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Enrico Solcia · Laura Villani · Ombretta Luinetti
Erminio Trespi · Elena Strada · Carmine Tinelli
Roberto Fiocca

Altered intercellular glycoconjugates and dilated intercellular spaces of esophageal epithelium in reflux disease

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Abstract *Background and aims:* The usefulness of histological diagnosis of gastroesophageal reflux disease (GERD) is limited by poor specificity or sensitivity of available diagnostic tools. Recently, ultrastructural morphometry showed interstitial space dilation (ISD) to be a reliable sign of reflux disease. Aims of this study were to (a) search for a light microscopy equivalent of ISD, (b) test its diagnostic value, and (c) look for a possible role of intercellular glycoconjugates in its genesis. *Methods:* Esophageal grasp biopsies were taken during endoscopy, 2–3 cm and 6–7 cm above the squamocolumnar junction, from patients under investigation for GERD symptoms. The biopsies were fixed in aldehyde solutions and embedded in resin for electron microscopy or in paraffin for routine histology, and the glycoconjugates underwent immunohistochemistry using 3-fucosyl-*N*-acetylactosamine antibodies. *Results:* Irregular intercellular space dilation was detected in the basal and prickle layers using both light and electron microscopy. Hematoxylin–eosin preparations showed ISD in 20 of 22 (90%) erosive esophagitis cases, 30 of 44 (68%) endoscopy negative GERD cases, and 1 of 12 (8%) controls, with good interobserver (K=0.75) and bioptic site reproducibility. ISD correlated with loss or rearrangement of intercellular glycoconjugates of the overlying layers and with granulocyte (eosinophil and/or neutrophil) infiltration. *Conclusions:* Light microscopy ISD is a suitable index of GERD. Alterations of intercellular glycoconjugates are likely to have a role in the genesis of ISD and GERD.

Key words Esophagus · Esophagitis · Reflux disease · Intercellular glycoconjugates · Intercellular space dilation

E. Solcia (✉) · L. Villani · O. Luinetti
Department of Human Pathology,
University of Pavia and IRCCS Policlinico San Matteo,
Via Forlanini, 16, I-27100 Pavia, Italy
Tel.: +39-382-528474, Fax: +39-382-525866

E. Trespi · E. Strada
Endoscopy Unit, University of Pavia
and IRCCS Policlinico San Matteo, Pavia, Italy

C. Tinelli
Epidemiology Units, University of Pavia
and IRCCS Policlinico San Matteo, Pavia, Italy

R. Fiocca
Department of Pathology, University of Genova, Genova, Italy

Introduction

Various histological changes of esophageal epithelium have been documented in patients with erosive and nonerosive gastroesophageal reflux disease (GERD), from basal cell hyperplasia to papillae elongation and intraepithelial infiltration of inflammatory cells [2, 14, 15]. The diagnostic value of these findings has been the subject of many investigations leading to conflicting results [4, 25, 30]. In general, histological lesions were well represented in the esophageal mucosa, showing erosive esophagitis at endoscopy. Unfortunately, they proved less predictive of endoscopically negative GERD, either because of low sensitivity (infiltration of neutrophils and eosinophils) or of poor specificity (lymphoid cells infiltration, basal cell hyperplasia, papillae elongation). Therefore, at present, the contribution of histology to the diagnosis of GERD is relatively limited, as it adds little to the diagnostic power of endoscopy and pHmetry.

Dilation of intercellular spaces (ISD) has been reported in electron microscopy investigations to be a specific change of acid-damaged esophageal epithelium, either in acute experiments on rabbits or in humans suffering from GERD [3, 12, 29]. Increased paracellular permeability to acid seems crucial in the pathogenesis of ISD, possibly through damage to epithelial cell membranes causing impaired sodium transport and water accumulation in the intercellular space [3, 18, 28]. Although the occasional occurrence of ISD in esophageal biopsies of human reflux esophagitis has been mentioned [20], no definite light microscopy equivalent of ultrastructural ISD has been characterized up to now.

An amorphous to finely fibrillar material has been shown, using ultrastructural studies, to fill the space in between cells of the squamous and prickle (spinosum) layers of the esophageal epithelium [10, 17, 19]. The glycoprotein nature of the most part of such material is supported by a number of light and electron microscopy investigations showing its reactivity with periodic acid-Schiff (PAS), periodic acid-thiocarbohydrazide-silver proteinate, alcian blue, concanavalin A, or by immunohistochemical tests for 3-fucosyl-*N*-acetyllactosamine (hapten X, Lewis X, CD15) and carcinoembryonic antigen (CEA) [6, 9, 11, 19, 21] and by the extraction of a 180-kDa glycoprotein [22]. Tracer experiments suggest that the intercellular glycoconjugates (IGs) have a crucial role in sustaining the barrier function of the epithelium against penetration of fluids and solutes from the lumen, by working as an impermeable "cement" sealing the intercellular spaces, especially in the squamous cell layer [19]. It may be anticipated that any damage to this protective intercellular cement represents a potential cause of increased paracellular permeability, ISD and, with more severe acid permeation, epithelial cell damage. However, no information is available on the actual changes of IGs in reflux esophagitis.

In this study, esophageal biopsies from erosive reflux esophagitis, non-erosive reflux disease and control subjects have been investigated (1) to search for light microscopy equivalents of ultrastructural ISD, (2) to identify alterations of IGs or superficial and prickle epithelium possibly associated with increased permeability to acid and (3) to evaluate the potential diagnostic power of histological findings compared with symptoms, pHmetry, and endoscopy.

Materials and methods

One hundred patients suffering from heartburn, acid regurgitation, or various dyspeptic symptoms underwent clinical evaluation for possible reflux disease. Twenty-four-hour pHmetry was performed according to DeMeester et al. [5] and Schindlbeck et al. [24]. Patients were considered pathologic when the percentage time with a pH of less than 4 was above 4.2% of the total time, above 6.3% of the time in upright position, or above 1.2% of the time in supine position. Reflux symptoms were evaluated according to severity and frequency with the help of a questionnaire, as previously reported [30]. Only cases with heartburn or acid regurgitation occurring regularly or interfering with the patient's ordinary life were considered significant for GERD. The patients underwent esophagogastroduodenal endoscopy, during which esophagitis was assessed according to the Savary-Miller criteria, and two routine grasp biopsies were taken 2–3 cm above the Z-line. To test for site-related variability of histological findings [31], additional biopsies were taken at 6–7 cm in 30 cases negative for erosive esophagitis [30]. In erosive cases, biopsies were taken on the border of the erosion as well as from non-erosive mucosa.

Fourteen patients were dropped from the study because they refused or failed to complete pHmetric investigation, symptoms were inappropriately assessed or remained undefined, lesions other than esophagitis were found (peptic ulcer, cancer), and/or less than two bioptic specimens of esophageal mucosa suitable for histological evaluation were available. Of the 86 subjects investigated, 12 endoscopy-negative cases were taken as controls. They complained of various reflux-unrelated dyspeptic symptoms (nausea, early satiety, post-prandial fullness or bloating of the upper abdomen, and food-relieved epigastric pain) while being free of reflux symptoms (heartburn, acid regurgitation, dysphagia, non-cardiac chest pain, respiratory or throat symptoms) and showing a pH less than 4 for less than 4.2% of total recording time, less than 6.3% of time in upright position, and less than 1.2% in supine position [5, 24, 30]. Among the remaining 74 symptomatic patients, 22 showed endoscopic evidence of erosive reflux esophagitis, while 44 had reflux symptoms as well as evidence of pathologic reflux during the 24-h pHmetry coupled with negative endoscopy (endoscopy-negative GERD). Eight "borderline" cases included five patients with reflux symptoms coupled with negative pHmetry and three patients with moderately pathologic pHmetry not associated with symptoms diagnostic for GERD.

Tissue specimens from all cases were fixed with 4% formaldehyde in 0.4% sodium acetate plus 0.4% calcium acetate for 24–48 h, dehydrated in ethyl alcohol, and embedded in paraffin according to the routine procedure of our laboratory [7]. Five-micron deparaffinized sections were stained with hematoxylin–eosin, Giemsa and alcian blue-PAS-hematoxylin methods or immunostained using the avidin-biotin procedure [13] using anti-CD15 (leu-M1) mouse monoclonal antibodies (clone MMA, Becton Dickinson, San Jose, Calif.; or clone Tii 9, Biotest, Dreieich, Germany) directed against the lacto-*N*-fuco-pentose III/3-fucosyl-*N*-acetyllactosamine oligosaccharide. Hematoxylin–eosin and Giemsa sections were evaluated for granulocyte infiltration (intraepithelial eosinophils and intraepithelial as well as lamina propria neutrophils) and microscopic erosion-necrosis as previously reported [2, 26, 30]. CD15-immunostained sections were used to investigate the "cement" material filling the intercellular spaces; they were also of help in detecting intraepithelial neutrophils [23].

In 12 cases (7 patients with pHmetry-positive GERD, with or without endoscopic evidence of erosive esophagitis, and 5 pHmetry- and endoscopy-negative non-GERD subjects), two additional biopsies of esophageal mucosa were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 h at ice-cold temperature, post-fixed in 1% osmium tetroxide for 1 h at room temperature, and embedded in Epon-Araldite resin. Ultramicrotomic sections (0.5–1 μm) were either stained with toluidine blue and viewed in the light microscope or contrasted with uranyl/acetate and lead citrate and observed in a Zeiss EM 902 electron microscope (at ×250 to ×10.000 enlargement) equipped with an electron energy-loss spectrometer (Electron-Spectroscopic Imaging, ESI). In addition, ultrathin resin sections consecutive to the above semi-thin sections were contrasted with uranyl and lead and observed under ordinary transmission electron microscopy.

The Chi-square test was used to determine whether observed differences were statistically significant; a two-tail P value <0.05 was considered to be significant. Interobserver variation was evaluated by Kappa (K) statistics and 95% confidence interval [8].

Results

CD15 (Hapten X; 3 fucosyl-*N*-acetyllactosamine) immunoreactive glycoconjugates

Normal esophageal epithelium

CD15-immunoreactive glycoconjugates were easily detected in the intercellular space of the prickle and squamous cell layers of human esophageal epithelium (Fig. 1A). A granular reactivity was also seen in the peripheral cytoplasm of the prickle cells, especially in the mid and upper part of their layer (Fig. 1B). Neither cytoplasmic nor intercellular reactivity were observed in the basal cell layer. The intercellular CD15 immunoreactivity frequently showed unequal distribution in the stratum

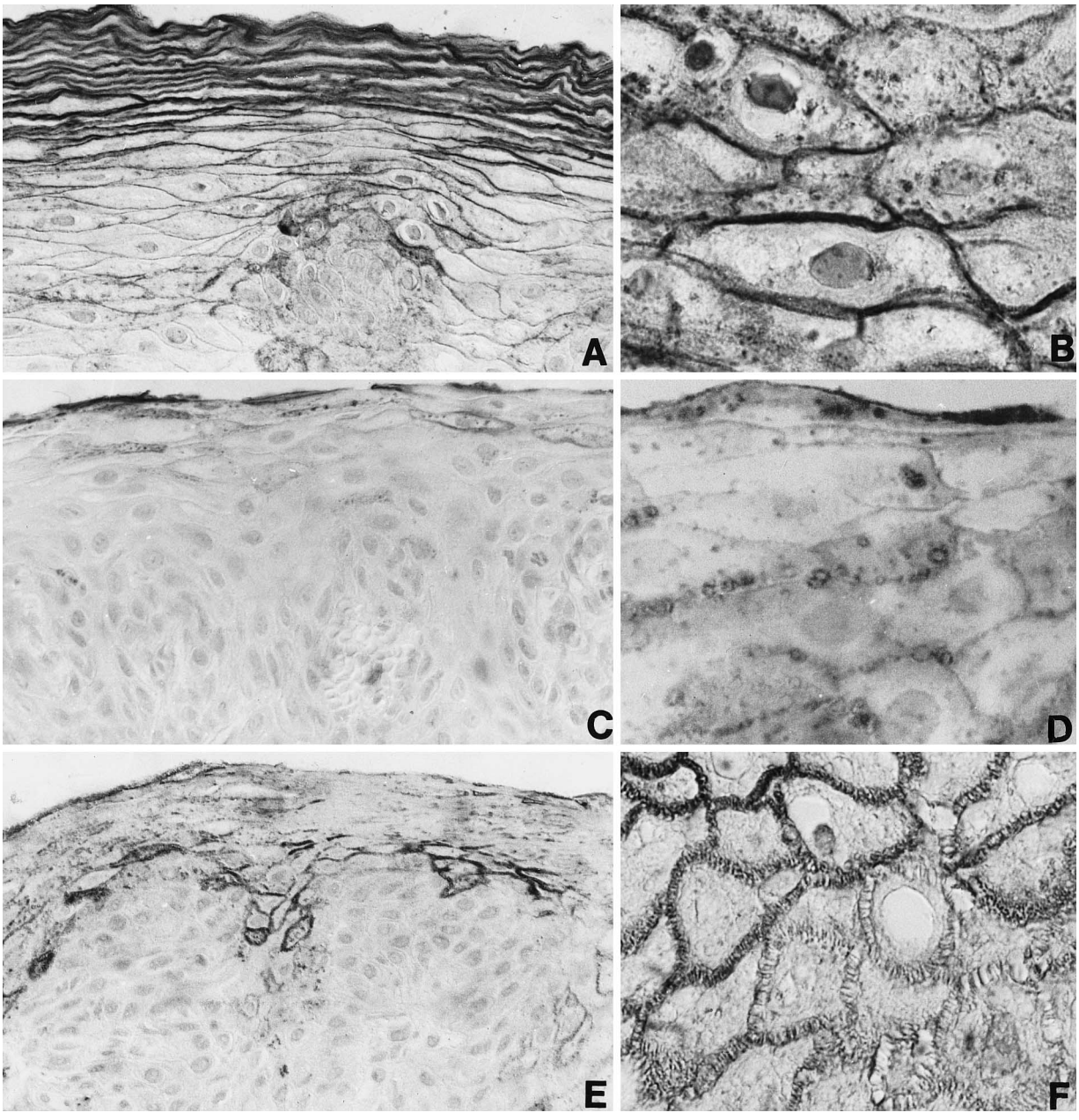

Fig. 1 Esophageal epithelium from control (**A**, **B**) and gastroesophageal reflux disease (GERD) cases (**C**–**F**) immunostained with CD15 antibodies, lightly counterstained with hematoxylin to identify cell nuclei, and observed under Nomarski optic. **A** Normal epithelium showing compact reactive laminae running parallel to the luminal surface and filling the intercellular space in between flattened superficial cells. The reactive intercellular material is less dense in the underlying prickle cell layer, though it increases around a papilla. **B** High enlargement of the prickle layer to show reactive cytoplasmic granules and incomplete intercellular laminae. **C** Disappearance of compact laminae due to loss of the superficial layer; only scarce immunoreactive material survives in the exposed prickle layer. **D** Small vesicles appear in poorly reactive interstitial spaces of the upper prickle layer denuded of superficial layer. **E** Marked loss of reactive laminae in a morphologically preserved superficial layer; reactive glycoconjugates are better recognized in the upper prickle layer, whereas the hyperplastic deeper prickle and basal epithelium surrounding two papillae remains unreactive. **F** Spongious enlargement (compare with **B**) of the intercellular material in the prickle layer, which in the deeper part gives way to dilated empty spaces partly crossed by prickles. Magnifications: **A**, **C**, **E**, ×350; **B**, **F**, ×900; **D**, ×1100

spinosum, sometimes with a discontinuous, either granular or interrupted, pattern. It often increased in quantity and became more continuous in pattern from the lower to the upper cellular layers. This process was especially prominent in the epithelium covering the distal half of the papillae, where a sort of CD15-immunoreactive peripapillary basket was observed. In the squamous layer, the intercellular reactivity formed more compact, continuous laminae running tangentially to the epithelial surface. The laminae were especially prominent and compact in the thin intercellular spaces in between flattened superficial cells, which were sealed off by the CD15 reactive material.

Esophageal epithelium of GERD patients

Changes in CD15 immunoreactivity of esophageal epithelium from endoscopy-negative GERD patients and from non-erosive areas of endoscopy-positive patients were qualitatively similar. In the intercellular space, they ranged from focal or diffusely reduced amounts to complete disappearance of reactive IGs (Fig. 1C–F). Loss and/or change in morphology and distribution of IGs, with special reference to lack of continuous laminar pattern, were frequent and extensive in the epithelial strata underlying altered or lost superficial layer. Loss or interruption of CD15-positive intercellular laminae were frequently observed also in structurally preserved, non-erosive superficial layers (Fig. 1E); sometimes the IGs formed small reactive spots or short bands dispersed in an otherwise unreactive or poorly reactive intercellular space. In addition, minute clear microvesicles or bubbles were found to be intercalated into the intercellular material; at sites, they were abundant enough to confer a cribriform or spongious pattern to the lamina (Fig. 1D, F). Sometimes, cell membrane invaginations and cytoplasmic vacuoles bordered by a thin rim of CD15-reactive material were also seen.

IG changes were often associated with changes in intracellular immunoreactivity, ranging from complete loss, to reduced amounts of peripheral granules, to the appearance of a perinuclear, Golgi-type reactive spot. Sometimes, reactive granules were increased and filled most of the cell cytoplasm, occasionally in combination with disappearance of the intercellular reactivity.

Focal separation of superficial cells leaving a clear fissure between confronting cell membranes, still covered by a thin CD15-positive surface coat, was also found. Occasionally, this resulted in epithelial vesiculation, i.e., formation of a closed space with respect to the esophageal lumen, accumulating serous fluid with or without a few inflammatory cells. More often, epithelial fissures in communication with the lumen or increased epithelial desquamation resulting in more or less extensive loss of the superficial layer were seen. In ordinary hematoxylin–eosin preparations, the latter changes were difficult to distinguish from artifactual epithelial detachment caused by bioptic trauma. In CD15-immunostained sections, reactive IGs were usually found to accumulate in the intercellular spaces underlying vesiculation, fissuring or desquamation, thus helping to distinguish true from artifactual epithelial separations. In addition, an abrupt keratotic change (cytoplasmic hyperchromatism and densification) of the juxtaluminar cells often marked surviving deep squamous or upper prickle cells that had been denuded of their superficial layer. These changes, which in more severe cases were associated focally with microscopic superficial erosion-necrosis and granulocyte infiltration, were observed in both endoscopy-positive and endoscopy-negative GERD cases, although they were more frequent in the former patients.

At the site of frank erosion of endoscopically positive patients, surviving epithelia usually showed prominent hyperplasia of CD15-unreactive basal cells and immature prickle cells, in addition to loss of squamous cells and mature prickle cells, often resulting in complete disappearance of both intercellular and intracellular immunoreactive glycoconjugates. Often, extensive loss of immunoreactivity occurred despite apparent maturation of prickle cells, suggesting the possibility of some selective damage to glycoconjugate biosynthesis and secretion.

Intercellular space dilation

Electron microscopy

The ultrastructure of normal and pathologic human esophageal epithelium has been described in a number of previous papers [1, 10, 12, 29]. Small (around 150 nm in diameter) secretory-type granules known as “membrane-coating” granules with a mostly amorphous or finely fibrillar core enclosed by a distinct membrane were found in the cytoplasm of prickle cells, often marginated close to their cytoplasmic membrane. Such granules have been shown to be discharged into the intercellular space and to represent a likely source of IGs [11, 19]. Accordingly, material with amorphous to finely fibrillar structure resembling the content of cytoplasmic granules was seen in the intercellular space, partly adhering to the external leaflet of cell membranes (Fig. 2). In control biopsies, the intercellular spaces were apparently kept open by numerous finger-like cell protrusions connected to each other and with the opposing cell membrane by desmosomes. The cell protrusions were longer and more elaborate in the basal layer; they were shorter in the upper prickle and squamous layers, where the intercellular space became thinner and the confronting membranes more regularly parallel. In most preparations, the intercellular space was about 0.5 μm or less in width in the squamous layer, while being more variable in the basal and prickle layers, usually with values of less than 2.0 μm.

After careful morphometric analysis, Tobey and coworkers showed that clinically relevant ISDs, apparently diagnostic for GERD, were characterized by a diameter of more than 2.4 μm in at least one site [29]. As ultrastructural morphometry would be a rather demanding procedure to identify a lesion of potential diagnostic utility, we looked for more qualitative intercellular space changes which could allow a more easy characterization of the lesions illustrated by Tobey and coworkers. In 5 of 7 GERD patients investigated, we found areas where the interconnecting finger-like cell protrusions appeared to be stretched, thinned, or disrupted, while the intervening cell membranes were pushed away, apparently by excessive fluid accumulation in the intercellular space, so as to form multiple bullae or irregularly dilated sausage-like spaces (Fig. 3). In some cases, extensive infolding of cell membranes was found associated with cytoplasmic vacuoles containing the same amorphous to finely granular/filamentous material and/or clear fluid present in adjacent intercellular spaces. No such changes were found in

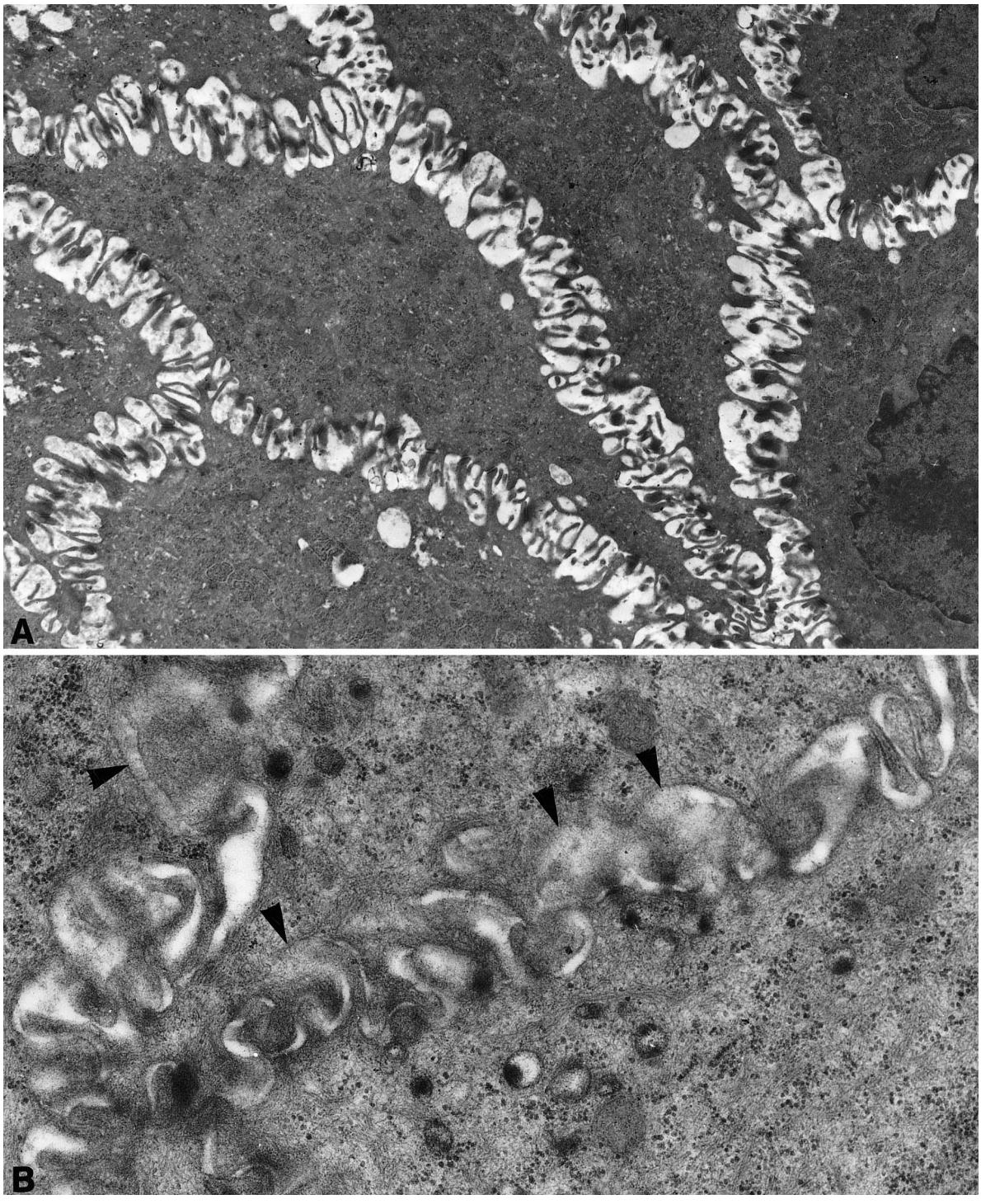

Fig. 2 Ultrastructure of normal esophageal epithelium. **A** Lower prickle layer showing intercellular spaces with elaborate cell processes joined by desmosomes; semi-thin aldehyde-osmium fixed, uranyl-lead stained, resin section viewed by means of electron-spectroscopic imaging. **B** Upper prickle layer: an amorphous-filamentous material (*arrowheads*) partly fills the intercellular space, possibly taking origin from extrusion of adjacent cytoplasmic granules. Comparison with light microscopy immunohistochemical findings suggests that both granules and intercellular material store CD15 reactive glycoconjugates. **B** Conventional electron microscopy of a thin section from the same resin block as of **A**, stained with uranyl-lead. Magnifications: **A**, ×5400; **B**, ×21,600

ultrastructural preparations from control non-GERD subjects, whose intercellular spaces were less prominent and, especially, more uniform and regular. Cellular edema was also a consistent finding in GERD patients.

Light microscopy

The irregular dilations of intercellular spaces identified under conventional electron microscopy were easily rec-

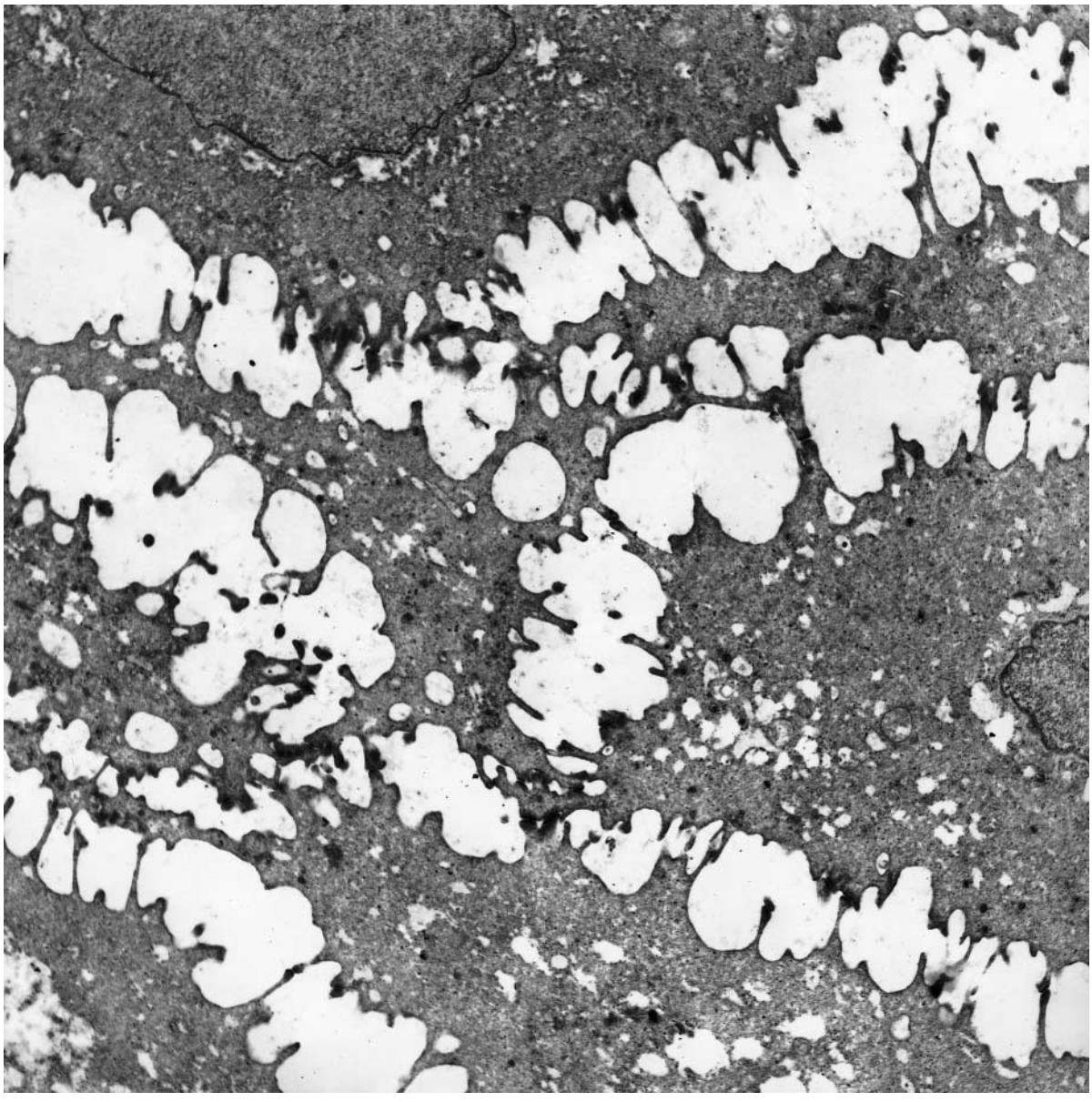

Fig. 3 Excessive fluid accumulation in the interstitial space of the prickle layer in a gastroesophageal reflux disease (GERD) case causes irregular dilation with cytoplasmic invaginations and occasional, membrane delimited, vacuoles, to be distinguished from focal cytoplasmic clarification due to glycogen deposits. Semi-thin resin section prepared and observed in the electron microscope as for Fig. 2A. Magnification ×5400

ognized also in semi-thin 0.5- to 1-μm thick sections from the same resin blocks, studied using the electron microscope equipped with the spectroscopic imaging device (at magnifications from ×250 to ×10,000) or in adjacent sections stained with toluidine blue and viewed under light microscope at magnifications from ×200 to ×1000 (Fig. 4). Similar changes were also recognized by light microscopy of hematoxylin-eosin stained, 5-μm-thick paraffin sections obtained from the same bioptic material from which resin blocks for ultrastructural studies were prepared (Fig. 5). Re-investigation of CD15-immunostained, hematoxylin-counterstained sections and of adjacent hematoxylin–eosin sections, allowed us to easily recognize ISDs also in these sections and to analyze their topographic distribution and relationship with IGs. ISD was mostly focal in distribution and occurred mainly in the basal and lower prickle layers (i.e., below the strata where usually IGs are more concentrated) as empty clear bullae or tracts intercalated in the intercellular material. An attempt to compare IG loss or rearrangement in immunostained sections and ISD in the same sections, or in adjacent hematoxylin–eosin sections from the same paraffin block, showed coexistence of the two types of changes in 48 of 80 cases investigated, absence of both in 13, discordant findings in 7 (3 with positive IGs changes and 4 with ISD), and inconclusive results in 12 cases. The inconclusive results were due to difficulty in assessing the pathologic status

Table 1 Interstitial space dilation (ISD) and other histological signs of esophagitis among 86 subjects investigated. *GERD* gastroesophageal reflux disease

	Controls (*n*=12)		Endoscopy-positive* reflux esophagitis (*n*=22)		Endoscopy-negative GERD (*n*=44)		Borderline cases (*n*=8)	
	n	%	*n*	%	*n*	%	*n*	%
ISD	1	8.3	20	90.1	30	68.2	3	37.5
Eosinophils, >1 per bioptic site	0		16	72.7	17	38.6	0	
Neutrophils, any	0		14	63.6	9	20.5	0	
Erosion/necrosis	0		13	59.1	4	9.1	0	

* Only biopsies of endoscopically non-erosive areas were considered

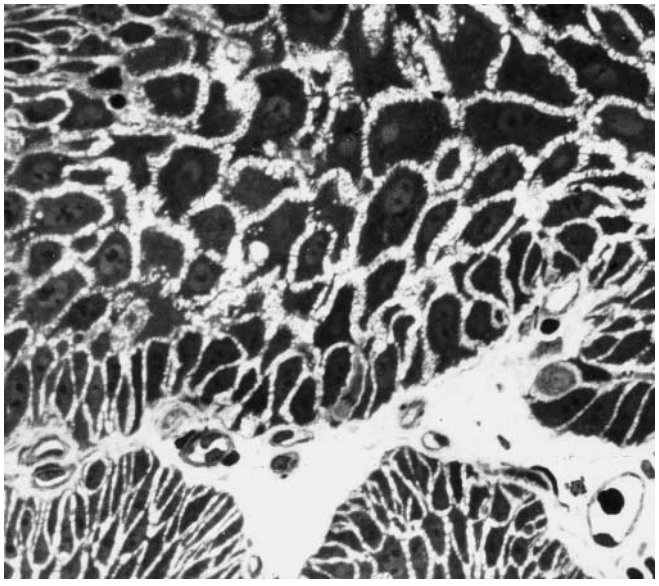

Fig. 4 Light microscopy of semi-thin, aldehyde-osmium fixed resin sections stained with toluidine blue. Irregular dilation of intercellular space with cytoplasmic invaginations and vacuole formation in the basal and deep prickle layers of a GERD patient, to be compared with non-dilated or uniformly dilated basal layer with regular cell borders (*bottom left* and *center*) of an adjacent epithelial sample in the same bioptic specimen. Note the hypertrophic pattern of cells bordering dilated intercellular spaces. Magnification ×450

of less prominent IG or ISD changes (agreement rate: 89.7% with χ^2=35.3, P<0.00001, considering only the 68 cases where both parameters could be assessed). In general, ISD could be evaluated more easily and with more confidence than IG changes, whose considerable polymorphism and variability of patterns sometimes caused problems in pathologic state assessment. Therefore, ISD appeared more promising as a potential diagnostic tool for GERD.

Comparison of ISD with endoscopy, pHmetry, symptoms, and other histological parameters

Table 1 outlines the distribution of ISD and some conventional histological signs of reflux disease in routine histological biopsies from the 86 subjects investigated. Only those ISDs that showed irregular borders, sometimes with interspersed empty bullae or associated with cytoplasmic invaginations and vacuoles, were considered as pathologic (Fig. 5). Uniform enlargement of the intercellular space in the basal layer delimited by regular borders and crossed by regularly spaced, parallel prickles were not considered significant, unless prominent and extensive. Changes occurring near to the cutting margin or ruptures of the bioptic specimen were also disregarded as being possibly artifactual. A small group of seven patients (one with erosive esophagitis, four with endoscopy-negative GERD and two borderline cases) showed only one or a few, limited foci of moderate dilation, mainly of regular type with scattered intercellular bullae or focal membrane invagination. These mild changes, mostly confined to the basal layer of main or papillary epithelium, were often difficult to separate from normal ranges of histological variability or from artifactual cellular detachment due to mechanical stress caused by bioptic trauma or histological procedures. Such cases were not included among the 54 ISD-positive cases of Table 1; none of them showed unquestionably pathologic changes of IGs or granulocyte infiltration.

Only histological parameters not requiring perfect orientation of tissue sections – difficult to obtain with ordinary grasp biopsies – were investigated for comparison with ISD. Given the occasional finding of an eosinophilic leukocyte in biopsies from non-GERD cases [2], we considered only cases showing two or more intraepithelial eosinophils per bioptic site. At least four sections from the two bioptic specimens taken 2–3 cm above the squamo-columnar junction were evaluated in all cases, with the exception of two patients with erosive disease, where three sections from the only suitable specimen available were studied. In the 30 cases with biopsies taken from two sites, eight sections from four specimens were investigated.

ISD was found in 53 of the 74 patients and in 1 of the 12 controls, from which figures a sensitivity of 71.6% and a specificity of 91.7% were calculated. Considering only the 66 patients with both pHmetry and symptoms unequivocally supporting the diagnosis of GERD, ISD was detected in 50 cases, with 75.8% sensitivity, i.e., 90.1% sensitivity for endoscopy-positive and 68.2% for

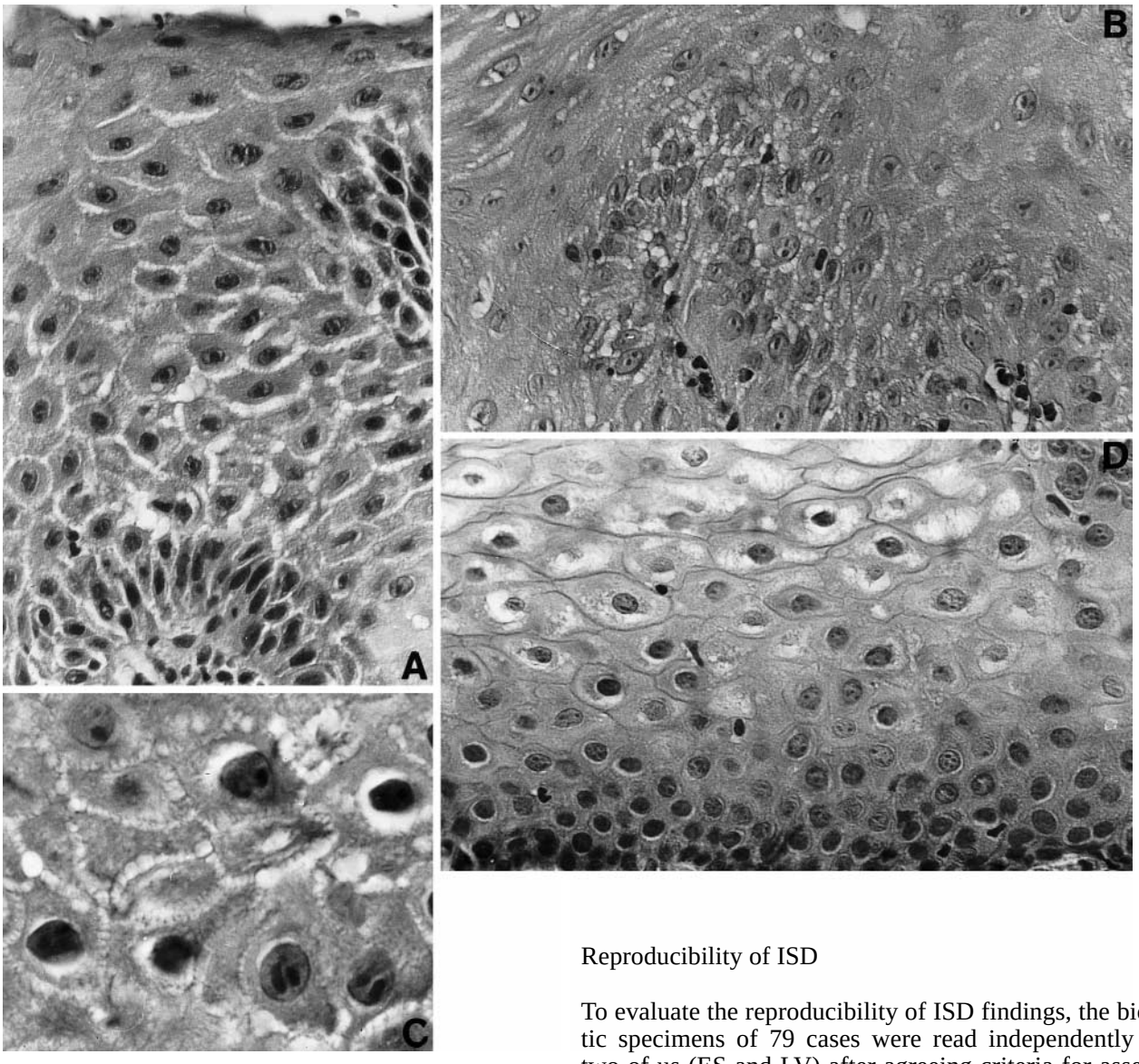

Fig. 5 Light microscopy of hematoxylin–eosin-stained paraffin sections from gastroesophageal reflux disease (GERD) (**A**, **B**, **C**) and control (**D**) cases. Prominent interstitial space dilation (ISD) involving the whole depth of superficially erosive epithelium (**A**) or, focally, the peripapillary part of non-erosive epithelium (**B**). Higher enlargement shows irregular pattern of ISD with partly confluent bullae and focal disappearance of prickles; note the enlarged, hyperplastic nuclei with prominent nucleoli (**C**). Control case (**D**). Magnifications: **A**, **B**, **D**, ×350; **C**, ×900

endoscopy-negative cases. Compared with other histological signs of GERD, it appears that ISD is significantly more sensitive than intraepithelial eosinophils (χ^2=9.39, P=0.002), neutrophils, microscopic erosion/necrosis, or a combination of these parameters in both endoscopically positive and (even more markedly) endoscopically negative GERD patients. Four of the 66 GERD patients showed more than 1 eosinophil in the absence of ISD, while all patients with neutrophils or microscopy erosion-necrosis also had ISD.

Reproducibility of ISD

To evaluate the reproducibility of ISD findings, the bioptic specimens of 79 cases were read independently by two of us (ES and LV) after agreeing criteria for assessing ISD positivity: 57 of the 79 cases were concordantly diagnosed positive and 15 were concordantly diagnosed negative, while 7 proved discordant, 5 cases being diagnosed positive by ES and two by LV (K=0.75).

In the 30 endoscopy-negative cases (inclusive of 21 GERD, 4 borderline and 5 control cases), where both higher (6–7 cm above the Z line) and lower (2–3 cm above the Zeta line) esophageal biopsies were evaluated, the two bioptic sites were concordantly positive (15) or negative (7) for ISD in 22 cases, while 4 were positive only in 2-cm and 4 in 6-cm biopsies. Eosinophils were detected in 7 of the former and 4 of the latter biopsies.

Discussion

Experimental studies showed that the superficial squamous layer represents the main barrier to acid permeation of the esophageal epithelium from the lumen and

suggested that the glycoconjugates sealing the intercellular space at this level may have an important role in the barrier function [18, 19]. Acid has been suggested to permeate the epithelium through the paracellular route, up to the intercellular space of the underlying prickle and basal layers, where it can be buffered by bicarbonate diffusing from the serosal side [28]. When this buffering capacity has been overcome, acid may attack and penetrate the cells through their basolateral membranes and damage them with resulting edema and necrosis [3, 27]. Thus, changes of the intercellular cement filling the interstitial space of the superficial/squamous layer are expected to be among the earliest signs of acid-mediated damage to a structurally preserved esophageal epithelium. In fact, our investigation of non-erosive esophageal mucosa from endoscopy-negative GERD patients showed peculiar changes of CD15-reactive intercellular material at this level, including focal spongious transformation due to the appearance in its context of minute clear microvesicles. The occurrence of these microvesicles also in proximity to the epithelial surface raises the possibility of a luminal origin of their clear apparently fluid content. Thus, the microvesicles may represent a morphologic counterpart of the acid refluxate permeation into the epithelium, which experimental studies have suggested to occur through the paracellular route [19]. Such an interpretation would explain the prominent and extensive spongious pattern often shown by the intercellular material at the level of the prickle layer, when this has been deprived of its protective superficial layer.

In other cases, the compact laminar pattern normally shown by the intercellular material of the superficial layer was lost, resulting in a poorly coalescent granular-interrupted material. In more severe cases, usually associated with superficial erosion, loss of intercellular material was found at both superficial and prickle layers, often associated with loss of intracellular CD15-reactive granules. The IG changes, some of which were strictly focal or rather subtle, might be indicative of an early, less severe epithelial damage, mostly undetectable by routine histological investigation. In addition to loss or rearrangement of IGs, other changes involving the superficial layer were observed in this and previous studies [3, 12], including keratotic transformation or excessive desquamation of the most superficial cells or vesiculation and fissuring of the superficial squamous layer. These changes may reflect acid-mediated damage and, in turn, facilitate penetration of acid into deeper epithelial strata, thus contributing to their damage.

Most changes found in the prickle and basal layers of GERD epithelium are likely to be secondary to acid permeation of intercellular space at this level [19, 27, 28]. From previous experimental evidence [3], ISD may be one of the earliest and most sensitive of such changes. The correlation we observed in GERD cases between IG loss or rearrangement at the superficial/upper prickle layers and ISD of the underlying lower prickle/basal layers fits with this conclusion. In addition, our study outlines the irregular pattern of intercellular space dilation, with stretching or detachment of desmosome-anchored finger-like cell protrusions by the excessive fluid accumulation, causing unequal separation of the confronting cell membranes with extensive membrane infolding, endocellular invaginations, and vacuoles. These changes are likely to reflect a profound acid-mediated perturbation of ions and fluid exchanges between cells and intercellular spaces [18, 27, 28], resulting in excessive fluid accumulation in the interstitial space, with or without cellular edema and more severe cellular damage [12, 29].

It is important to outline that the irregular, peculiar morphology of ISD proved of help in its identification, thus providing a simple qualitative electron as well as light microscopy alternative to the ultrastructural morphometry approach used by Tobey and co-workers [29]. This allows potential application in routine histological diagnosis. An advantage of ISD as a diagnostic tool for GERD stems from the fact that it does not require proper orientation of the mucosa in histological sections, a strict requirement for the assessment of papillae elongation and basal cell hyperplasia [14] which proved difficult to fulfil in routine grasp biopsies [4, 16]. In addition, we found no substantial difference between the results of biopsies taken at different distances from the squamo-columnar junction, a factor which has been suggested to affect the specificity of other histological tools [31]. Comparison with other histological signs of GERD not requiring critical tissue orientation, such as intraepithelial eosinophils and mucosal neutrophil infiltration or microscopic erosion and necrosis [2, 30, 32], suggests that ISD may prove of value to increase the sensitivity of routine histological diagnosis of GERD, including the diagnosis of endoscopy-negative cases which so far proved difficult and poorly reproducible [4, 25].

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