Quantitative study of mucosal structure, enzyme activities and phenylalanine accumulation in jejunal biopsies of patients with early and late onset diabetes

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SUMMARY A study of the three-dimensional structure of the upper jejunal mucosa in diabetics has been carried out. The structural findings were related to¹⁴C-L-phenylalanine uptake in vitro, sucrase activity in mucosal homogenates, and the enzyme content of the absorptive cells as measured cytophotometrically. A low grade mucosal transformation of the sprue-type was found, which was associated with decreased sucrase activity, and with no reduction in phenylalanine accumulation. On the other hand the specific activities of alkaline phosphatase, non-specific esterase, and succinic dehydrogenase in the surface cells remained unchanged.

Numerous studies have shown that experimental diabetes mellitus in the rat is associated with structural changes in the small intestine (Jervis and Levin, 1966; Nakabou et al., 1974; Lorenz-Meyer et al., 1974, 1977), these being accompanied by functional alterations (Crane, 1961; Aulsebrook, 1965; Fromm et al., 1969; Levinson and Engelst, 1970; Olsen and Rosenberg, 1970; Caspary, 1971; Olsen and Rogers, 1971; Schedl and Wilson, 1971; Younoszai and Schedl, 1972).

In the acute phase, an increase in the activities of brush border hydrolases and an enhanced absorption of actively transported hexoses and amino acids (Crane, 1961; Olsen and Rogers, 1971; Schedl and Wilson, 1971) has been reported. On the other hand, the mucosal histology remained unchanged in acute experimental diabetes mellitus (Schedl and Wilson, 1971).

In the chronic stage, structural modifications have been demonstrated in addition to altered function. The villi became hyperplastic and both cell proliferation and intestinal circumference increased (Nakabou et al., 1974; Lorenz-Meyer et al., 1974, 1977), leading to an enlargement of total absorptive surface (Lorenz-Meyer et al., 1977). Functionally, absorption is increased in vivo when related to total surface area and the accumulation of glucose, β-methyl-glucoside, and of some actively transported amino acids in vitro is also stimulated (Crane, 1961; Olsen and Rosenberg, 1970; Schedl and Wilson, 1971; Lorenz-Meyer et al., 1977).

Compared with these studies in the experimental animal functional investigations in diabetic patients are scanty (Vinnik et al., 1965; Genel et al., 1971; Gottesbürken et al., 1973, 1974) and no morphological investigations of the small intestine have hitherto been presented, except in secondary diabetes due to pancreatic disease (Caspary et al., 1974). Glucose, water and electrolyte absorption was found by our own group to be unaltered in vivo according to the triple lumen tube technique (Gottesbürken et al., 1973), but an increase was reported in the studies of Vinnik and coworkers (1965). These authors, however, used unphysiologically high glucose concentrations in their test solution. In a further study, Genel et al. (1971) failed to show an increased glucose accumulation rate in vitro in small intestinal biopsies from diabetic patients.

The purpose of this investigation was to explore the mucosal structure of the jejunum in diabetic patients by three-dimensional quantitative analysis to find out whether changes occur in man similar to those seen in the chronic diabetic rat. In addition, phenylalanine absorption was measured in vitro and the activity of sucrase—a brush border marker enzyme—in whole homogenates of the biopsies was determined biochemically. Finally, the activities of alkaline phosphatase, succinic dehydrogenase, and non-specific esterase were measured cytochemically using microdensitometric methods.
Methods

Subjects

Seventeen diabetic patients without evident gastrointestinal disease were investigated; seven suffered from early onset diabetes and were treated with insulin, while the remaining 10 received either a diet alone or diet plus sulphonylurea-derivatives. At the time of investigation, the patients were all in a well-balanced metabolic state and none suffered from exocrine pancreatic disease. The clinical data (body weight, diet, physical state, age, and the duration of diabetes mellitus) are given in Table 1. Twenty patients with an age range from 24 to 83 years served as controls. All these patients were without gastrointestinal disease (according to history, clinical examination, number, and aspect of faeces), and for three to four days before the investigation on a diet containing 160g-200g carbohydrates.

Jejunal Biopsies

After an overnight fast, specimens were obtained under radiological control from just beyond the ligament of Treitz by using Quinton’s hydraulic multi-biopsy tube. Informed consent was obtained in each case.

Two pieces of mucosa, 5-15 mg in weight, were fixed for microdissection as described by Clarke (1970); two pieces were used for immediate determination of phenylalanine accumulation in vitro and two other pieces for the parallel determination of the extracellular space using 14C-polyethylene glycol (MW=1000). Two pieces of tissue were shock-frozen in liquid nitrogen and stored at −80°C in air-tight sealed plastic bags for biochemical and quantitative histochemical investigations respectively. Another biopsy was fixed in formalin and embedded in paraffin. Five μm sections were cut and stained with haematoxylin and eosin for microscopic evaluation.

Microdissection and Quantitative Analysis of Jejunal Mucosa

Three-dimensional quantitative analysis of the mucosal biopsies was done by adapting the microdissection technique of Clarke (1970) for human tissue as described earlier (Riecken et al., 1976). Briefly, the biopsies were fixed in three parts of ethanol and one part of glacial acetic acid for 24 hours; thereafter a Feulgen stain was done en bloc. The specimen was then photographed under the stereomicroscope to determine the number of villi per unit serosal area. Thereafter it was photographed upside down for the determination of the number of crypts per unit area. Consequently the thicknesses of at least 10 individual villi per specimen were measured under the stereomicroscope; the villi were then isolated, floated in Schiff’s reagent on a cover glass,
and photographed under the Orthoplan microscope. Total villus surface could then be calculated from the photographically determined area of the isolated villus and its circumference, as follows:

villus surface = 2 × villus area + (villus – circumference × villus – thickness).

Finally, at least 10 crypts were isolated from 10 different villi of one specimen and the crypt length was measured under the stereomicroscope. The isolated crypts were floated on a slide and covered. Mitoses were counted under the microscope and given as counts per crypt.

In addition, the sections stained with haematoxylin and eosin were used to count the number of cells per 100 μm villus column and crypt column and the epithelial cell height under the light microscope using an eye-piece graticule.

**Measurement of 14C-L-phenylalanine absorption in vitro**

The accumulation rate of 14C-L-phenylalanine in biopsies was determined in vitro according to the method of Robinson et al. (1964) and Beck et al. (1976). Measurements were carried out by incubating the tissue for 30 minutes at 37°C in a solution consisting of 1 mM L-phenylalanine in Krebs-bicarbonate buffer containing 11:1 mM glucose, to which 2 μCi 14C-L-phenylalanine per 10 ml was added. Counts were measured after lysis of the tissue with 30% KOH in a Packard scintillation counter (type 3380). The extracellular space was determined using PEG (150 mg/10 ml) to which 2 μCi 14C-PEG per 10 ml and 20 mg glucose as a nutritional substance were added as above. Tissue uptake of phenylalanine was calculated from these data and expressed as nmol accumulated per μl intracellular space (ICR) assuming 80% tissue water.

**Determination of sucrase**

This was done in the whole homogenates of the biopsy specimens, using the method of Dahlqvist (1964). Enzyme activity was expressed in terms of the protein content of the homogenate, measured by the method of Lowry et al. (1951).

**Determination of enzyme activities in individual enterocytes**

Frozen sections were used for cytochemical determination of alkaline phosphatase, succinic dehydrogenase, and non-specific esterase as described earlier (Lorenz-Meyer et al., 1977). The reaction products were quantified by measuring the optical density using a Leitz microdensitometer (MPV II) (Gutschmidt et al., 1978). The results were corrected for the thickness of the sections and the time of incubation for development of the reaction product (optical density/min × mm).

**Statistical comparisons**

Throughout, the t test has been used.

**Results**

**Mucosal structure**

**Microscopic appearance**

No clear-cut changes in the mucosal surface of diabetics were observed under the dissecting microscope when compared with the controls, but the villi seemed to be somewhat broader. There was no definite evidence of alterations in the zones of villi and crypts or of changes in the structure of the surface epithelium or of the round cells in the lamina propria.

**Morphometric evaluation**

The measurements as obtained from haematoxylin-eosin-stained sections are given in Table 2. The number of villus cells per unit length was significantly increased in the biopsies from the diabetic patients, while epithelial cell height was unchanged. The number of crypt cells per unit length was not different from the controls, though the crypt cell height was reduced.

The measurements performed on the micro-dissected biopsy specimens are given in Table 3. Villus height was reduced and villus breadth increased in the diabetic mucosa, but this increase was not statistically significant. These data reflected

<table>
<thead>
<tr>
<th>Table 2  Morphometric data (X ± SEM): histology</th>
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</thead>
<tbody>
<tr>
<td>Diabetics</td>
</tr>
<tr>
<td>(n = 17)</td>
</tr>
<tr>
<td>No. of cells per 100 μm</td>
</tr>
<tr>
<td>Villus column</td>
</tr>
<tr>
<td>Crypt column</td>
</tr>
<tr>
<td>Height (μm)</td>
</tr>
<tr>
<td>Villus cells</td>
</tr>
<tr>
<td>Crypt cells</td>
</tr>
<tr>
<td>Mitoses per crypt (no.)</td>
</tr>
</tbody>
</table>

NS: not significant
Table 3  Morphometric data (± SEM): microdissection

<table>
<thead>
<tr>
<th></th>
<th>Diabetics (n = 17)</th>
<th>Controls (n = 20)</th>
<th>Statistical comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villi per unit area (no.)</td>
<td>21.9 ± 1.65</td>
<td>20.8 ± 1.06</td>
<td>NS</td>
</tr>
<tr>
<td>Surface of individual villus (mm²)</td>
<td>0.55 ± 0.043</td>
<td>0.47 ± 0.019</td>
<td>NS</td>
</tr>
<tr>
<td>Mucosal surface per unit serosal area (mm²/mm²)</td>
<td>11.4 ± 0.84</td>
<td>11.0 ± 0.82</td>
<td>NS</td>
</tr>
<tr>
<td>Villus height (µm)</td>
<td>380 ± 13.8</td>
<td>437 ± 10.7</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Villus breadth (µm)</td>
<td>360 ± 23.7</td>
<td>306 ± 11.0</td>
<td>NS</td>
</tr>
<tr>
<td>Ratio of villus height to villus breadth</td>
<td>1.2 ± 0.10</td>
<td>1.5 ± 0.07</td>
<td>P &lt; 0.025</td>
</tr>
<tr>
<td>Crypt height (µm)</td>
<td>165 ± 5.5</td>
<td>150 ± 3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Ratio of villus height to crypt length</td>
<td>2.4 ± 0.18</td>
<td>3.0 ± 0.16</td>
<td>P &lt; 0.025</td>
</tr>
<tr>
<td>No. of crypts per unit serosal area (mm²)</td>
<td>205 ± 9.7</td>
<td>198 ± 9.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

a change in villus shape from finger-like to broad leafy villi (as seen under the dissecting microscope). As a result, the ratio of villus height: villus breadth was significantly reduced in the diabetic mucosa (Table 3). There was a tendency towards an enlargement of the surface of the single villus but mucosal surface per unit area remained unchanged. Similarly, the number of villi and crypts per unit area was not statistically different between the two groups. Crypt length, on the other hand, was longer in the diabetic mucosa; although this difference was not significant, the altered height of the villus and crypt regions was convincingly reflected by a reduction in the ratio of the heights of villi and crypts (P < 0.025) (Table 3). These differences in morphometric findings were accompanied by a greater number of mitotic figures in the diabetic mucosa (Figure); this difference was most pronounced in the insulin-dependent group where the results differed significantly from those in the patients under oral antidiabetic drugs.

14C-L-PHENYLALANINE ACCUMULATION in vitro

The accumulation rates of 14C-L-phenylalanine (expressed as the 'distribution ratios' of the substrate between intra- and extracellular water) were not statistically different (Table 4) between the two groups of mucosal biopsies.

Table 4  Biochemical and microdensitometric data (± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Diabetics (n = 15)</th>
<th>Controls (n = 19)</th>
<th>Statistical comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accumulation of 14C-L-phenylalanine*</td>
<td>4.9 ± 0.34</td>
<td>5.5 ± 0.19</td>
<td>NS</td>
</tr>
<tr>
<td>Specific activity of sucrase (U/g protein)</td>
<td>41.3 ± 4.40</td>
<td>61.8 ± 6.15</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Protein contents of mucosal homogenates (g prot./g mucosa)</td>
<td>0.221 ± 0.0173</td>
<td>0.219 ± 0.0149</td>
<td>NS</td>
</tr>
<tr>
<td>Alkaline phosphatase (optical density/min × mm)</td>
<td>17.3 ± 0.95</td>
<td>18.0 ± 0.75</td>
<td>NS</td>
</tr>
<tr>
<td>Succinic dehydrogenase (optical density/min × mm)</td>
<td>1.1 ± 0.08</td>
<td>1.2 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Non-specific esterase (optical density/min × mm)</td>
<td>12.9 ± 0.73</td>
<td>10.7 ± 0.75</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Transport expressed as the 'distribution ratio' of the substrate between intracellular and extracellular water.

ENZYME STUDIES

The specific activity of sucrase in whole homogenates of mucosal biopsies was significantly lower in the diabetic group than in the controls (Table 4). On the other hand, the levels of alkaline phosphatase, succinic dehydrogenase, and non-specific esterase in the individual absorptive cells, assessed cytochemi-
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...cally, did not differ between diabetics and controls (Table 4). No difference was observed in the protein contents of the mucosal homogenates.

Discussion

The results of this study have shown that the human mucosal structure of the upper jejunum is distinctly altered in diabetes mellitus. It is characterised by a change in the shape of the villi with a reduction in villus height and an increase in villus breadth. Simultaneously, the crypts are slightly enlarged with a very marked increase in mitotic counts. As a result of this altered zoning of villi and crypts, the ratio of villus height: crypt length was significantly reduced. In addition, the increased number of epithelial cells per unit villus length revealed mucosal hyperplasia. Thus a low grade transformation of the mucosa has been shown and this was, as judged by the mitotic counts, more pronounced in insulin-dependent diabetics than in those who received oral hypoglycaemic drugs or simply maintained a diet.

Along with these morphological changes, a decreased sucrase activity in whole homogenates was observed. This change occurs characteristically in association with sprue-like pattern of the mucosa (Riecken and Martini, 1973; Menge et al., 1976) and is therefore in good agreement with the morphology but contrasts with the normal disaccharidase activities in human diabetes mellitus reported by others (Chaudhary and Olsen, 1973; Ruppin et al., 1974; Caspary et al., 1974). Alimentary influences as a cause of these differences are unlikely in view of the similar carbohydrate content of the food ingested by the control group. On the other hand, the enzyme contents of the individual cells as measured cytochemically were within the normal range in this investigation. Absorptive function as judged by $^{14}$C-L-phenylalanine accumulation in vitro tended to follow the decrease in sucrase activity, being slightly but not significantly reduced.

In contemplating these various findings, two major questions arise: first, how do these mucosal alterations compare with those occurring in the chronic experimental diabetic animal, and, second, what is the mechanism by which these changes are brought about? In chronic diabetes of the rat, analysis of jejunal structure and crypt cell kinetics has demonstrated an enlargement of the villi and an enhanced cell proliferation (Lorenz-Meyer et al., 1977). Similarly, the mucosa of the diabetic patients is characterised by increased mitotic counts, though this change is less pronounced in man than in the rat. This change in mitotic activity need not necessarily be associated with an increase in crypt length, as the region of mitotic activity may be expanded into the upper third of the crypt. In contrast with the animal model, however, the villi in the diabetic patients were not elongated but altered in shape with enlarged villus base and reduced villus height. Thus, in chronic diabetes of the rat, the mucosal response is quantitatively and qualitatively different from that seen in the upper jejunum of diabetic man.

When comparing these differences, it must be borne in mind that there are specific reasons which render difficult any comparison of diabetes in the experimental animal with that in man. The diabetic patients were in a well-balanced metabolic state at the time of investigation, while the diabetic rats were not. Blood sugar levels were well controlled in the patients but they were markedly increased in the animals. Nonetheless, an influence of a raised blood sugar level has so far been demonstrated only on mucosal function but not on structure; this was in acute experiments where continuous intravenous glucose infusion in the rat resulted in a significant increase in the mucosal-serosal flux-rate of glucose (Csáky and Fischer, 1977). On the other hand, no correlation was found between blood sugar levels and villus height (Lorenz-Meyer et al., 1977), whereas a positive correlation between food intake and villus height could be demonstrated. Furthermore, intestinal hyperplasia did not develop when the animals were only allowed normal food intake (Nakabou et al., 1974). From these experiments it is evident that the intestinal changes in the chronic diabetic rat result primarily from hyperphagia, which is very pronounced in chronic experimental diabetes and which has been shown to produce equivalent mucosal alterations in a number of different experimental conditions (Riecken and Menge, 1977). In the diabetic patients, on the other hand, a normal or low food intake was maintained.

Although these differences between diabetes in man and the experimental animal may explain the divergent adaptive responses of the small intestinal mucosa, the mechanism of the development of the mucosal transformation observed in diabetic patients remains speculative. A more or less pronounced sprue-pattern is usually associated with luminal stress due to a variety of damaging factors (Menge et al., 1977). An altered intestinal motility characteristic of human diabetes (McNally et al., 1969) could be associated with altered intestinal bacterial growth, but this has not been hitherto explored properly. However, bacterial overgrowth has been assumed in diabetic enteropathy on the basis of a therapeutic response due to tetracycline treatment (Malins and French, 1957).

Hormonal factors have also to be taken into account, among these hyperglucagonaemia, which is characteristic of diabetes mellitus. In fact, a sprue-
like pattern has been produced in the rat on chronic application of glucagon at pharmacological doses (Lorenz-Meyer et al., 1977). Glucagon reduces intestinal motility (Nechelel et al., 1966) and markedly influences intestinal blood flow (Varró and Csernay, 1966) and could, in the long term, lead to bacterial overgrowth in the intestine of the diabetic and a consequent transformation of the mucosa.

Finally, an influence of antidiabetic drugs on intestinal structure and function must be considered. The effect of biguanides on intestinal function has been explored in man previously (Bloch et al., 1973) and no alterations in absorption and mucosal morphology have been detected. In this study the data were obtained, as in the present investigation, after an overnight fast. Similarly, no changes have been reported as a result of treatment with sulphonylurea-derivatives. Furthermore, the data between the diabetic patients on antidiabetic drugs and diet exclusively did not differ significantly. Thus oral antidiabetic medication along with insulin is very unlikely to be responsible for the observed changes, in view of the fact that insulin-dependent patients revealed the same or even a slightly more pronounced mucosal response as the patients under oral medication.

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