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A new morphological classification during follow-up in patients with celiac disease. A three-dimensional observation by scanning electron microscopy

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Summary. The structure and ultrastructure of the villi of small intestinal mucosa was examined in 237 duodenal or jejunal biopsies taken from children with active celiac disease and during gluten-free diet. All biopsies were processed for light and scanning electron microscopy.

Conventional histology showed four different morphological aspects: total and subtotal villous atrophy in patients on unrestricted diet, partial villous atrophy and normal mucosa during gluten-free diet. Scanning electron microscopy demonstrated that in active celiac disease the severity of the intestinal lesions was related to individual vulnerability to gluten. Our results showed that during dietary treatment the process of mucosal healing was constant and strictly time-dependent. Furthermore, the ultrastructural examination has been relevant in evaluating the evolution of the villous regeneration. In this study a classification regarding the healing process of the small intestinal mucosa correlated with the time of start of dietary therapy is proposed.

Key words: Celiac disease, Scanning electron microscopy, Ultrastructure of jejunal mucosa

Introduction

A provisional diagnosis of Celiac Disease (CD) may be made when severe histological lesions involving upper intestinal mucosa are found. These alterations, consisting of total, subtotal or partial villous atrophy, crypt hyperplasia and increased chronic inflammatory infiltration of the lamina propria, are caused by the presence of gluten in the diet and improve when gluten is withdrawal (Dicke et al., 1953; Shiner, 1959; Shiner and Doniach, 1960; Yardley et al., 1962).

Clinical diagnosis is made via biopsy of small intestinal mucosa and is usually examined under dissecting and light microscopy (LM) (Walker-Smith, 1967; Loehry and Creamer, 1969). Scanning electron microscopy (SEM) has supported light and transmission electron microscopy (TEM) in studying the morphology of the intestinal mucosa (Demling et al., 1969; Marsh and Swift, 1969). In particular, SEM is considered to be the best method available today for studying surface details of biological samples; in fact, SEM permits three-dimensional study of a wide tissue surface using high resolution power with a great depth of field (Carr et al., 1981).

SEM descriptions of human intestinal mucosa were first reported twenty years ago (Toner and Carr, 1968; Demling et al., 1969; Marsh and Swift, 1969). Other papers soon followed, some of them being ultrastructural evaluations of lesions present in the small intestine of patients with CD (Asquit et al., 1970; Marsh et al., 1970). In spite of these studies, it has only been recently that the regenerative morphological process of the villi has been described once gluten was removed from the diet (Marsh, 1981; Halter et al., 1982; Poley, 1983; Carpino et al., 1985) and that it has been possible to evaluate the specific ultrastructural lesions of the enterocytes and microvilli (Stenling et al., 1984; Hardoff et al., 1986; Poley, 1988, 1992).

Numerous studies on this topic have been published but these have concerned a small number of patients. In this paper we evaluate and propose a specific classification of the intestinal mucosa during the healing process in 149 children with CD on the basis of SEM observations. Biopsies from children with constitutional short stature or chronic diarrhoea served as controls.

Materials and methods

Patients

From 1983 to 1993 a total of two hundred and thirty-seven biopsies were taken from 149 children (88 girls and 61 boys); their ages at the time of presentation ranged from 10 months to 16 years. They were referred to our department for chronic protracted diarrhoea, malabsorption, abdominal pain and short stature. In

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order to study the initial injury and the degree of mucosal regeneration, the patients were examined at the time of their first visit and at various times during their follow-up. The diagnosis of CD was made following the ESPGAN criteria (Meewisse, 1970; Walker-Smith et al., 1990).

The control group included 18 patients (7 girls and 11 boys); all underwent jejunal biopsies for short stature or chronic diarrhoea. Their ages ranged from 14 months to 13 years. Suction biopsies were performed on all patients in order to exclude organic intestinal disease (Groll et al., 1980).

Peroral biopsy

The specimens were taken using a pediatric double-port Kilby capsule or by endoscopy. The capsule was localized by fluoroscopy and all specimens were obtained from the area of the distal duodenum. After examination and orientation under dissecting microscope one biopsy specimen was processed for classical histological evaluation, the other was prepared for SEM.

Sample preparation

The specimens, prior to conventional LM, were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. They were then orientated and serially cut in a plane parallel to crypts and villi; each section contained the muscularis mucosae; all were stained by hematoxylin-eosin and periodic acid Schiff (PAS) reaction.

The biopsies prepared for SEM were fixed in 2.5% glutaraldehyde (0.1M cacodylate buffer at pH 7.4) for 48 to 72 hours. The specimens were washed for four hours in the same buffer and post-fixed for two hours with 1.3% osmium tetroxide and rewashed in the same buffer. Tissues were dehydrated by gradually increasing concentrations of acetone and then critical point dried in liquid CO_2. They were then mounted on aluminium stubs, coated with gold or gold-palladium and viewed under a Cambridge stereoscan 150 and a Hitachi S-4000 SEM, using an accelerating voltage of 5-20 W.

Results

Control patients

In all examined biopsies, LM revealed a normal mucosa, the intestinal villi were prominent, thin and of uniform height. Few lymphocytes, eosinophils and plasmacells were present in the mucosal lamina propria. The glandular crypts of Lieberkhün were straight with regular glandular architecture (Fig. 1).

The figures observed by SEM were basically similar to others previously described (Carpino et al., 1985). Briefly, their mucosa was characterized by villi, usually tongue-shaped, mitten and finger-like in appearance. Villi crests were tightly arranged and the crypt orifices were hidden by the villi; mucus and debris were absent from the epithelial surface (Fig. 2). At medium magnification the cell borders were easily identifiable, the enterocytes were dome-like and showed a hexagonal or pentagonal shape, the goblet cells were irregularly interspaced between the enterocytes and rare lymphocytes over the mucosal surface were also noted (Fig. 3). At high magnification, the microvilli presented a uniform and regular appearance and were not easily recognized because they were generally covered by a glycocalyx network probably mixed with residual mucus (Fig. 4).

Patients with celiac disease

Biopsy fragments evaluated by means of LM allowed the identification of four different morphological types. The aspects were always related to the dietary regimen adopted by the patients. Based upon our observations, and in accordance with observations made by the majority of authors, we divided the four types as follows: total, subtotal, partial villous atrophy and normal villous structure.

Ultrastructural studies by SEM permitted us to identify more morphological aspects related to regenerative processes that began once gluten was removed from the diet. The aspects of the intestinal alterations and mucosal recovery observed by SEM were divided into different stages as reported in Table 1.

Table 1. Morphological aspects of specimens examined by LM and SEM after gluten-free diet and classification by SEM.

No. CASES	SEX		GLUTEN-FREE DIET	LM ASPECTS	SEM ASPECTS	SEM CLASSIFICATION
	M	F				
44	19	25	Untreated	TA/STA	Flat mucosa	Total atrophy
26	10	16	14-28 days	TA/STA	Circular crest	Severe atrophy I
15	7	8	30-60 days	TA/STA	Semicircular crests	Severe atrophy II
17	7	10	3-5 months	STA/PVA	Parallel crests	Moderate atrophy I
19	7	12	6-8 months	PVA	Convoluted crests	Moderate atrophy II
46	19	27	9-16 months	PVA/NM	Short villi	Mild atrophy
70	31	39	17-24 months	NM	Finger-like villi	Normal mucosa

TA: total villous atrophy; STA: sub-total villous atrophy; PVA: partial villous atrophy; NM: normal mucosa; LM: light microscopy; SEM: scanning electron microscopy.

The most severe form of mucosal atrophy was seen only in patients on an unrestricted diet. The mucosa was flat with an aspect similar to that observed by LM. The openings of the glandular crypts or crypt wells were easily recognized and were irregularly spaced or gathered in groups of 2-4 at the bottom of small mucosal depressions (Fig. 5). At high magnification, the surface epithelium showed prominent enterocytes of different shapes and sizes. Only a few enterocytes retained their hexagonal or pentagonal shape. In the majority of epithelial cells the microvilli were short and scattered having free margins due to the absence of glycocalyx, the goblet cells preserved their morphology. An increased number of lymphocytes was clearly seen on the luminal surface (Fig. 6).

In patients on a gluten-free diet for more than 14 to 28 days, SEM revealed slight elevations in the mucosa that surrounded the openings of the glandular crypts. These collar-like structures were found around the periphery of the crypts (circular crests). The mucosa

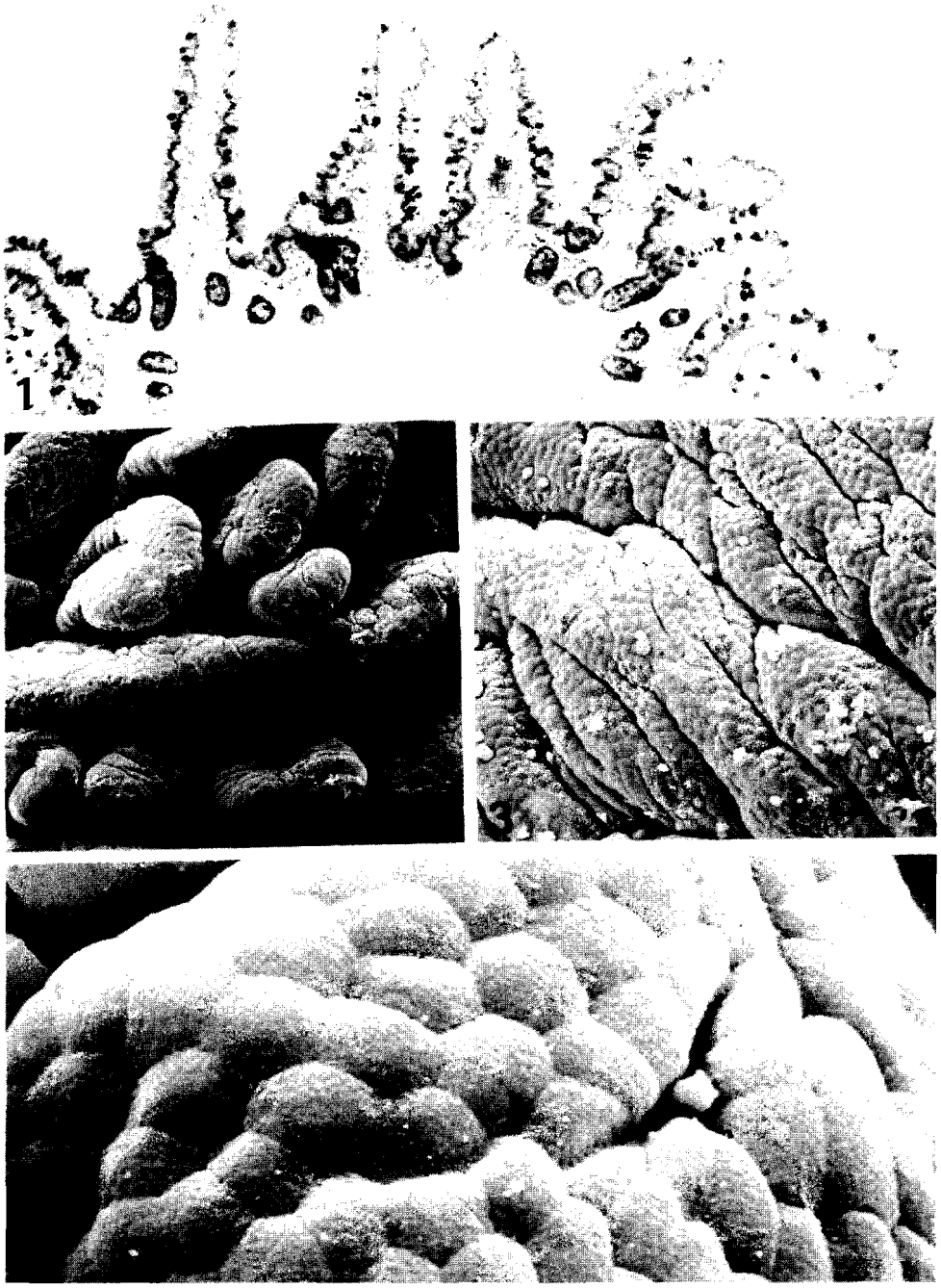

Fig. 1. Control: normal pattern of small intestinal mucosa. PAS, x 150

Fig. 2. Control: architecture of small intestinal mucosa shows finger-like and mitten-like shaped villi. SEM, x 200

Fig. 3. Control: the enterocytes are regular in shape and show a polygonal profile. Goblet cells are well defined, and occasional lymphocytes are also evident on the surface. SEM, x 1,500

Fig. 4. Control: a dense glycocalyx network covered the epithelial cells like a carpet. SEM, x 4,000

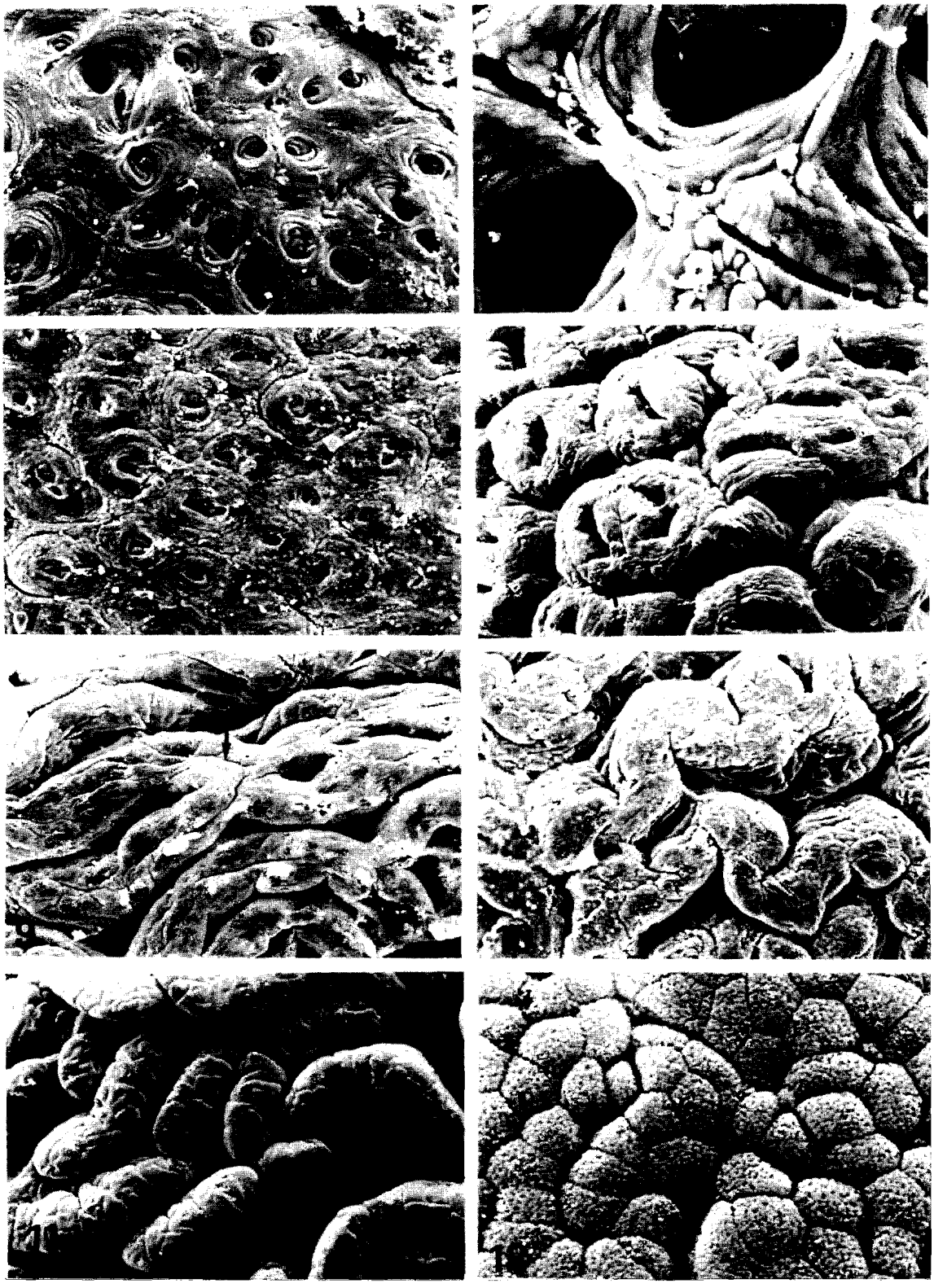

Fig. 5. Untreated celiac patients: total absence of villi, the mucosa is completely fat. SEM, x 200

Fig. 6. Untreated celiac patients: the enterocytes are irregular, convex and the mucosal surface has a cobblestone appearance; the goblet cells are preserved. SEM, x 2,000

Fig. 7. Treated celiac patients (21 days): the openings of the crypts are surrounded by collar-like crests. SEM, x 200

Fig. 8. Treated celiac patients (40 days): high semi-circular crests exhibit a cleft in two opposite points (arrows). SEM, x 200

Fig. 9. Treated celiac patients (4 months): linear mucosal elevations parallel to each other and joined by transverse bridges are seen (arrows). SEM, x 200

Fig. 10. Treated celiac patients (7 months): the mucosal surface is characterized by a low cerebriform aspect. The openings of glandular crypts are no longer visible SEM, x 200

Fig. 11. Treated celiac patients (14 months): the mucosa is elevated and shows villous ridges close to each other with a wall-like shape. SEM, x 200

Fig. 12. Treated celiac patients (14 months): the epithelial cells on the crest of the short villi are identical to those observed in the control subjects. SEM, x 3,000

interspaced between the glandular orifices appeared depressed, probably due to the loss of cell mass (Fig. 7).

In patients on a restricted diet for 28 to 60 days, the intestinal mucosa was raised and formed well defined circular crests along the crypts. These crests exhibited a cleft in two diametrically opposed points so that they appeared as two semicircles (semicircular crests) (Fig. 8). At this stage the enterocytes were less prominent with absent glycocalyx and showed ill defined cell borders.

The ultrastructural analysis of the mucosa in patients on diet therapy for 3 to 5 months exhibited semicircular crests surrounding the crypts. The crests were joined to those of the contiguous crypts by linear mucosal elevations which were parallel to each other and were often fused by transverse bridges (parallel crests) (Fig. 9).

After 6 to 9 months of diet a different stage of

Fig. 13. Treated celiac patients (20 months): in restored mucosa the newly-formed villi are finger-like or mitten-like in appearance. SEM, x 400

Fig. 14. Treated celiac patients (20 months): at the tip of the villi a patchy area of alterations of the enterocytes and microvilli are seen (arrows). SEM, x 2,000

intestinal recovery was evident. The mucosa was raised in long thick pleats giving a cerebriform appearance to the tissue (convoluted crests). At this time the glandular crypt openings were not easily identified, due to the growth of mucosal crests (Fig. 10). At high magnification, modifications of glycocalyx surface as well as of microvillous dimensions and arrangement were still present. Basically these consisted of microulcerations, decreased glycocalyx and reduced length and density of microvilli.

The intestinal morphology of patients on dietary limitations for a period of 9-10 and up to 16 months, presented a pattern characterized by an elevated mucosa arranged in regular ridges; the villi were elongated and assumed a wall-like shape, while others showed a typical «mitten-like» appearance. Nevertheless, in all biopsies examined the villi were lower when compared with the villi of normal mucosa (short villi) (Fig. 11). At medium magnification the cell borders were easily identifiable, almost all the apical surfaces of enterocytes being convex and assuming a cobblestone appearance (Fig. 12).

In patients on a gluten-free diet for more than 16 months a normal mucosa was the most frequently observed morphological pattern (92%), the others showed aspects of short villi. At low magnification the villi were practically indistinguishable from those found in the control group; they were finger or mitten-like, covered by enterocytes and goblet cells of normal shape and size (Fig. 13). However, in some biopsies, at the tip of the villi, a partial extrusion of cell cytoplasm was observed and also patchy alterations of the glycocalyx. Further microvilli appeared similar to those previously described in first degree of mucosal regeneration (compare Fig. 14 with Fig. 6). Moreover, these lesions were never spread over the surface, but alternated with zones of normal surface epithelium. They formed only slight lesions in normal intestinal mucosa (Fig. 14).

Discussion

In many studies (Marsh et al., 1970; Marsh, 1981; Halter et al., 1982; Poley, 1984, 1992; Stenling et al., 1984; Carpino et al., 1985; Hardoff et al., 1986; Moroni et al., 1989; Magliocca et al., 1992) SEM has been used to evaluate the general structure of specimens taken from normal and celiac jejunal mucosa. On the basis of these reports there is a general agreement that this method is useful in revealing morphological lesions of the mucosal surface as well as the aspects of villi regeneration while following a gluten-free regimen. Nevertheless, the studies available at this time have been made on a low number of patients with active CD and during recovery, and probably for this reason the results from different papers are not similar.

With the aim of unifying the data in the literature, we revised all the biopsies examined by SEM at our laboratory in the last ten years and compared the findings observed by LM with those by SEM, describing the most important surface alterations located in the small bowel mucosa in a large patient population (149 children) with CD.

The morphological diagnosis, performed by light and dissecting microscopy showed three major degrees of mucosal involvement, such as total, subtotal or partial villous atrophy (Maffei et al., 1979; Walker-Smith, 1979). In children with active CD, we observed a substantial agreement between findings obtained by LM and SEM; in fact, both techniques usually revealed the most severe form of mucosal injury. These results are in accordance with a recent report by Poley (1992) who described, in untreated CD patients, lesions of variable severity recognized by SEM and concluded that the severity of the initial lesion may depend on individual vulnerability of small bowel mucosa to gluten.

This opinion has already been proposed in a previous study (Magliocca et al., 1992) in which no relationship between duration of exposure and degree of mucosal involvement in patients undergoing gluten-challenge was found.

In patients treated on a gluten-free diet the mucosal morphology is in constant flux and the many features observed with SEM have been relevant in evaluating the evolution of the regenerative process. The data in our paper confirm the hypothesis of Loehry and Creamer (1969) and of Marsh et al. (1970). These authors represented the morphological events involved in the process of mucosal atrophy and villous recovery in CD patients with a diagram.

Our study demonstrated that the process of villi regeneration, secondary to gluten withdrawal, is strictly time-dependent and that it commences in the first days, as previously revealed (Carpino et al., 1985; Magliocca et al., 1987; Petrozza et al., 1987). The pronounced collar-like structures described around the crypt vestibules may be related to more rapid cell maturation with an increase of migration from the crypt wall to the mucosal surface; these results, confirmed by histological evidence of crypt hyperplasia associated with an elevated mitotic activity index, suggest a continuous restoration mechanism for the healing mucosa. The next phases brought about a growth of the circular crests to form brain-like structures still with dismorphic and low villi. Only after nine months did the villi, although incomplete, assume a regular pattern and could the mucosa be considered as almost normal. Most enterocytes also appeared normal and the microvilli and glycocalyx were well preserved.

By contrast, Halter et al. (1982), in 5 children on a dietary regimen for different periods of time, described in all a regenerative process of the mucosa, but they concluded that the degree of improvement did not always correspond to the length of time on the diet. Marsh et al. (1970), in one patient after 12 months of gluten-free diet, observed a scarse improvement of the mucosa which was demonstrated by the presence of collar-like structures. Stenling et al. (1984) in 19 of 21 biopsy specimens examined by low power SEM reported

a complete normalization of the mucosal architecture after dietary treatment for one year. Our results disagree with these authors. In fact, in all the cases of our study we noted a constant improvement of mucosal morphology which was always time-related suggesting a continuous process of villous regrowth. It is possible that these differences in the process of morphological improvement sometimes depend on poor compliance of patients in maintaining a strict gluten-free diet over a long period (Colaco et al., 1987).

At high magnification our SEM images permitted us to identify lesions of the enterocytes and alterations of microvilli and glycocalyx. These findings were most likely due to an altered cell turnover rate and changes were noted on vast areas of the villi. Enterocyte and microvilli lesions, also noted in patients with complete villous regeneration, were distinguished from simple artifacts (Phillips et al., 1979; Poley, 1988). These lesions combined with the loss of glycocalyx could be the entry-way for macromolecules (Menzies et al., 1979; Udall and Walker, 1982; Stenhammar et al., 1989), these, in turn, by immunological mechanisms may initiate or perpetuate the surface alterations (Walker, 1975; Bramble et al., 1985; Moroni et al., 1989).

Based on our ultrastructural observations, summarized in Table 1, it is possible to propose a classification regarding the healing process of small bowel mucosa correlated with the start of a gluten-free diet. Severe atrophy type I, present in the initial phase of diet therapy, is characterized by circular crests, and type II by semicircular crests which represent the first expression of morphological healing. Moderate atrophy type I represents continuous modifications present in the following grade of mucosal recovery and consists of parallel crests, and type II in convoluted crests. Mild atrophy is the better form of regrowth and is characterized by villi of a normal aspect but lower in height when compared to normal intestinal mucosa. Moreover, the observations by SEM of biopsies taken from patients with gastrointestinal disorders allowed the identification of microorganisms on the epithelial surface (Antonakopoulos et al., 1982; Rolston et al., 1986; Phillips et al., 1992) as well as Giardia Lamblia infestation (Scirè et al., 1991), which usually escapes identification by conventional histology.

Although, the regenerative process showed by SEM in our study is characteristic for patients with CD, this is to be considered non specific; in fact other authors have described similar aspects in patients with soy-protein intolerance (Poley and Klein, 1983) in children with IgA deficiency (Giorgi et al., 1986) and in other childhood enterophaties (Poley, 1983).

In conclusion, we can state that SEM appears more sensitive than LM in assessing the evolutionary phases of the healing process and that a good correlation between the degree of villous regrowth and the length of the dietary treatment, at the ultrastructural level, could be established. However, we believe that the histopathological evaluation by LM remains the method of choice for routine diagnosis of CD, and the use of SEM may be an aid in evaluating doubtful and borderline case in which it is difficult to make a correct diagnosis based on LM findings alone (Maki et al., 1990).

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