms. In the latter category, those which use tissue from coeliac patients with active disease, where the toxicity is measured by failure of the mucosa to recover in the presence of low concentrations of peptide (0.1–0.5 mg/mL of medium) are more specific than those which are animal models of the disease (e.g. foetal rat and foetal chick mucosa), although the latter are excellent as screening assays. Assays which use tissue from coeliac patients in remission have recently been reported (Fais et al., 1992; Maiuri et al., 1994) and focus attention on the phenomena which occur when coeliac patients are challenged by gluten, such as the expression of adhesion molecules.

It has been possible to make several important conclusions on the basis of the *in vitro* assays. Townley et al. (1973) studied the toxicity of a number of

fractions from ion-exchange of a peptic-tryptic-pancreatinic digest of wheat gliadin and found that Cornell's fraction 9 consistently prevented morphological improvement, assessed by electron microscopy, of atrophic coeliac mucosa in culture. Some damage to control tissue was noted, but the other fractions of the digest all allowed recovery of the coeliac mucosa and did not damage any of the control tissue. De Ritis et al. (1979) confirmed that fraction 9 was very active in inhibiting *in vitro* development and morphogenesis of small intestine from 17-and 18-day rat foetuses and thus showed that gliadin is intrinsically toxic to the developing enterocyte, although this is not necessarily the case for mature intestinal epithelial cells.

Jos et al. (1975) using jejunum from patients with active CD, showed that the noxious effects of peptic-tryptic digests of gliadin were also observed with digests of casein. These results indicated that there is a need to further investigate the use of tissue from patients in remission in order to obtain coeliac-specific toxicity data. Although assays based on the two main animal models may be criticised because they are not directly related to CD, they provide an excellent basis for screening peptides for activity in CD as the results are very reproducible and the assays require only small quantities of peptides. Auricchio's group, using the foetal rat intestinal assay, found that activity was present in one of the sub-fractions of fraction 9 after digestion by remission coeliac mucosa, but not after digestion by normal mucosa (Cornell et al., 1988). This shows that the assay is able to detect specific differences between the products of digestion of toxic fractions by coeliac mucosa and normal mucosa and points to a mucosal defect in CD.

The foetal chick assay (Mothes et al., 1985) is a less costly assay than the foetal rat assay and makes use of changes in certain biochemical parameters with sucrase activity, in particular, being a very useful indicator of changes in the tissue. Like the rat assay, there is probably less dependence upon immunologically mediated reactions with the foetal chick assay. It has been particularly useful in detecting differences in activity between closely related peptides, which would be difficult *in vivo*, due to the action of larger amounts of digestive enzymes.

(ii) Immunological assays

There is need to study activity of this type in CD, particularly in regard to understanding mechanisms of pathogenesis. However, there is still the possibility that an abnormal immunological reaction may be the basic cause of the disease. It is known that the cell-mediated immune response to gluten proteins plays a role in pathogenesis of CD.

Leucocyte migration inhibition factor (LIF) (Ashkenazi et al., 1980) and macrophage pro-coagulation activity (Devery et al., 1991) are useful indicators of mediators released by these cells on contact with certain gluten components.

Devery et al. (1991) using an indirect LIF assay and a macrophage procoagulant assay, found that synthetic peptides in α/β gliadin molecules located in the proline-rich domain (near the N-terminus) were the most active

of those tested. Their peptide A contained the PSQQ and QQQP motifs located at residues 13–16 and 15–18 respectively and their peptide B the PSQQ and QQPY motifs located at residues 50–53 and 52–55 respectively. Using the indirect LIF assay; Penttila et al. (1991) found no response to wheat protein fractions in control subjects compared with the high response in coeliac patients.

(iii) In vivo assays

After appropriate *in vitro* assays have been performed, confirmation of toxicity by direct instillation *in vivo* can then be used to define the smallest part(s) of the A-gliadin molecule which damage(s) the small intestine. Some will question the ethics of this procedure without *in vitro* evidence of activity.

8. Conclusions

Although the aetiology of CD remains unclear, the enzymopathic and immunological hypotheses remain the most tenable based on evidence to date. There is a need to separate the aetiology from the pathogenesis of the disease, particulalry as far as the immunological data are concerned as there is no doubt that much of these data relate to possible pathogenetic mechanisms. The main question to be answered is whether CD is the result of a defect in mucosal digestion of peptides which are known to damage the small intestine or whether it is the result of an abnormal immune response.

There is evidence of direct cytotoxicy in CD, which is in agreement with the effects of undigested peptides on organelles such as lysosomes and on undifferentiated cells. However, the contribution of various concomitant immunological mechanisms makes it very difficult to discern the relative contributions to damage without a thorough study of the effects on the various cellular and sub-cellular components in isolation. There is evidence that these mechanisms depend upon other factors, many of which could be studied using *in vitro* systems.

The most promising new *in vitro* systems are the ones in which remission coeliac mucosa is used instead of atrophic mucosa. These systems will help us to better understand the factors involved in the damage and whether the underlying mechanisms are of a primary or secondary nature.

Progess with such new assays and the use of synthetic peptides promise to elucidate the structures of the peptides which go to make up the A-gliadin structure. So far, the use of assays with foetal rat and foetal chick intestine in culture have served us well in screening for activity of peptides. When coupled with the use of human coeliac mucosal cultures, these techniques have focused attention on certain sections of the A-gliadin structure which seem to relate to toxicity in CD. Experiments using small synthetic peptides are helping to define the importance of key motifs to their toxicity. These peptides may differ in their mode of action as to whether they are cytotoxic and/or

immunogenic. They thus provide an important impetus for a break-through in the understanding of this fascinating, but troublesome, disease.

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Author's address: Assoc. Prof. H. J. Cornell, Royal Melbourne Institute of Technology, Melbourne, Australia.

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