

Early biochemical responses of the small intestine of coeliac patients to wheat gluten

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SUMMARY The pathogenesis of coeliac disease has been investigated by studying the response of small intestinal hydrolases in patients with coeliac disease subject to gluten challenge. Small intestinal biopsies taken before and two and a half hours after a gluten challenge in five patients with coeliac disease who had been maintained on a gluten free diet were examined by a combination of electron and light microscopy, organ culture, pulse chase biosynthetic labelling, SDS-PAGE and autoradiography. Before the challenge, the small intestinal biopsies showed nearly normal morphology. Two and a half hours after the challenge there was deterioration in villus architecture, distortion of microvillus structure, disorganisation of the intermicrovillus pit region, an increase in lysosome like bodies in the apical cytoplasm of the luminal enterocytes and pronounced hypertrophy of the rough endoplasmic reticulum of these cells. SDS-PAGE of small intestinal biopsies from four treated coeliac patients before gluten challenge revealed normal microvillus membrane and hydrolase composition. There was a generalised reduction but no specific alteration in the pattern of polypeptide synthesis in the mucosa of the small intestine in these subjects two and a half hours after the gluten challenge. These results suggest that the generalised reduction in small intestinal brush border enzymes in coeliac patients is not the primary pathogenetic mechanism and represents a secondary effect.

Three possible mechanisms have been proposed for the pathogenesis of coeliac disease. These include lack of a specific small intestinal enzyme,¹ toxic lectin-like binding of gluten to small intestinal enterocytes² and immunological hypersensitivity to specific cereal protein epitopes.³ The primary aims of this study were to investigate whether coeliac disease is caused by either a missing small intestinal enzyme or a gluten induced loss of a specific small intestinal hydrolase. This was undertaken by examining the early morphological and biochemical responses of the small intestine of coeliac patients in remission and subject to gluten challenge by a combination of electron and light microscopy, organ culture, pulse chase biosynthetic labelling, sodium dodecyl sulphate poly-

acrylamide slab gel electrophoresis (SDS-PAGE), and autoradiography. A combination of these techniques permits investigation of the earliest previously noted damage to the villus morphology of the small intestine of treated coeliac patients subject to gluten challenge. Light and electron microscopy were used to study the early morphometric and ultrastructural changes of the small intestinal mucosa in these patients, whilst SDS-PAGE and biosynthetic labelling were used to look for specific abnormalities in the hydrolases of the small intestinal brush border membrane of treated coeliac patients subject to a gluten challenge. Comparison of the small intestinal mucosa of treated coeliac patients before and two and a half hours after a gluten challenge was undertaken as this was the earliest reported time at which gluten induced morphological damage had previously been noted.⁴

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Methods

PATIENTS

Five patients, three women and two men, aged 25 to 57 years with coeliac disease, treated with a gluten free diet for between two and 30 years were investigated. In each case, the diagnosis had previously been established by standard criteria.⁵ Informed consent was obtained from the patients before the study which was approved by the Ethical Committee of St Thomas' Hospital.

EXPERIMENTAL DESIGN

In all five subjects, a Quinton peroral hydraulic multiple biopsy instrument was positioned under fluoroscopic control in the proximal jejunum. Seven jejunal biopsies were taken at time zero and two and a half hours after the subjects had ingested 10 g wheat gluten suspended in 150 ml tap water. In one of the five volunteers, additional biopsies were taken at four and six hours after the challenge.

In all five subjects one biopsy before and another two and a half hours after the gluten challenge were fixed in formal saline. These biopsies were processed in wax to 5 μ m sections, stained with haematoxylin and eosin and examined morphometrically under a light microscope. One of the biopsies taken from each patient before and two and a half hours after the gluten challenge was fixed in 4% glutaraldehyde, buffered with 0.1 M sodium cacodylate pH 7.4 and processed for electron microscopy according to criteria described by Mann *et al.*⁶ Formalin fixed sections were used to measure villus height to crypt depth ratio, epithelial surface cell height and intra-epithelial lymphocyte count per 100 enterocytes.⁷ The results are expressed as the mean \pm 1 standard deviation of 10 observations and compared statistically by a paired Student's *t* test.

In four of the subjects five of the jejunal biopsies taken before and two and a half hours after the gluten challenge were used to compare brush border composition and membrane synthesis using a combination of organ culture, pulse chase biosynthetic labelling, SDS-PAGE, and autoradiography. These five biopsies from each patient at both time zero and two and a half hours after the challenge were cultured separately and pulse chase labelled with ³⁵S-methionine by incubating them on the grids of organ culture dishes, as described by Browning and Trier⁸ with 20 μ Ci of ³⁵S-methionine (Amersham International, High Wycombe) in 2.5 μ l PBS placed on the upper mucosal surface of each biopsy for 10 minutes. Just sufficient tissue culture medium was then added to the organ culture dishes to come into contact with the stainless steel grids, and the biopsies cultured for a further 110 minutes. The tissue culture

medium comprised 6 ml Trowell's T8 medium, 2 ml NCTC 135 medium, 0.2 ml 200 mM L-glutamine, 0.1 ml 1 M HEPES, 0.2 ml penicillin (5000 U/ml), plus streptomycin (5000 μ g/ml) and 1.5 ml fetal calf serum to 10 ml, all the reagents for which were obtained from Gibco Limited, Paisley. The incubations were at 37°C in an atmosphere of 95% O₂ and 5% CO₂. The biopsies were then frozen separately in thawing isopentane over liquid nitrogen and stored in liquid nitrogen for up to a fortnight before analysis as described below. In one of the five subjects 10 additional biopsies were taken, five at four and five at six hours after the gluten challenge to assess semi-quantitatively by SDS-PAGE the amount of microvillus membrane present at these times compared with the biopsies taken before and two and a half hours after the challenge.

FRACTIONATION OF MUCOSAL BIOPSIES

The five biopsies from each subject both before and two and a half hours after the challenge were independently pooled for each subject at the two time points before fractionation. These mucosal biopsies were fractionated at 4°C into cytoplasmic and membrane preparations by homogenisation in 300 mM mannitol 50 mM HEPES buffer using 20 strokes of a loose fitting Dounce homogeniser. Membranes were pelleted from the homogenate by high speed centrifugation at 100 000 g for 60 minutes in a Beckman TL100 centrifuge.

MICROVILLUS MEMBRANE PREPARATION BY CATION PRECIPITATION

Preparation of the adult coeliac microvillus membrane vesicles by Mg²⁺ precipitation was as previously described,⁹ based upon the procedure of Kessler *et al.*¹⁰ as modified by Gains and Hauser.¹¹ Final purification was by sucrose density gradient centrifugation. Aminopeptidase activity was used routinely as a marker of enzyme activity for the microvillus membrane. Purified adult small intestinal microvillus membrane vesicles banded at an isopycnic sucrose density of approximately 1.2 g/ml and showed an approximately 15-fold rise in aminopeptidase activity.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS
Microvillus membranes were prepared from pre-challenge biopsies with a combined wet weight of 60 mg and directly compared with those obtained from post challenge biopsies of the same combined wet weight by SDS-PAGE. One dimensional sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) was carried out using slabs of 7.5% polyacrylamide as described by Laemmli¹² under reducing conditions. Protein molecular weights were

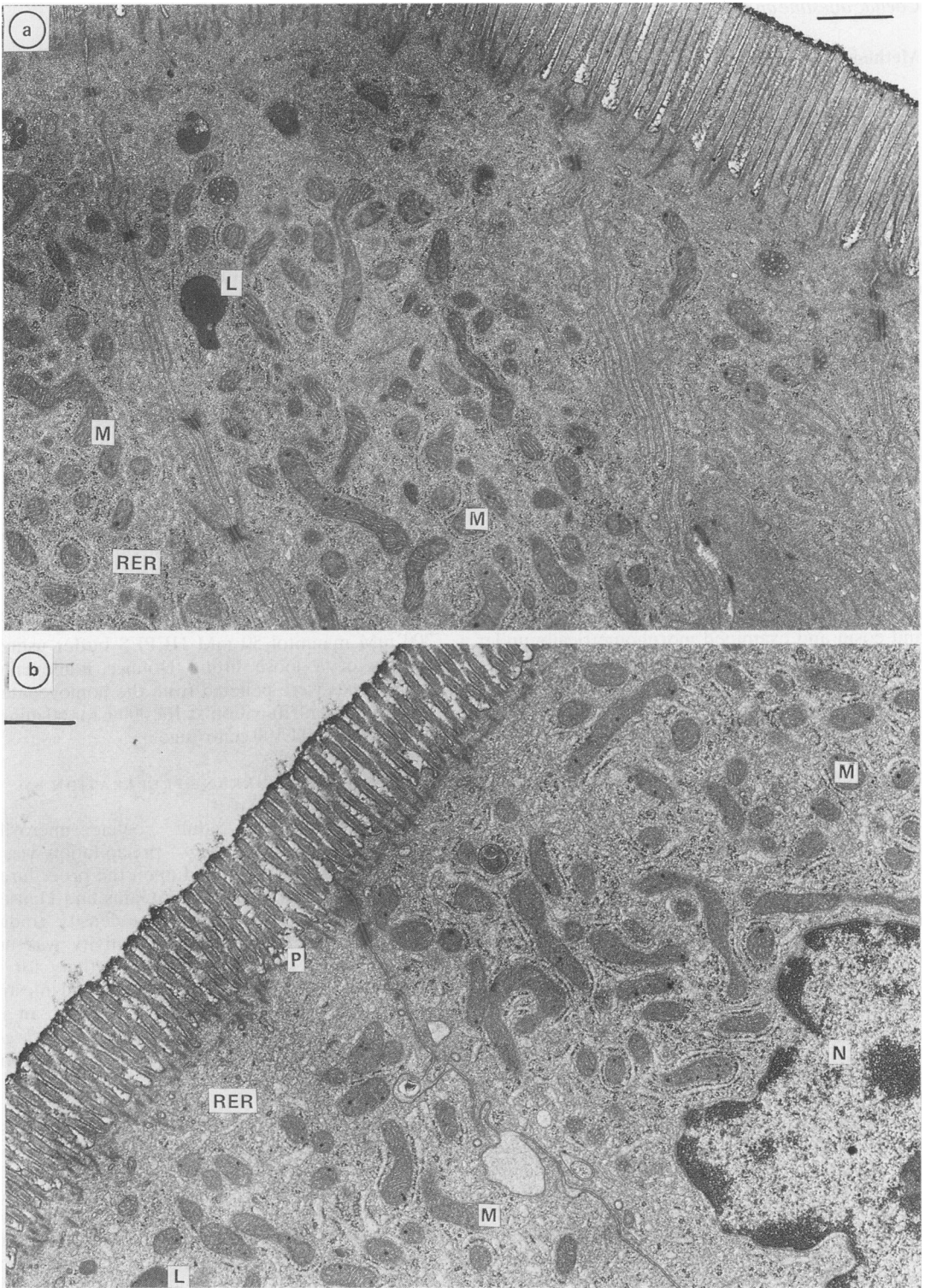


Fig. 1 Electron micrographs of enterocytes from one treated coeliac patient (a) before and (b) two and a half hours after gluten challenge. L=Lysosome; M=Mitochondria; N=Nucleus; P=Microvillus pit; RER=Rough endoplasmic reticulum. —represents 1 μ m.

estimated by reference to migration of standard proteins (carbonic anhydrase, 29 kD; ovalbumin 45 kD; bovine serum albumin, 68 kD; rabbit muscle phosphorylase b, 97.4 kD; *E Coli* beta-galactosidase, 116 kD and rabbit muscle myosin 205 kD, all obtained from Sigma Chemicals, Poole, Dorset). Proteins were detected by Coomassie blue and silver staining.¹³ ³⁵S-methionine incorporation was investigated by gel autoradiography.

ENZYMATIC DETERMINATIONS

Aminopeptidase N (EC 3.4.11.2) was assayed as described by Wachsmuth *et al.*,¹⁴ and protein was measured by the method of Lowry *et al.*¹⁵

Results

STRUCTURE AND ULTRASTRUCTURE OF COELIAC SMALL INTESTINAL MUCOSA

Before challenge, examination of the small intestinal biopsies from the five coeliac patients on a gluten free diet by light and transmission electron microscopy revealed that the jejunal mucosa of the coeliac patients possessed normal or nearly normal villus architecture with the exception of patient No 2 in whom the intraepithelial lymphocyte count was raised at 64/100 enterocytes. In all five subjects the biopsies revealed a well developed brush border, with elaborate microvilli – an example of which is shown in Figure 1a. The results of the light microscopy, morphometry are presented in the Table.

Table Morphometric variables of jejunal biopsies from five coeliac patients before (A) and two and a half hours after (B) a gluten challenge

Patient No	Villus height: crypt depth		Epithelial surface cell height (μm)		Intraepithelial lymphocytes/100 enterocytes	
	A	B	A	B	A	B
1	1.85 (0.1)	1.57 (0.1)	42.0 (2.1)	30.3 (1.5)	23.4 (3)	35.1 (2.9)
2	1.51 (0.2)	1.34 (0.2)	31.1 (1.9)	27.6 (2.8)	64.0 (4.7)	71.3 (4.4)
3	2.88 (0.2)	2.27 (0.2)	35.5 (1.9)	35.1 (1.2)	22.6 (3.6)	31.8 (3.2)
4	3.16 (0.2)	1.9 (0.2)	35.9 (2.4)	35.1 (2.2)	30.7 (1.9)	62.9 (4.2)
5	2.01 (0.1)	1.37 (0.1)	38.2 (1.6)	25.3 (1.9)	28.2 (3.2)	46.8 (3.0)
Mean (1SD)	2.3 (0.7)	1.7 (0.4)	36.5 (4.0)	30.7 (4.4)	33.8 (17)	49.6 (17)
Significance	p<0.05		NS		p<0.05	

Two and a half hours after the gluten challenge in all five subjects there was mildly abnormal villus architecture as shown in Fig. 2b, with a fall in villus height: crypt depth ratio ($p<0.05$), a fall in epithelial surface cell height, which failed to reach significance and a rise in intraepithelial lymphocyte count ($p<0.05$), the results of which are shown in the Table. Electron microscopy of the biopsies taken two and a half hours after the gluten challenge, revealed microvillus membrane perturbation, extensive intermicrovillus pit hypertrophy, hypertrophy of the rough endoplasmic reticulum and the presence of numerous lysosome like organelles in the apical part

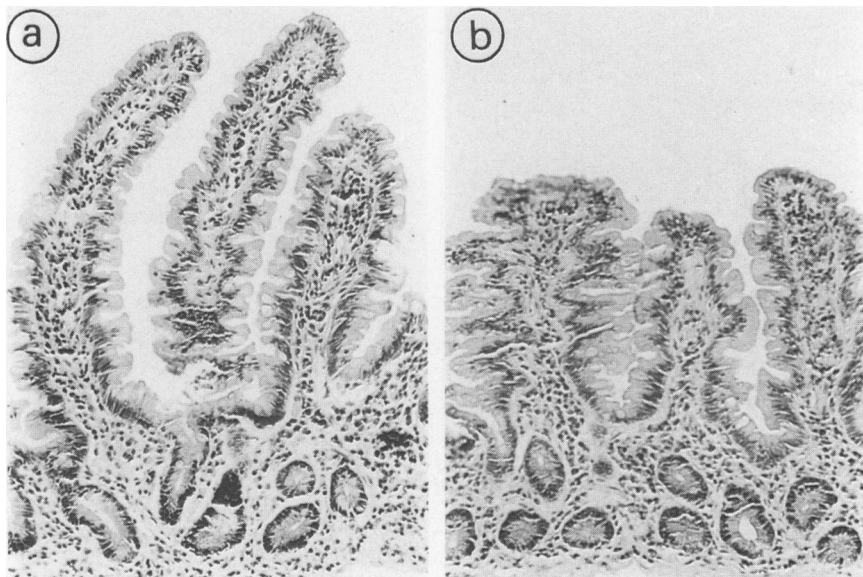


Fig. 2 Light microscopy of small intestinal mucosa from one treated coeliac patient – (a) before and (b) two and a half hours after gluten challenge.

of the surface enterocytes (Fig. 1) in all five subjects. No direct continuity between the apical membrane and the cytoplasmic lysosomes was evident in any of the sections examined.

POLYPEPTIDE COMPOSITION OF THE PRECHALLENGE MICROVILLUS MEMBRANE

The small intestinal microvillus membrane from the four coeliac patients before gluten challenge possessed a normal complement of proteins with a dominant actin band at 47 kD and a group of high molecular weight proteins at 127 to 164 kD. The latter correspond to the microvillus hydrolases.¹⁶ An

example of this in one of the patients, documented by one-dimensional SDS-PAGE is shown in the first track of Fig. 3. These constituents closely resemble the polypeptide components of normal jejunal microvillus membranes.^{17,18}

MICROVILLUS MEMBRANE AFTER GLUTEN CHALLENGE

Examination of Coomassie Blue staining of SDS-PAGE of microvillus membranes from a pooled quantity of 60 mg of both pre and post challenge biopsies taken from each subject before and after the challenge revealed no change in their composition

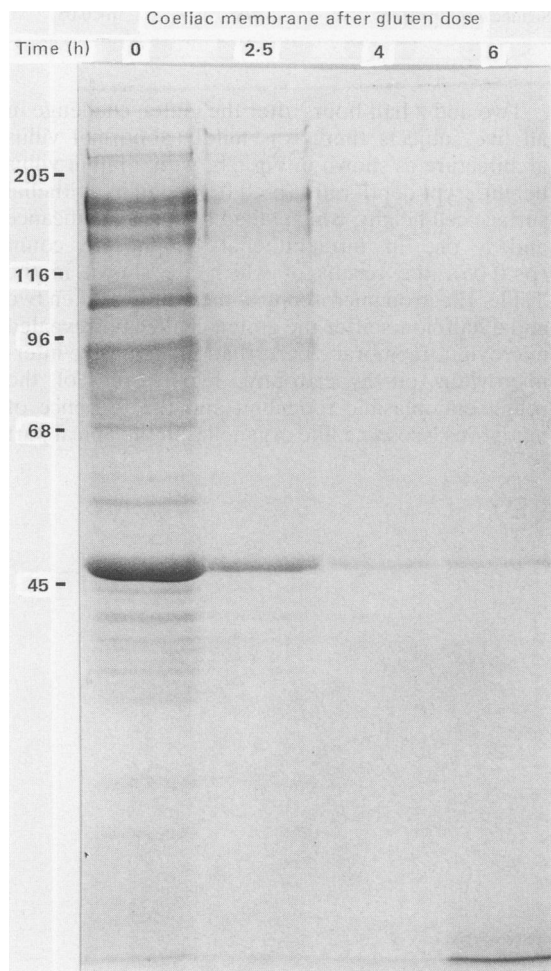


Fig. 3 Seven and a half per cent SDS-PAGE stained with Coomassie blue of microvillus membranes prepared from biopsies with a combined wet weight of 60 mg taken from one coeliac patient before (0 h), two and a half, four, and six hours after gluten challenge. Molecular weights of 205, 116, 96, 68, and 45 kilodaltons are marked at the side.

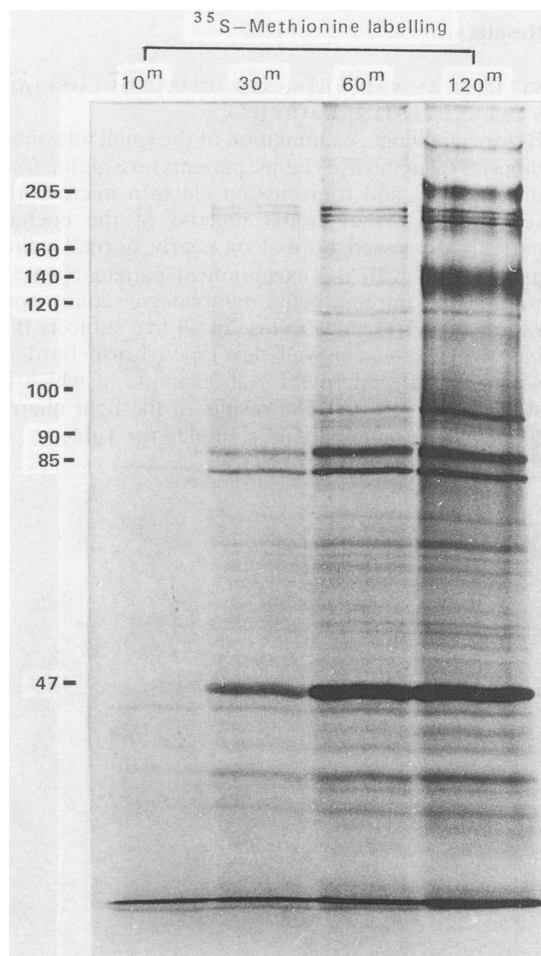


Fig. 4 Gel autoradiograph of microvillus membrane preparations from one coeliac patient who had been taking a gluten free diet before gluten challenge, after pulse labelling the biopsies for 10 min with ³⁵S-methionine followed by 10, 30, 60, and 120 min cold chase with methionine in tissue culture medium. Molecular weights of 205, 160, 140, 120, 100, 90, 85, and 47 kilodaltons are marked at the side.

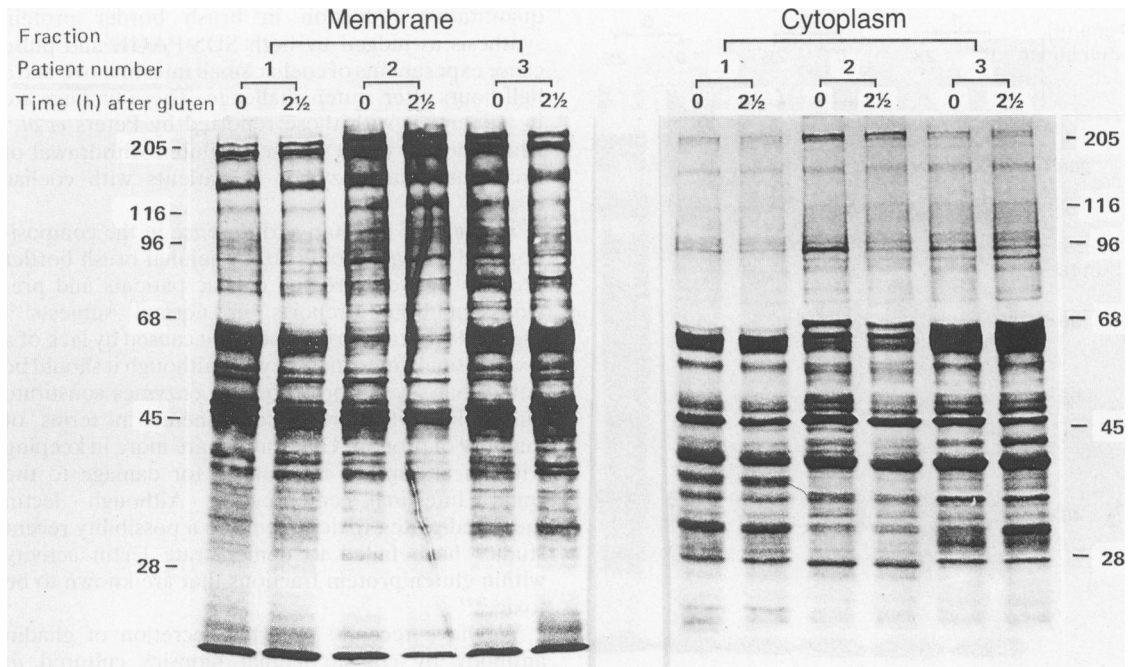


Fig. 5 Gel autoradiographs of total membrane and cytoplasmic fractions of biopsies taken before and two and a half hours after gluten challenge from three coeliac subjects who had been taking a gluten free diet. The biopsies were pulse labelled with ^{35}S -methionine and chased with cold methionine in tissue culture medium for 120 minutes. Molecular weights of 205, 116, 96, 68, 45, and 28 kilodaltons are marked at the side.

but a marked decrease in yield in the four subjects examined in this way as shown in Fig. 3 track 2. The progressive loss of microvillus membrane at four and six hours after challenge in the single patient who had biopsies at these times is shown in Fig. 3 tracks 3 and 4. Any defect in the apical membrane therefore seems to reflect diminished microvillus membrane synthesis rather than abnormal structure.

BIOSYNTHETIC STUDIES

The results of ^{35}S -methionine pulse chase SDS-PAGE autoradiography studies using mucosal biopsies pooled separately for each subject from three of the four subjects investigated in this way are shown in Figs 4–6. Purification of the microvillus membrane from biopsies taken before gluten challenge showed that synthesis of a complete spectrum of microvillus polypeptides was obtained only after a 120 minute cold chase after 10 min ^{35}S -methionine biosynthetic labelling (Fig. 4). This time period was therefore used in all the subsequent biosynthetic studies. Although clear differences in the labelled polypeptide constituents were observed between individual patients, no changes in labelled polypeptides were seen in either the total membrane or cytoplasmic

fractions two and a half hours after gluten challenge (Fig. 5).

To complement these studies, microvillus membrane preparations from the same pooled biopsies for each subject before and after the gluten challenge were also examined (Fig. 6). Again, no changes in labelled microvillus polypeptides were seen subsequent to gluten challenge in any of the subjects examined. The cytoplasmic and total membrane extracts prepared from mucosal biopsies contained spectra of polypeptides which differed markedly in molecular weight (Fig. 5).

Discussion

Our results confirmed normal or near normal light and electron microscopic appearances of jejunal biopsies taken from coeliac patients in remission on a gluten free diet, in agreement with Bramble *et al.*¹⁹ We also found a normal complement of, and normal synthesis of microvillus membrane components in these biopsies. Two and a half hours after a 10 g oral gluten challenge in five treated coeliac patients we found morphological and ultrastructural damage to the jejunal mucosa confirming the findings of Bayless

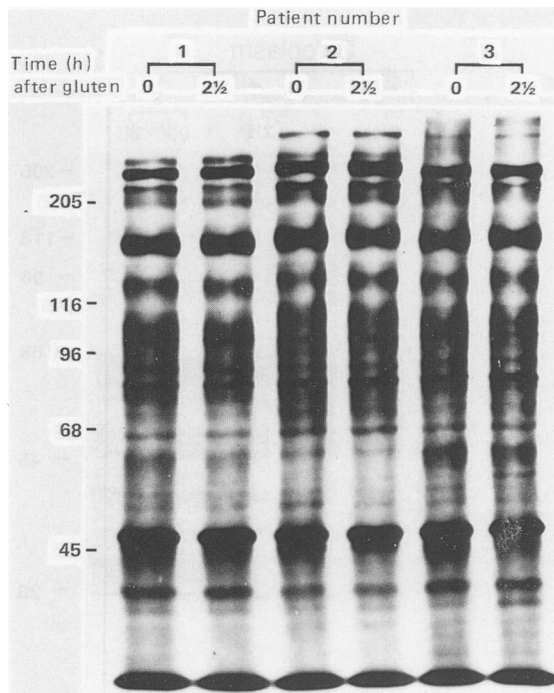


Fig. 6 Gel autoradiographs of microvillus membrane preparations from biopsies taken before and two and a half hours after gluten challenge from three coeliac subjects who had been taking a gluten free diet. The biopsies were pulse labelled with ^{35}S -methionine and chased with cold methionine in tissue culture medium for 120 minutes. Molecular weights of 205, 116, 96, 68, and 45 kilodaltons are marked at the side.

*et al.*²⁰ The increased number of lysosome like particles in the apical cytoplasm of surface enterocytes has previously been reported²¹ and is believed to represent impending cell dissolution. It is unlikely that any of the observed changes in villus morphology were because of the presence of the bioRx capsule alone as no changes in villus morphology were noted in serial jejunal biopsies taken hourly over six hours from a treated coeliac patient challenged with 150 ml tap water alone in a previous study.²² It is also unlikely that excessive quantities of gluten could cause non-specific toxic small bowel changes, as serial jejunal biopsy specimens obtained from normal volunteers in three previous studies showed no changes in their small intestinal villus morphology in serial jejunal biopsies after gluten challenge.²³⁻²⁵ Moreover it was previously established that up to 150 g of gluten given daily for at least eight weeks had no demonstrable histological effect on the small bowel in normal volunteers.²⁶

Coincident with the morphological damage to the

brush border microvilli we observed a generalised quantitative reduction in brush border protein synthesis as judged by both SDS-PAGE and pulse chase experiments of coeliac small intestine two and a half hours after gluten challenge. These findings are in agreement with those reported by Peters *et al.*,²⁷ who reported the response to gluten withdrawal of small intestinal enzymes in patients with coeliac disease.

The lack of qualitative difference in the composition and *in vitro* production of jejunal brush border proteins between treated coeliac patients and previous published reports in normal subjects^{17,18} suggests that coeliac disease is not caused by lack of a specific small intestinal enzyme, although it should be noted that several brush border enzymes constitute only minor membrane components in terms of quantity of protein. Our findings are more in keeping with an alternative mechanism for damage to the small intestinal enterocytes. Although lectin mediated gluten toxicity remains a possibility recent studies have failed to demonstrate lectin activity within gluten protein fractions that are known to be toxic.^{28,29}

We have recently reported secretion of gliadin antibody by coeliac jejunal biopsies cultured *in vitro*,³⁰ infiltration of coeliac small intestinal mucosa by T lymphocytes²² and aberrant expression of HLA-class II antigens by small intestinal crypt enterocytes of treated coeliac patients within two hours of an acute gluten challenge.³¹ These findings suggest a possible pathogenetic role for local cellular and antibody mediated immune mechanisms in coeliac disease. The present study has shown both morphological and biochemical damage to the enterocyte brush border membrane over a similar time course and therefore adds further weight to this hypothesis.

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