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Further evidence of a primary mucosal defect in coeliac disease

In vitro mucosal digestion studies in coeliac patients in remission, their relatives, and control subjects

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SUMMARY Subfractions of fraction 9, obtained from a peptic-tryptic-pancreatinic digest of wheat gliadin, were subjected to *in vitro* mucosal digestion and the filtrates examined for residual peptides. Small-intestinal mucosa from four groups of individuals were studied—eight patients with coeliac disease in remission; eight healthy controls; nine first degree relatives of patients with coeliac disease, and six children with recurrent diarrhoea investigated for possible coeliac disease, but in whom the diagnosis was excluded. The highest amounts of residual peptides (measured by scanning densitometer) were detected after digestion with mucosa from patients with coeliac disease and the lowest amounts with the control groups. The results obtained with the group of relatives fell between those of the coeliac disease and control groups, while the recurrent diarrhoea group overlapped the relatives and controls. The residual peptides were derived chiefly from the B-type subfractions of subfractions 1 and 2, obtained by ion-exchange chromatography of fraction 9. These subfractions are rich in glutamine/glutamic acid and proline and have a molecular weight (apparent) of not greater than 1500 Daltons. The results lend further support to the hypothesis of an enzyme deficiency in coeliac disease. A partial enzyme deficiency may exist in some first-degree relatives and in some children with recurrent diarrhoea but with histology of the small intestine within normal limits. HLA-B8 antigen is not correlated with this deficiency, but, when the two factors are associated, they could be related to the manifestation and severity of coeliac disease.

The demonstration that an ultrafiltrate of a peptic-tryptic-pancreatinic digest of wheat gliadin was toxic to patients with coeliac disease (Bronstein *et al.*, 1966) has led to mucosal digestion studies *in vitro* on fractions of this digest in an attempt to define the toxic substances (Cornell and Townley, 1973a). These latter studies have pointed to the possibility of an enzyme deficiency in the small intestine of patients with coeliac disease and this is linked to evidence that fraction 9 of this digest contains a large proportion of the toxic components of gluten. This is supported by the results of similar experiments with rye gliadin and by the ability of fraction 9 to cause significant reduction in D-xylose absorption in coeliac patients in remission (Cornell and Townley, 1974). Organ culture of small intestinal mucosa (Townley *et al.*, 1973) provides further evidence that fraction 9 contains the causative agents in coeliac disease.

Further mucosal digestion studies on the purified components of fraction 9 could therefore lead to a knowledge of their basic chemical structure and to the development of a more sensitive tool to detect subtle variations in the ability of mucosal specimens to digest these components. Hence, subfractions of fraction 9 were tested with small bowel mucosae of coeliac patients in remission and control subjects, and also of first-degree relatives of coeliac patients and a group of children who were thought to have coeliac disease, but in whom the diagnosis was positively excluded.

Bearing in mind the association between the HLA-B8 antigen and coeliac disease (Falchuk *et al.*, 1972; Stokes *et al.*, 1972), it was of interest to see whether there was also an association between the HLA-B8 antigen and the inability of mucosa completely to digest certain subfractions of fraction 9. Such data could thus provide the basis for a hypo-

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thesis of the aetiology of the disease which might reconcile the evidence for the biochemical and immunological abnormalities in patients with this condition.

Methods

PREPARATION OF SUBFRACTIONS OF FRACTION 9

Subfractions of fraction 9 were prepared as previously described (Cornell and Townley, 1973b), except that a larger column of QAE Sephadex was used (23 × 1·4 cm) to which was applied about 400 mg of fraction 9.

Subfractions 1 and 2 (150 mg each) were chromatographed on a column of SP Sephadex-C25 (Pharmacia Fine Chemicals, Uppsala, Sweden), 15 × 1 cm, equilibrated in a pH 3·6 tris-citrate buffer (0·01 M with respect to tris) and run at 28 ml/cm²/h. After collection of the unabsorbed fraction (subfraction A) subfraction B was obtained by application of a tris-citrate buffer pH 6 (0·02 M with respect to tris) and subfraction C by application of a pH 8 buffer of the same type. Lyophilised subfractions were desalted on 25 × 0·9 cm columns of Biogel P2, 200-400 mesh (Bio-Rad Laboratories, California, USA) and again lyophilised. Amide and total nitrogen contents were determined on each sample as previously described (Cornell and Townley, 1973a). Finally, portions of the B-type subfractions (15 mg) from subfractions 1 and 2 were purified on columns of Sephadex G50—fine (45 × 1 cm) in 0·05 M ammonium carbonate buffer and lyophilised. Estimates of molecular size were obtained by the method of Andrews (1964) using Dextran Blue 2,000, cytochrome C, insulin, glucagon, bacitracin, and glutathione as standards. The chromatographic scheme is shown in Fig. 1. Amino acid analyses of the important subfractions were carried out and determinations of the N-terminal amino acids were made *via* their dansyl derivatives as described by Smith (1969).

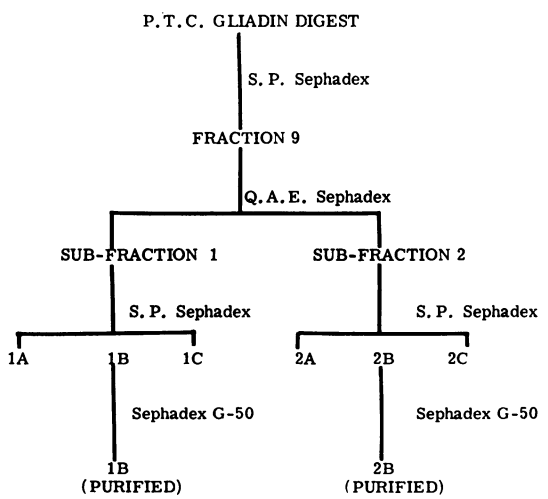

Fig. 1 *Chromatography of toxic peptides present in peptic-tryptic-pancreatinic (PTC) gliadin digest.*

SMALL INTESTINAL BIOPSY SPECIMENS

Peroral biopsy specimens were taken from the area of the duodenal-jejunal junction using a paediatric (Watson) capsule both in adults and children. Specimens were sealed in Parafilm and foil, immediately frozen to −20°C, and stored at this temperature until ready for use (maximum period three weeks). Biopsy material was obtained from 31 individuals derived from four groups.

PATIENTS

Group A (eight children with coeliac disease in remission)

These children were all clinically well and on a gluten-free diet. After the biopsy procedure involved in this study, they were challenged with gluten and subsequent mucosal specimens displayed subtotal villous atrophy. All biopsy specimens obtained in remission were indistinguishable from normal on light microscopy. These gluten-challenged children have been described elsewhere (Rolles and McNeish, 1976).

Group B (nine first-degree relatives of patients with proven coeliac disease)

These relatives (two adults and seven children) were all on a normal gluten-containing diet and mucosal histology was normal by light microscopy. Three of the children (12, 14, 15) were alleged to have symptoms with gluten, including diarrhoea and abdominal discomfort. These children were challenged with added gluten (20 g/day) with exacerbation of symptoms, but follow-up investigations including repeated biopsy on all three were still normal.

Group C (six children in whom coeliac disease had been considered but in whom no histological abnormality was found in the small bowel)

Four of these children who had a negative response to gluten challenge have been described elsewhere (Rolles and McNeish, 1976).

Patient number 18 had dermatitis herpetiformis and minimal non-specific mucosal changes following gluten challenge. Her biopsy specimen, used in this study, was histologically normal.

Group D (eight control subjects)

These included six children who had no evidence

clinically or biochemically of malabsorption or small bowel disease and who had a jejunal biopsy as part of a work-up for failure to thrive. In none was any abnormality found and 'failure to thrive' was attributed to constitutional and emotional factors. Two healthy young adult volunteers were also used.

HLA TYPINGS

HLA typings were performed on 24 of the subjects including some from each of the four groups.

MUCOSAL DIGESTION EXPERIMENTS

Biopsy specimens were homogenised, incubated with subfractions of fraction 9 and portions of the filtrates subjected to high voltage electrophoresis at pH 1·9 as previously described (Cornell and Townley, 1973a). A ratio of 0·45:1 of mucosal protein (Lowry *et al.*, 1951) to substrate was employed for all incubations and the portion of filtrate applied contained 120 μg of subfraction in all cases.

Densitometric scans were then made on all strips cut from electrophoretic runs. These scans utilised a Joyce-Loebel Chromoscan (Newcastle upon Tyne, England) and were carried out on duplicate strips. Particular attention was paid to the region between the pyroglutamic acid and glutamic acid markers. The area under the curve between these two positions was estimated and used as a means of measurement.

Experiments were run in batches; in each batch one or more control specimens were tested along with a mucosal sample from group A and one or more samples from groups B and C. Results were then expressed as a ratio of the area under the curve of the test sample to the area under the curve of the control sample. With subfractions 1 and 2 there was sufficient material to run duplicates in all but four experiments. Because of the small amount of B-type subfraction available, only single experiments were performed using four coeliac and four control mucosal samples and one sample from a first-degree relative.

Results

YIELDS OF FRACTION 9 AND ITS MAJOR SUBFRACTIONS

Fraction 9 constitutes about 8% of the gliadin nitrogen usually applied (1·1 g) to the SP Sephadex column. After desalting, the actual yield of this fraction is normally about 5% of the gliadin. Rechromatography of fraction 9 on QAE Sephadex gave the major subfractions (1 and 2) in combined actual yield of 2% of the original gliadin. Type-B and type-C subfractions were the major ones obtained by further purification on SP Sephadex and each was obtained in similar yield (about 0·3% of the original gliadin).

Purification of the B-type subfractions of subfractions 1 and 2 on Sephadex G50 revealed a major peak in each, corresponding to an apparent molecular weight of 1400-1500 Daltons, well separated from other minor peaks.

AMINO ACID ANALYSIS OF FRACTION 9 AND ITS MAJOR SUBFRACTIONS

Results are shown in Tables 1 and 2. Subfractions 1 and 2 are particularly rich in glutamine/glutamic acid and proline, as are also subfractions 1A and 1B.

Table 1 *Major amino acids of fraction 9 and its major subfractions (from QAE Sephadex)**

	Fr 9	*Subfr 1*	*Subfr 2*	*Subfr 3*
Asp				5·4
Thr				4·8
Ser	5·9	4·0	5·4	6·3
Glu	30·7	41·7	32·5	27·6
Pro	15·0	20·6	18·7	13·4
Ala	4·2			6·7
Val	5·9		5·4	7·6
Ile	5·2		4·3	5·5
Leu	7·1	4·8	6·0	6·3
Phe		4·0		
His	6·6	4·3	5·6	

*Figures are molar percentages.
For brevity, only those amino acids present at 4% or greater are shown.

Table 2 *Major amino acids of subfractions of fraction 9 (from QAE Sephadex and SP Sephadex)**

	1A	*1B*	*1C*	*2A*	*2B*	*2C*
Asp						4·2
Ser		5·9	5·6	6·0	6·1	6·1
Glu	44·6	45·0	28·2	31·5	35·7	22·9
Pro	28·7	24·0	12·4	14·9	19·4	11·6
Gly		4·8			7·3	4·0
Ala			5·4	4·6	5·8	8·3
½ Cys			4·5			
Val			6·6	6·9		7·2
Ile			4·7			5·0
Leu			9·4	6·8	5·2	9·1
Phe	6·9	7·0				
Arg						4·5

*Figures are molar percentages.
For brevity, only those amino acids present at 4% or greater are shown.

MUCOSAL DIGESTION EXPERIMENTS: ELECTROPHORETIC PATTERNS

1. *Comparisons of subfractions digested with mucosa from remission coeliacs and controls.*

There were appreciable amounts of undigested peptides remaining from remission coeliac mucosal digestion of subfractions 1 and 2, contrasting with lower amounts with subfractions 3, 4, and 5.

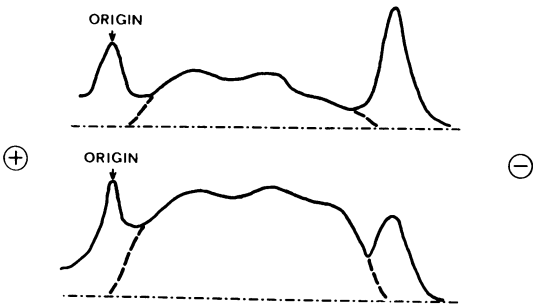

Fig. 2 *Scanning densitometer profiles after electrophoresis of digests of subfractions 1 and 2 (pooled). Digests prepared using homogenates from coeliac patients in remission* (bottom) *and control subjects* (top). *Estimates of area corresponding to peptides provided by extension of relevant profiles to baselines. Amino acids of higher mobility than glutamic acid are not shown.*

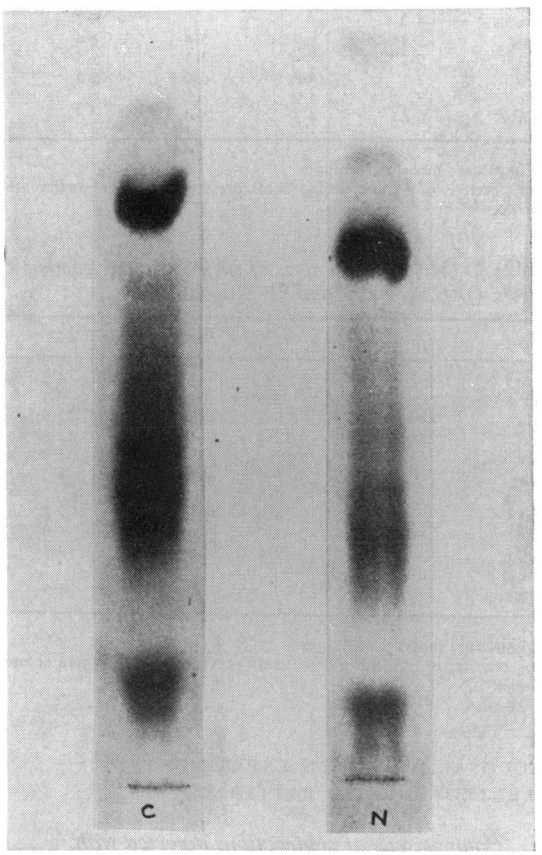

Fig. 3 *HVE of mucosal digests of subfraction 2B with coeliac (C) and control (N) mucosa. (Buffer: acetic acid—formic acid—water (pH 1·9). Voltage: 60 V/cm. Time: 40 minutes. Paper: Whatman 3MM. Stain: KI-starch after chlorination.)*

There were correspondingly lower amounts of undigested material from the controls with subfractions 1, 2, and 3, whereas subfractions 4 and 5 were equally well digested by both coeliac and control mucosa. Scans of digestions of subfractions 1 and 2 (pooled) by mucosa from four coeliac children in remission compared with mucosa from five controls showed that there was almost twice the amount of residue remaining from the coeliac digestions. (The means were 27·7 cm^2 and 14·7 cm^2 respectively). This was a highly significant difference ($P < 0.001$ by Student's t test on original areas). Figure 2 shows the scan profile obtained with a coeliac digestion compared with that of a control digestion. Residues obtained from subfractions 4 and 5 (pooled) were much less than those from 1 and 2, and there was no significant difference between coeliacs and controls.

B-type subfractions obtained from subfractions 1 and 2 were less completely digested by remission coeliac mucosa than by the controls. The most marked difference was seen with subfraction 2B (Fig. 3).

2. *Comparison of mucosal digestions from clinical groups using selected subfractions*

The subfractions selected for these experiments were those which showed the greatest differences between digestion by mucosa from coeliacs in remission and controls in terms of residual material. These were subfractions 1 and 2 (pooled) and the B-type subfractions.

Figure 4 shows the relative amounts of residual material (expressed as a ratio compared with controls) obtained from scans of mucosal digests subjected to electrophoresis. The high ratios obtained with the remission coeliacs (group A) contrast with the controls (group D) as previously described. Between these two extremes were the first-degree relatives, group B (mean 20·6 cm^2 with subfractions 1 + 2) in which results were sometimes nearer to those of the coeliacs than the controls. The relatives were found to be significantly higher than the latter ($P < 0.005$), while the patients with recurrent diarrhoea (group C) overlapped the relatives and the controls. There were subjects of both HLA-B8 and non-HLA-B8 typing in all groups, but there was no discernible correlation between HLA typing and the ability to digest the subfractions.

N-TERMINAL AMINO ACIDS OF B-TYPE SUBFRACTIONS

Glutamine/glutamic acid was the major N-terminal amino acid in both subfractions 1B and 2B. The high amide nitrogen content of these subfractions (35% and 30% of the total nitrogen, respectively), suggests that both are rich in glutamine, but there is

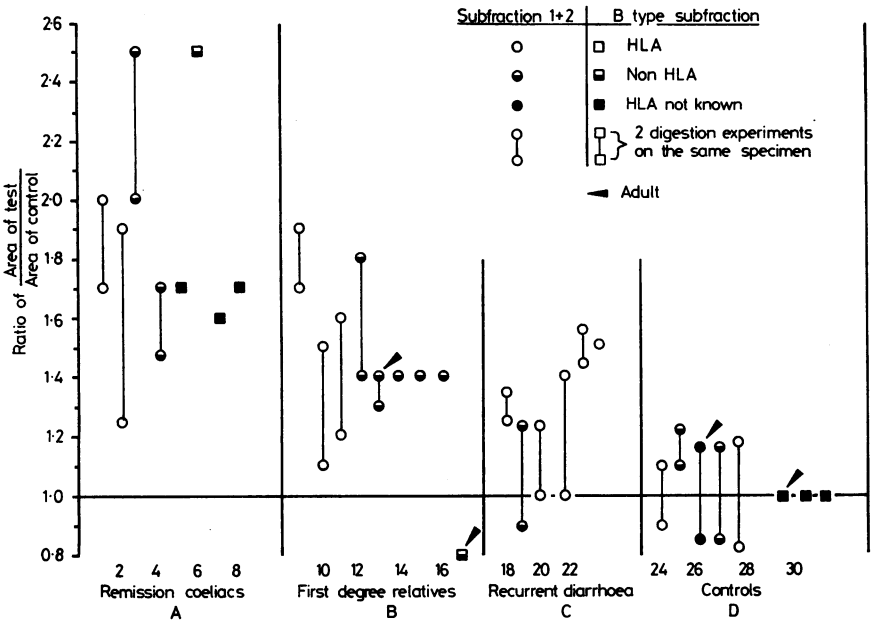

Fig. 4 *Mucosal digestion studies on clinical groups. Residual peptides from selected subfractions after electrophoresis. Subjects have been assigned the numbers 1-31, but for clarity only even numbers are shown.*

doubt as to whether the N-terminus is glutamic acid or glutamine.

Discussion

From this study, two important points arise. Firstly, that mucosal digestion studies are meaningful for testing subfractions of gluten in studies of the aetiology of coeliac disease. Secondly, by means of the purified fractions employed, it has now been demonstrated, in a direct manner, that some of the relatives of coeliac patients have a partial deficiency in their ability to digest these fractions. Indirectly, an abnormality had already been indicated (Cornell, 1974). Thus, in some of these relatives, there appear to be mucosal biochemical features which have more in common with remission coeliac mucosa than with normal control mucosa.

Toxic subfractions of gluten

Progress in the identification of the toxic components of gliadin has been hampered by the complexity of this enzymic digest. However, such digests are near to the form in which gliadin is present in the upper small intestine, and there is interest in the smallest molecular fragment of gliadin produced under physiological conditions that is still toxic to the coeliac mucosae.

The separation of peptide-containing residues (which give discernible electrophoretic bands) from remission coeliac mucosal digests of certain subfractions of this digest suggests a specific enzyme deficiency in the small intestinal mucosa of individuals with this disease, supporting our previous findings (Cornell and Townley, 1973a, b, 1974; Townley *et al.*, 1973). Peptides present in subfractions 1 and 2 (of fraction 9), and in the B-type subfractions of these, appear to be of greatest importance to these studies. Although subfractions 1B and 2B were each obtained in yields corresponding to about 0·3% of the original gliadin, their true content could be about one order of magnitude higher because of losses on the ion exchangers and desalting columns. Obviously, confirmation of toxicity of these subfractions of fraction 9 in patients will be necessary in support of our contention that they represent the polypeptides implicated in coeliac disease.

Hypothesis of aetiology of coeliac disease

In recent years, there has been an increasing tendency towards an immunological argument for coeliac disease (reviewed by Asquith, 1975). A new look at skin tests (Anand *et al.*, 1977) and *in vitro* activation of peripheral-blood lymphocytes (Sikora *et al.*, 1976) with gluten subfractions may lead to a better understanding of the relationship between antigenicity and clinical toxicity and of the possible role of lymphocyte sensitisation in the pathogenesis of coeliac disease. However, to view these and other data from coeliac research as supporting one of the

two major hypotheses of aetiology is probably an oversimplification. It had become increasingly apparent during these studies that the evidence on both sides was complementary. It was interesting to note that Weiser and Douglas (1976) postulated an alternative mechanism for gluten toxicity, in which they regarded gluten as acting as a lectin. This hypothesis was entirely compatible with the work which showed that certain gluten subfractions were cytotoxic in different degrees to a variety of cells in tissue culture (Hudson *et al.*, 1976). Based on the evidence to date, a hypothesis can now be outlined as follows:

1. There is in gluten a substance (perhaps a protein or substance acting as a lectin) which may have non-specific cytotoxicity. There is evidence that low molecular weight enzymic digests of gliadin are still toxic to individuals with coeliac disease, suggesting that fragments of this substance are involved.

2. Normal human mucosa is in some way resistant to this cytotoxic effect, possibly because of some surface factor, or, more likely, because it contains an enzyme (but not necessarily a peptidase) which can digest the toxic substance and thus render it harmless.

3. Some individuals have a genetically determined lack of the protective local factor, thus making their intact mucosa susceptible to the cytotoxic effect of ingested gluten. This effect would be dose-dependent and might produce local symptomatic changes. It was noted that three of the first-degree relatives studied in this project had marked gastrointestinal symptoms when added gluten was ingested, although their mucosae were histologically normal.

4. Another genetically determined factor, which is independent of the preceding one, is an immunological predisposition to produce an inappropriate response to the toxic fraction. This factor is the one associated with HLA-B8 or, as more recently described, HLA-DW3 (Keuning *et al.*, 1976). This is also the factor which has a genetic association with other immunological conditions.

5. Individuals with bowel diseases that disrupt the small intestinal mucosa may still develop adverse reactions to gluten (Weiser and Douglas, 1976). Individuals with the immunological predisposition but with the normal complement of mucosal surface protective factor could develop gluten intolerance following damaging factors such as gastroenteritis. However, after full recovery on a gluten-free diet, they might be able to tolerate gluten providing their mucosa remained intact. Some cases of so-called 'temporary gluten intolerance' might come from this group (McNeish *et al.*, 1976).

6. Individuals having both the immunological predisposition and the mucosal defect could have symptoms varying considerably in severity depending upon the balance of the two and also upon environmental factors. The latter include the age at which gluten is first encountered, the total gluten load, and concurrent gut-damaging factors such as intolerance to cow's milk protein and gastroenteritis.

The possibility that there are two quite separate (possibly dominant) genetic components which need to combine may help to explain some of the difficulties that have been encountered up to the present in interpreting the findings of family studies. For example, it is possible that most coeliac patients who have not got a coeliac relative have inherited one of these two genetic components from each parent and that where there is a very high incidence of coeliac disease within a single family, both components are inherited from the one parent. Two subpopulations of coeliac patients with high and low familial incidence have been described (Rolles and Mackintosh, 1974).

The environmental factors described can probably be used to explain the lack of concordance in some identical twins. One first-degree relative in this study (no. 9) was HLA-B8 and showed an apparent mucosal defect similar to coeliacs in remission. Such individuals may be at risk under adverse environmental conditions.

Before the problem of coeliac disease is finally solved, the exact structure of the toxic substance in gluten will have to be defined and the precise role of the various genetic and environmental factors determined.

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