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SCANNING ELECTRON MICROSCOPY OF THE SMALL INTESTINE MUCOSA IN CHILDREN WITH CELIAC DISEASE AFTER LONG-TERM DIETARY TREATMENT

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Abstract

Jejunal mucosal specimens from twenty children with celiac disease were studied by light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) after one year of dietary treatment. An ultrastructural morphometric study was performed in five patients who had an intestinal permeability (IP) test. Seventeen patients were tested for serum antigliadin antibodies (AGA). In ten children, in whom LM showed partial villous atrophy, SEM and TEM examination confirmed the lesion. In the second group (10 children) with normal morphology at routine LM, SEM showed lesions of variable degree in 70% of cases. The morphological ultrastructural investigation showed good correlation with the immunological and functional data (IP test): ultrastructural damage of the jejunal mucosa after one year of a gluten-free diet was found in patients with positive serum AGA and an abnormal IP test. Furthermore, the morphometric study of the ultrastructural alterations allowed a quantitative, closer correlation between morphological and functional data. Our results suggest: 1) SEM and TEM investigations offer additional and more complete information on celiac patients, over LM alone. 2) The morphometric evaluation of the ultrastructural alterations highlights quantitative and reproducible correlations between morphological and clinical data, not strengthened by the subjective, qualitative study.

KEY WORDS : celiac disease, jejunal mucosa, light microscopy, transmission electron microscopy, scanning electron microscopy, morphometry, intestinal permeability test.

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Introduction

Gluten-sensitive enteropathy (GSE, celiac disease) is a disease in which the small intestine mucosa of susceptible persons is damaged by the presence of gluten in the diet (Shiner and Shmerling 1970, Shiner, 1973). In patients with the classical form of GSE, the intestinal mucosa shows stunted villi or a subtotal villous atrophy (Shiner, 1974, Poley, 1984); the surface cells display striking morphological abnormalities (i.e., irregular microvilli, shortening of the cells, increased vacuolation, swelling of the mitochondria) associated with functional defects (Rubin et al. 1966, Rubin, 1971). On removal of dietary gluten, both the clinical manifestation and the pathologic abnormalities revert towards normal: within three to six days of removal of gluten from the diet, the surface epithelial cells are already reverting towards normal (Falchuk, 1979). In children maintained on a gluten-free diet, the architecture of the mucosa frequently achieves complete normality with return of villous structure (Falchuk, 1979). Nevertheless, Stenling et al. (1984a) demonstrated that scanning electron microscopy (SEM) is able to detect lesions in 75% of patients on a gluten free diet for 1 year, although routine histological investigation revealed lesions in only 25% of patients.

We have performed a histological, ultrastructural, morphometric, immunological and functional study on 20 children affected by GSE and maintained on a gluten-free diet, to evaluate whether ultrastructural analysis can be useful in the follow-up of the alterations of the small intestine mucosa in this condition.

Patients and Methods

The study group consisted of 20 children of both sexes, aged 18 months to 16 years (TABLE 1). In all cases the diagnostic criteria of celiac disease were fulfilled (McNeish et al.,1979). The diagnostic criteria included the finding of flat jejunal mucosa on free diet associated with a clinical response to gluten withdrawal. Concerning the diet, patients and their parents were informed after the first intestinal biopsy was done, before starting the gluten-free diet. Explanations were usually given by a doctor; furthermore, a list of gluten-free products were given to the patients. The patients dietary habits were checked by the 24 h-recall method every two months together with a clinical evaluation. The current studies were performed after about 1 year on a gluten free diet (range 12-15 months).

Seventeen subjects were tested for serum antigliadin antibodies (AGA), IgA and IgG by an enzyme-linked immunosorbent technique (ELISA) (Savilahti et al.,1983).

Small intestinal permeability (IP) on five patients was determined by the cellobise/mannitol test (Cobden et al., 1980, Hamilton et al., 1982, Ford et al., 1985, Lifschitz, 1985, Pearson et al., 1985, Catassi et al., 1988). Twenty jejunal biopsies at the ligament of Treitz were obtained with a pediatric Watson capsule. After the sampling, the mucosal specimens were immersed in the fixative (2% glutaraldehyde in 0.1M cacodylate buffer pH 7.4) and divided into portions for light microscopy (LM), for SEM (sizes 1-2 mm^3) and transmission electron microscopy (TEM) (sizes 1-2 mm^3). For SEM, specimens were immersed in the fixative for 2h, postfixed with 1% osmium tetroxide in the same buffer for 1h, dehydrated in ethanol gradients, critical-point dried (cdp 010 Balzers) and coated with 20 nm gold. The gold coating has performed with sputter coater S150A Edwards which allows the control of the temperature excluding every tissue damage due to overheating. The specimens were examined with a Philips 505 SEM using an accelerating voltage of 20 kV ; in all specimens standard views were obtained at low (50x, 150x), medium (500x, 1500x) and high (4000x, 10000x) magnifications. For TEM, after fixation for 2h, the specimens were postfixed with 1% osmium tetroxide in the same buffer for 1 h. Specimens were then dehydrated with a gradient of ethanol solutions and embedded in an Epon-Araldite mixture. Thin sections were stained with uranyl acetate-lead citrate. Observations were made in a Philips 301 transmission electron microscope; in all specimens microvillous length and glycocalyx thickness were estimated on micrographs of the middle third of villi at the enlargement of 7100x. Seven specimens of intestinal mucosa in children with constitutional short stature were used as control.

TABLE 1. Clinical data of the study group

Pat/sex/ age	D m	AGA/IgA	AGA/IgG	IP Ce/Ma nv= 0.014±0.007
1/f/24m	12	neg	neg	-
2/f/9y	15	neg	pos (1:160)	-
3/f/24m	15	neg	neg	-
4/m/20m	12	neg	neg	-
5/m/28m	15	neg	neg	-
6/f/30m	12	neg	pos (1:640)	-
7/f/31m	14	neg	neg	-
8/f/31m	13	neg	neg	-
9/m/28m	12	neg	neg	-
10/f/36m	14	neg	pos (1:160)	p (0.066)
11/m/30m	12	-	-	-
12/m/24m	12	-	-	-
13/m/24m	13	-	-	-
14/f/72m	15	neg	neg	-
15/f/12y	14	pos (1:40)	pos (1:160)	-
16/f/18m	13	pos (1:40)	pos (1:640)	-
17/f/56m	12	neg	neg	p (0.057)
18/m/24m	12	neg	pos (1:640)	n (0.019)
19/f/16y	14	pos(1:160)	pos (1:160)	p (0.076)
20/m/49m	13	neg	neg	n (0.019)
c1/m/13m		neg	neg	n (0.019)
c2/f/20m		neg	neg	n (0.020)
c3/f/21m		-	-	-
c4/f/39m		-	-	-
c5/f/20m		neg	neg	n (0.014)
c6/f/26m		-	-	-
c7/m/11y		neg	neg	n (0.014)

Pat = patient; c = control
AGA = serum antigliadin antibodies
IP = intestinal permeability test
D = duration of gluten free diet
m = months; y = years
p = pathologic; n = normal; nv = normal value

A morphometric study was performed on the 5 patients with available data from the IP test and on 2 out of 7 control cases (i.e., children with constitutional short stature). In these specimens the amount of area without glycocalyx was studied by SEM: ten cobblestone areas (4000 μm^2)in the middle third of villous structure were examined in each patient at the magnification of 2500 x using a SEM IPS Kontron

in semiautomatic mode. The density of microvilli (No. microvilli/µm) was studied by TEM: ten areas from the apical part of the enterocyte in the middle third of villi for each patient were examined at the final magnification of 7100 x. Areas for TEM and SEM morphometric study were chosen according to a randomized systematic method (Weibel and Bolender,1973) excluding the peripheral parts of the specimens.

Results

Immunological and functional (IP) data of the study group are reported in TABLE 1.

TABLE 2. Morphological data of the study group

Pat	LM	TEM			SEM			
		MICROVILLI		GCD	V	CSA	ECI	MVI/GCD
		l	ir					
1	N	d	+	+	PVA	+	+	+
2	PVA	d	+	-	PVA	+	+	+
3	PVA	d	+	-	PVA	+	+	+
4	PVA	d	+/-	-	PVA	+	-	+
5	PVA	d	+/-	-	PVA	+	+	+
6	PVA	n	+/-	+	PVA	-	-	+
7	N	n	+/-	+/-	PVA	+	-	+
8	N	n	-	-	N	-	-	-
9	N	n	-	-	N	-	-	-
10	N	d	+	+	N	+	+	+
11	sPVA	fd	+	+	sPVA	+	+	+
12	N	n	+	+	N	+	+	+
13	N	d	+	+/-	PVA	-	+	+
14	N	n	+	+/-	PVA	+	+	+
15	PVA	n	+/-	+	PVA	+	+	+
16	PVA	n	+/-	-	PVA	+	+	+
17	N	fd	+	+/-	PVA	+	+	+
18	PVA	n	+/-	+	PVA	+	+	+
19	sPVA	d	+	+/-	sPVA	+	+	+
20	N	n	-	-	N	-	-	-

Pat= patient; GCD = glycocalyx distortion
CSA = cobblestone appearance
ECI = enterocyte irregularity
MVI = microvillous irregularity
V = villi; l = length
ir = irregularity; n = normal (1.0 µm)
d = decrease (<1.0 µm); fd = focal decrease
N = normal; PVA = partial villous atrophy
sPVA= severe partial villous atrophy
+ = present; - = absent

In controls, routine histology, SEM, and TEM showed the normal aspects of the small intestine mucosa from healthy children (Stenling et al., 1984b) (fig. 1a).

The results of LM, TEM and SEM study of children with celiac disease after dietary treatment are reported in TABLE 2 (SEM and TEM data are referred to the middle third of the villi). In light microscopy 10 out of 20 specimens showed alterations of the villus-crypt ratio, which were classified as partial villous atrophy (PVA)(Stenling et al., 1984a). The alterations were mild in 8 cases and more severe in 2 cases (sPVA).

Low power SEM of all specimens with PVA showed blunted villi or villous ridges of reduced height (fig.1b): the 2 cases which appeared severe by light microscopy showed a marked depression of villous ridges with a semicircular arrangement surrounding the crypt openings.

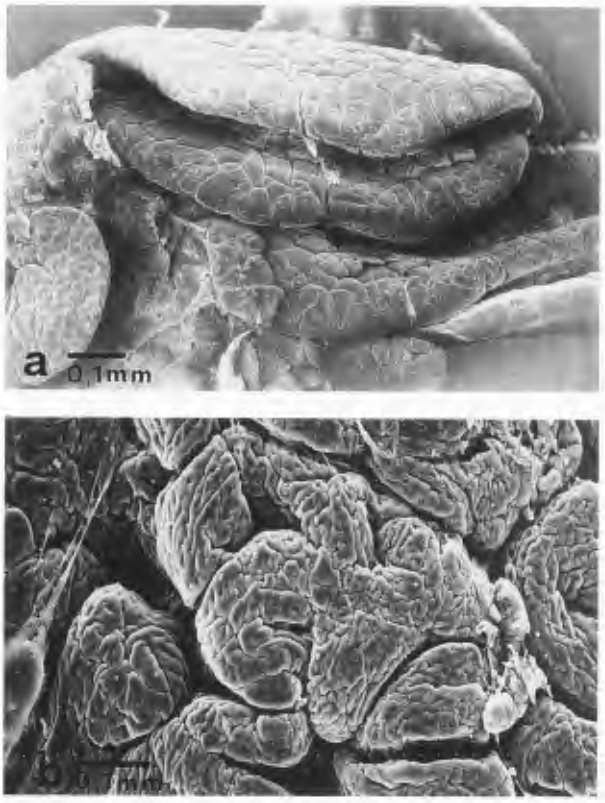

Fig.1. a) Normal jejunal mucosa with tongue-shaped villi of a child with constitutional short stature. SEM b) Jejunal mucosa with partial villous atrophy (PVA). SEM

At higher magnification by SEM, in all specimens with PVA , extensive alteration of the surface epithelium was visible.

Enterocytes with variation of size and shape or a "cobblestone" appearance (enterocytes with dome-shaped apical surfaces) were frequent

(fig.2). The glycocalyx was missing over wide areas, and many enterocytes showed frayed microvilli (fig.3a). In one case (n.19) an increased number of goblet cells was present. An excess of bacterial colonization was seen in one case (n. 4).

Examination by TEM showed a variable degree of damage both of the brushborder (with rarefied and shortened microvilli) and glycocalyx (fig.3b).

Enterocytes showed apical lysosomes and multivesicular bodies.

Among the 10 cases classified as normal on routine histological examination 7 showed alterations at the ultrastructural level. In 5 cases morphological alterations were evident under low power SEM (PVA, sPVA); in the other 2 cases, enterocyte irregularity and glycocalyx distortion, as described above, were present but usually were of a less severe degree. These observations were confirmed by TEM analysis.

The results of morphometric studies are reported in TABLE 3.

TABLE 3.Morphometry of microvilli and glycocalyx by TEM and SEM

Pat	MNv (No./µm) TEM	% cobblestone area without glycocalyx SEM	IP Ce/Ma nv = 0.014±0.007
10	3.481 ± 1.101	14.00	0.066
17	3.032 ± 0.125	12.33	0.057
18	2.292 ± 0.224	3.15	0.019
19	1.869 ± 0.259	50.14	0.076
20	2.776 ± 0.141	0.88	0.019
C5	4.176 ± 0.256	0.00	0.014
C7	5.095 ± 0.339	0.08	0.014

Pat=patient ; MNv=numerical density of microvilli
C =control subject ; IP =intestinal permeability
nv = normal value

The 3 patients with an altered IP test showed the greatest percentage of surface without glycocalyx on the cobblestone area in the middle third of villi (glycocalyx decrease,GD). In control patients, the GD was virtually absent on the middle third of the villi. All five patients in dietary treatment showed a lower density of microvilli when compared with control subjects.

Discussion

LM data overlap electron microscopy (EM) data in studies of the jejunal mucosal alterations in celiac patients during acute illness and after challenge with dietary gluten. These lesions range between slight (PVA) and subtotal villous atrophy. Less correlation is evident between electron and light microscopic appearances in celiac patients with gluten-free dietary intake (Poley, 1984, Stenling et al., 1984a). In the present work, we studied electron microscopic modifications in 20 patients after gluten elimination. The specimens obtained from the Treitz region were divided for LM, SEM, and TEM processing.

In theory, because the lesions in celiac patients are homogeneously diffuse, the same alterations should be present in each of the three blocks, but SEM, combined with TEM, was more sensitive also for lesions that can be seen usually also by LM as PVA. As a matter of fact, PVA was detected, not only, in the positive cases by LM (10 cases) but also in five cases in which LM failed to reveal PVA. Furthermore, in 2 cases which were classified as normal, also at low magnification by SEM and TEM, EM revealed minor alterations when examined at higher magnification.

A second result that should be stressed, is the agreement between ultrastructural and functional data: two out of three patients with an abnormal IP test, did not show light microscopic alterations, while ultrastructural investigation demonstrated morphological damage.

When ultrastructural morphometric data are added to the qualitative data, more interesting correlation can be done. As a matter of fact the more abnormal IP tests were found in those cases in whom a higher percentage of GD was found and patients with a normal IP test had only a small area of GD. It should be pointed out that case n.18 had a normal IP test but LM and EM disclosed morphological alterations. In this case morphometry showed low levels of GD (see TABLE 3). Therefore it seems that the IP test is correlated more to the glycocalyx appearance than to the integrity of the villi; but this correlation should be confirmed in a larger case series. Moreover EM correlated well with immunological data : all seven cases with antigliadin antibody positivity had ultrastructural alterations, while one of them showed a normal light microscopic appearance.

Our results suggest that SEM and TEM investigation, in addition to routine histology, offers more complete diagnostic information on celiac patients. This is in agreement with the results of other authors in this and other pathologies (Dvorak et al., 1979, Poley and Rosenfield, 1982, Stenling, 1984a). The addition of morphometry at EM level reveals interesting

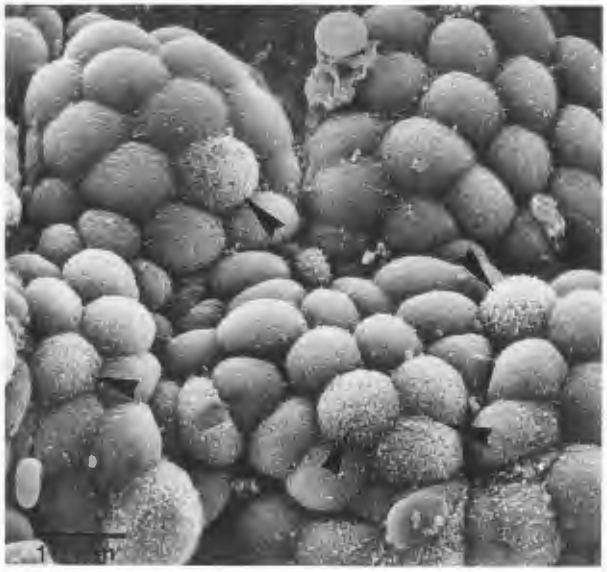

Fig.2. Cobblestone appearance: enterocytes display a dome-shaped apical surface and glycocalyx absence is visible in some cell (arrowheads). SEM

correlations between morphological and functional data that cannot be appreciated by a qualitative study alone.

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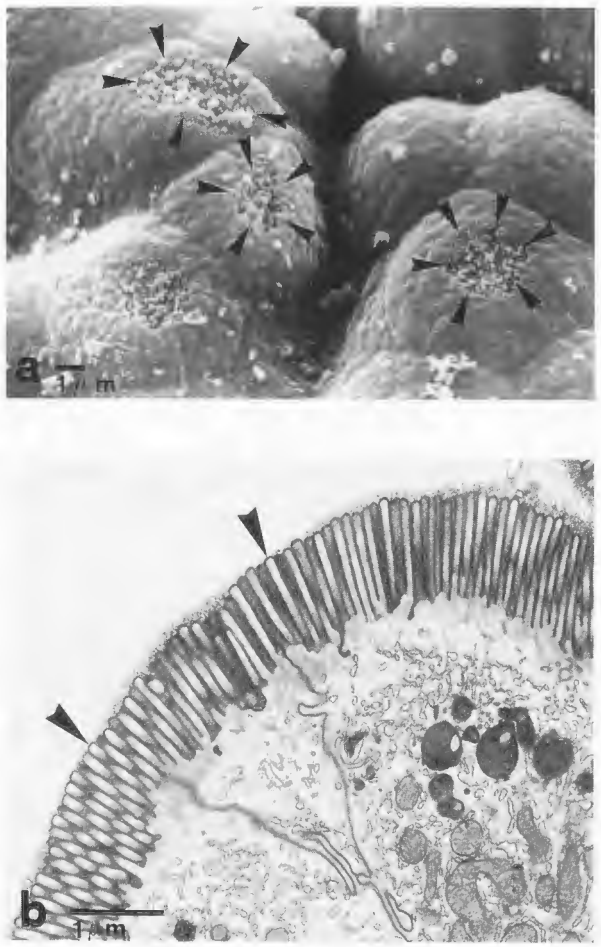

Fig.3. a) "Crater"-like appearance of the dome-shaped surface in a cobblestone area (arrowheads). This alteration is due to focal reduction of the glycocalyx disclosing the bare microvilli. SEM b) Transmission electron microscopic appearance of the crater-like surface of the enterocytes showed in Fig. 3a (arrowheads). TEM

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Discussion with Reviewers

S. Siew: What were the morphological findings in the small intestine mucosa of these 20 children before the gluten free diet was instituted?
Authors: Intestinal biopsy performed before the gluten-free diet showed an uniform aspect: in all 20 patients the mucosa was flattened.

S. Siew: What was the degree of regression of the pathology after one year of dietary treatment?
Authors: After about one year of gluten - free diet the morphology of the mucosa improved in all patients; but the degree of regression of the pathology was variable and it is described in details in the "Results" section. It looks unrelated to the time of recovery.

R. Stenling: The new information and major result of this study is the possible correlation between the increased permeability and the damage or structural alteration of the glycocalyx layer of the enterocytes. The definition, visualization and extension of this alteration within the mucosa surface are therefore important. Was there a qualitative variation of the damage of the glycocalyx? Did you also observed a glycocalyx decrease outside cobblestone areas? Was there a difference between the crests and middle regions of the villi? Can damage to parts of the apical surfaces of the enterocytes as shown in fig. 3a be excluded from preparative influence?
Authors: With the method used we can only verify the absence or reduction of the glycocalyx. Both these alterations were evident mainly in the cobblestone areas. Alterations of glycocalyx are regularly present at the apex of the villi in normal subjects: in pathologic conditions they extend to the middle region of the villi, even in absence of cobblestone areas. Preparation artefacts cannot be ruled out in specimens processed for EM: in our study, however, the controls always showed a good preservation of the glycocalyx ,furthermore the same lesion is visible in the same patients with both the ultrastructural techniques (TEM and SEM).

R. Stenling: The high frequency of PVA, 10/20 by LM and 15/20 by SEM contradict several other reports including the study by Stenling et al. referred to in the text. Are there possible explanations? Could factors such as definition of the structural deviation for PVA or control of the dietary intake of gluten influence this?
J.R. Poley: Why do so many patients show PVA or sPVA, after having been on a gluten-free diet?
Authors: Our definition of structural deviation for PVA agrees with the one used by Stenling et al. and its SEM appearance is shown in fig.1 b. This picture shows only a part of a specimen but it expresses the diffuse damage through the specimen. It is true that in our study the frequency of PVA was higher than in other studies including the one by Stenling et al., but in our study all the cases classified as PVA at LM level showed the same appearance illustrated in fig.1b

when examined by SEM. Therefore in our study SEM confirmed LM diagnosis. SEM, in our opinion, is more accurate in detecting PVA because it shows typical convoluted arrangement of the villi together with the shortened villi and as a matter of fact, SEM detected five further cases of PVA classified as normal by LM. In our study,diet was carefully explained to all patients' parents. Subjects suspected of gluten assunction were excluded from the study group but it should be remembered that these studies were performed with groups living in different countries. In our opinion, "restitutio ad integrum" is not so frequent as usually thought in children with celiac disease after one year of dietary treatment.

J.R. Poley: Why are these peculiar changes just on the apex of enterocytes? What explanation may be forwarded? The patients were on a gluten-free diet, and two out of five patients with focal loss of glycocalyx had a normal villous architecture by LM and SEM.
Authors: Generally, this type of lesion is present in enterocytes with dome-shaped surface. The cause is unknown: we think that an increased susceptibility to mechanical factors could be important.

J.R. Poley: Eight/twenty patients by LM had PVA, two of twenty had sPVA, whereas a 13 of 20 by SEM had PVA and 2 of 20 sPVA by SEM. Five of twenty were normal by LM but had PVA by SEM. Is this due to patchy lesions? We always assume that mucosal lesions in celiac disease are homogeneous.
Authors: All patients with PVA or sPVA by LM showed PVA or sPVA at ultrastructural examination. Besides , TEM and SEM showed lesions also in patients with normal LM appearance of the gut mucosa. We think that this difference is due to the higher sensitivity of ultrastructural examination rather than to the existence of patchy lesions.

J.R.Poley: What was the percentace of cellobiose and mannitol recovery?
Authors: The percentage of cellobiose and mannitol recovery is shown in this TABLE:

Pat	IP Ce/Ma	% cellebiose	% mannitol
10	0.066	0.90	13.62
17	0.057	0.32	5.6
18	0.019	0.14	7.62
19	0.076	0.57	7.55
20	0.019	0.11	5.84

J.R.Poley: Hodges et al. (Archivies of Disease in Childhood 1989; 64: 853) have shown that cellobiose/mannitol differential permeability in individuals with iron deficiency were much higher as compared to those who were not iron deficient. In this regard, were any of your patients listed in TABLE 3 iron deficient to explain higher permeability ratios?
Authors: In our study patients with iron deficiency were excluded.