Acute gluten challenge in treated adult coeliac disease: a morphometric and enzymatic study

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SUMMARY Using a Quinton hydraulic biopsy tube, jejunal biopsies were obtained from 10 patients with adult coeliac disease in remission and four healthy volunteers before and after administration of gluten fraction III into the proximal duodenum. The biopsies taken at hourly intervals for four hours, were analysed for changes in brush border enzymes, light microscopic appearances, and villous and crypt population counts. The results indicate that mucosal damage occurs within three to four hours of gluten administration with significant falls in brush border enzyme concentrations and villous population counts. The absence of any change in control biopsies indicates that gluten sensitivity is specific to the mucosa of patients with coeliac disease, the timing of the changes being consistent with a type III immune response or direct toxicity. Some recovery of the brush border enzymes but not the villous population was evident 24 hours after gluten administration while the crypt population showed evidence of a compensatory hyperplastic reaction.

It is well known that a fraction of gluten is toxic to the small bowel mucosa of patients with adult coeliac disease. The mechanism of damage has not been fully elucidated but evidence is accumulating that an abnormal immune response may be involved.¹ The effects of prolonged administration of gluten on jejunal morphology in coeliac disease are well recognised² and indeed such challenges have been particularly valuable in the diagnosis of childhood coeliac disease.³ The acute effects of gluten administration, however, have received much less attention although early ultrastructural abnormalities have been noted⁴ and brush border enzyme changes apparently develop within 10 hours of gluten administration.⁵ It is now possible to undertake quantitative morphological studies on jejunal mucosa and such techniques combined with light microscopy and disaccharidase estimations have been used in the present study to examine the acute changes in jejunal mucosa after gluten administration in both coeliac patients in remission and control subjects.

Methods

PATIENTS

Ten patients, six men and four women, aged 21-58 years with subtotal villous atrophy on jejunal biopsy were studied at the time of follow up biopsy having been on a gluten free diet for a minimum of six months. Jejunal morphology had improved to normal or 'near normal' histology (villous height >350 μ m) in five, while the remaining five showed improvement to partial villous atrophy. Four healthy volunteers, two men, two women, aged 20-36 years, were also studied and acted as a small control group. Coeliac patients agreed to have their follow up biopsy combined with administration of gluten fraction III after a detailed explanation of the study. Ethical committee approval was obtained and all subjects gave written informed consent for the procedure to be carried out.

BIOPSY TECHNIQUES

Patients were admitted to hospital on the morning of the test after an overnight fast. A paediatric nasogastric tube was attached to the outer sheath of a Quinton hydraulic biopsy tube which was then positioned at or just beyond the ligament of Treitz under fluoroscopic control. Baseline biopsies were

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Received for publication 4 May 1984

then obtained before intraduodenal instillation of a peptic tryptic digest of 25 g gluten⁶ via the nasogastric tube 15 cm proximal to the biopsy port. Repeat biopsies were obtained from differing sites within the region of the ligament of Treitz at one, two, three, and four hours. Four control subjects were studied in an identical fashion. Further biopsies at eight and/or 24 hours were obtained from the majority of coeliac patients but not controls, using a Crosby capsule or guided biopsy tube.

ANALYSIS OF SMALL BOWEL MUCOSAL CHANGES

Brush border enzymes

Mucosa obtained at each time period was homogenised in 0.9% sodium chloride and stored at -20°C for up to five days before estimations of alkaline phosphatase,⁷ lactase and maltase⁸ concentrations. Values are expressed as activity per milligram or gram of protein.⁹

Light microscopy

After fixation in buffered formal saline, biopsies were embedded in paraffin wax before sectioning and staining with haematoxylin and eosin. Villous height and crypt depth were measured, using an ocular micrometer and the mean of 10 counts obtained.

Population counts

Biopsies were fixed in Carnoy's fluid for one hour before storage in 70% ethanol. The biopsy specimens were then transferred to 50% ethanol for 15 minutes, followed by 15 minutes in water before hydrolysis in hydrochloric acid (1 mol/l) for 6 minutes at 60°C . After hydrolysis the specimen was stained with Feulgen for one hour. Finally the biopsy was dissected in 40% acetic acid¹⁰ and the number of cells counted in each of 10 villi and 10 crypts using a squash technique.^{11 12} All counts were carried out by an observer who was unaware of the times at which the biopsies were taken, timed specimens from individual patients being randomly labelled.

STATISTICS

Tests of significance refer to the Wilcoxon's matched pairs, signed rank test, or Student's paired *t* test where appropriate.

Results

CONTROLS

Brush border enzymes

All four controls had baseline values of alkaline phosphatase, lactase and maltase within the normal range¹³ (alkaline phosphatase 431–1406 $\mu\text{mol/min/g}$

mg; maltase 98–367 $\mu\text{mol/min/g}$; lactase 7.0–60.2 $\mu\text{mol/min/g}$). After administration of gluten fraction III no significant differences in enzyme concentration were observed over the four hour period of the study (Fig. 1).

Light Microscopy

There were no changes in villous height or crypt depth.

Population counts

Adequate material for full analysis was only available for three of the four controls studied. No changes in villous population counts were observed. Crypt cell counts also remained unchanged. Full results are shown in Table 1.

COELIAC PATIENTS

Brush border enzyme

In the whole group ($n=10$) alkaline phosphatase fell from 531 ± 66 to 337 ± 42 $\mu\text{mol/min/mg}$ at three hours ($p<0.05$) and 357 ± 51 at four hours ($p<0.05$). The fall in maltase was not significant but lactase concentrations fell from 5.3 ± 1.9 to 2.5 ± 0.6 $\mu\text{mol/min/g}$ at four hours ($p<0.05$). Figure 2 shows these changes as a percentage fall from baseline values. If

Fig. 1 Brush border enzyme changes in four healthy controls with normal small bowel mucosal morphology before and after intraduodenal instillation of gluten fraction III. All values fell within the normal range (shown in the text).

Table 1 Jejunal villous and crypt population counts expressed as numbers of cells per crypt or villous in three control subjects before and after administration of gluten fraction III into the proximal duodenum

Controls	Population	Prechallenge	1 h	2 h	3 h	4 h
1	Villous	7078	6875	6689	7032	6701
	Crypt	555	546	581	533	602
2	Villous	4394	—	4751	4756	4460
	Crypt	673	—	686	581	704
3	Villous	6248	5905	6264	5470	5879
	Crypt	560	530	557	571	569

only those five patients with normal or 'near normal' mucosa are considered the falls become much more obvious (Fig. 3). In this subgroup alkaline phosphatase fell from 686 ± 83 to 387 ± 98 $\mu\text{mol/min/mg}$ at four hours ($p < 0.025$) and maltase fell from 151 ± 23 to 92 ± 17 $\mu\text{mol/min/g}$ at four hours ($p < 0.025$).

Some recovery was evident at 24 hours, although lactase remained significantly below the baseline value ($p < 0.01$).

Fig. 2 Brush border enzyme changes in 10 patients with adult coeliac disease on a gluten free diet before and after intraduodenal instillation of gluten fraction III. Values for alkaline phosphatase (●—●), lactase (○—○) and maltase (▲—▲) are expressed as a percentage change from the prechallenge values. The significance of the changes are shown in the text.

Fig. 3 Brush border enzyme changes after gluten challenge in five patients with adult coeliac disease and normal or 'near normal' histology on a gluten free diet. Values are expressed as a percentage change from the prechallenge value. Symbols as in Fig. 2. The significance of the changes are shown in the text.

Light microscopy

No statistically significant changes in villous height or crypt depth occurred during the 24 hour period of study after gluten administration. Full results are shown in Table 2.

Population counts

Sufficient biopsy material for detailed study was available from seven of the 10 patients with treated coeliac disease. In this group, the villous population count fell from 3310 ± 145 to 2633 ± 135 cells per villus at four hours ($p < 0.02$) and to 2660 ± 211 cells per villus at eight hours ($p < 0.02$). At 24 hours the villous population count was still significantly below the pre-challenge value at 2525 ± 182 cells per villus ($p < 0.05$). The crypt population was significantly increased at 24 h ($p < 0.05$). Full results are shown in Table 3.

Discussion

In this study 10 patients with coeliac disease who had been on a gluten free diet for a minimum of six

Table 2 Measurement of villous height and crypt depth in seven patients with adult coeliac disease on a gluten free diet before and after administration of gluten fraction III into the proximal duodenum

	Patient							
Time (h)	CL	DW	EL	ST	AE	RP	WT	Mean±SEM
Villous height (μm)								
0	363	334	273	501	404	266	300	349±32
1	359	310	308	375	—	249	—	320±22
2	315	290	—	419	399	282	353	343±23
3	—	317	349	346	459	228	300	333±31
4	327	290	—	385	—	232	312	309±25
8	315	—	378	365	356	194	269	312±29
24	—	—	349	382	—	240	343	328±31
Crypt depth (μm)								
0	390	385	414	276	261	271	303	329±25
1	440	329	380	259	—	256	—	333±35
2	382	399	—	269	295	286	281	318±23
3	—	368	358	288	278	283	312	314±16
4	395	421	—	300	—	290	300	341±28
8	419	—	385	242	286	290	303	320±27
24	—	—	419	288	—	302	341	337±29

months underwent a single exposure to gluten at the time of rebiopsy. Control volunteers were also studied as gluten toxicity might be induced by exposing normal mucosa to high doses of gluten.¹⁴ In this context Doherty and Barry showed that gluten could affect the mucosa of patients with 'immune deficiency' syndromes and some first degree relatives of coeliac patients, producing changes similar to those seen in coeliac disease. Normal controls did not show this change although there was a significant fall in xylose absorption and

intra-epithelial lymphocytes significantly increased. In an earlier study of 14 subjects exposed to a high gluten diet, Levine *et al* were unable to show any deleterious effect.¹⁵ Their study did not, however include measurements of brush border enzymes or villous population counts. It was therefore necessary to perform enzyme determinations and villous population counts on controls in order to confirm the absence of short term gluten toxicity. No statistical differences were observed four hours after gluten challenge and no fall in brush border enzymes

Table 3 Changes in villous and crypt population counts in seven patients with adult coeliac disease on a gluten free diet before and after administration of gluten fraction III into the proximal duodenum

	Patient							
Time (h)	CL	DW	EL	ST	AE	RP	WT	Mean±SEM
Villous population (cells/villous)								
0	3502	3295	3773	2870	3215	3718	2802	3310±145
1	3192	3263	3619	3114	—	3412	—	3320±90
2	3119	3072	—	2754	3120	2657	2994	2953±82
3	—	3015	3923	2075	3123	2531	2639	2885±259
4	3092	2766	—	2408	—	2535	2365	2633±135+
8	3071	—	3337	2155	2938	2286	2177	2660±211+
24	—	—	3042	2455	—	2418	2186	2525±182*
Crypt population (cells/crypt)								
0	576	527	854	236	309	558	375	490±78
1	569	507	839	246	—	643	—	560±97
2	579	658	—	269	318	540	375	457±64
3	—	646	703	295	271	618	418	492±77
4	633	736	—	316	—	557	410	530±76
8	688	—	760	231	245	451	437	469±90
24	—	—	904	313	—	583	470	567±125*

* $p < 0.05$; + $p < 0.02$

was observed by Anand *et al.*¹⁶ in a single control subject eight hours after acute challenge with a gluten subfraction.

In coeliac patients however, acute challenge with a similar dose of gluten as used in controls led to a rapid fall in mucosal brush border enzyme concentrations with a parallel fall in villous population count. It appears that simple morphometric analyses are insensitive to these early changes as no differences were observed in villous height during the four hours of the study. Previous studies¹²⁻¹⁷ have shown that such simple morphometric measurements are not reliable indicators of the enterocyte population in the mouse when villi are abnormal in shape as seen during mucosal regeneration; however, in populations of normal shaped villi, correlation between their measurement and population counts is good.¹²⁻¹⁷ The villous population remained low at 24 hours while some recovery of crypt population and brush border enzymes were evident at this time the latter possibly because of maturation of enterocytes rather than cell replication or recovery of damaged cells. The crypt population results are interesting: there was a significant increase in crypt population size at 24 hours after gluten. Previous studies on crypt regeneration after damage induced by cytotoxic drugs¹⁸ have shown that crypt regeneration does not occur until depletion of the villous cell population has taken place supportive of a negative feedback control mechanism. In the present situation, the villous population is directly depleted by the reaction to gluten; the crypt population responds to this depletion by hyperplasia, again consistent with the negative feedback hypothesis.

In a study designed to determine the toxicity of different gluten fractions Dissanayake *et al.*⁵ were able to show abnormalities on light microscopy amounting to partial villous atrophy, an increase in intraepithelial lymphocytes, shortening and irregularity of microvilli shown on electron microscopy and falls in jejunal disaccharidases in two patients 10 hours after challenge with gluten fraction B. All the abnormalities noted were more pronounced in the three patients studied at 24 hours. In a further study using the same fraction¹⁶ changes of severe partial villous atrophy, an increase in IgA and IgM immunoperoxidase staining cells and a fall in disaccharidases was found in one patient three and a half and six hours after challenge. Mild partial villous atrophy and increased immunoperoxidase staining cells were found in a further patient four and eight hours after challenge while immunofluorescence staining was observed at eight hours. The changes improved at 24 and 48 hours. From this study the authors concluded that there

was immunologically mediated injury within the mucosa which was maximal three and a half to six hours after challenge. The findings were consistent with an Arthus type reaction while the improvement at 24–48 hours suggested that a type IV delayed hypersensitivity reaction was unlikely. These authors findings of early abnormalities on light microscopy which we were unable to confirm may be because of their use of a more toxic gluten subfraction (fraction B).

In the present study the rapid fall in villous population counts and mucosal enzymes also implies cell destruction probably because of the humoral immunity of the type III or Arthus reaction, although direct toxicity is also possible. In type III hypersensitivity reactions, immune complexes are formed which are ingested by neutrophils resulting in cell disruption and release of lysosomal enzymes.¹⁹ This usually takes four to six hours. The presence of circulating immune complexes, complement fixation reactions²⁰ and changes affecting immunoglobulin levels in the serum²¹ are all well recognised in untreated coeliac disease, while in the mucosa IgM containing cells are increased,^{22–24} findings which would be anticipated in a type III immune response.

Damage to the mucosa as judged by both enzyme estimations and morphometric study was more pronounced in those patients with normal or near normal mucosa. Whether this represents a greater potential for damage, because of total recovery, or some resistance to damage for those with partial villous atrophy is not clear.

In conclusion this study shows that gluten sensitivity is specific to the mucosa of patients with coeliac disease and that gluten toxicity results in rapid cell loss from the villi, which is paralleled by a fall in brush border enzyme concentrations. The timing is compatible with either direct toxicity to a susceptible population of enterocytes or enterocyte damage mediated through an abnormal type III immune response. Early mucosal damage mediated through a type IV immune response now seems less likely to be of primary importance in the pathogenesis of coeliac disease.

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