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SCANNING ELECTRON MICROSCOPY APPLICATION IN CLINICAL RESEARCH

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Abstract

Our personal experience on the application of scanning electron microscopy in cardiology, gastroenterology and ophthalmology is reviewed.

SEM has not yet significantly contributed to myocardium pathology. However, in the near future, SEM could be a reliable technique to complete the information available from other sources. As to atherosclerosis, SEM allowed us to improve our knowledge of the early stages of the disease; some pathological features, not always detected by conventional morphological examinations, can be documented. An important contribution to gastrointestinal pathology was made by SEM investigations both in the staging of some important diseases (i.e., coeliac disease, peptic ulcer, Crohn's disease, ulcerative colitis) and in the follow-up of mucosal changes during therapy. In the ophthalmological field,SEM provided three-dimensional new information to clinicians, who are familiar with the biomicroscopic images. Our experience in hematology is still limited. However, in the last few years SEM joined to immunocytochemistry allowed us to characterize cell populations in several blood diseases. Some procedures of particular interest in the management of human bioptic specimens are stressed in order to get to a complete correlative microscopy.

We conclude that continuous and simultaneous correlations have to be carried out between SEM and other methods and instruments available for morphological investigation.

KEY WORDS: Scanning electron microscopy, clinical application, cardiology, atherosclerosis, gastroenterology, ophthalmology, hematology, correlative techniques.

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Introduction

The application of scanning electron microscopy in clinical research has, in the past years, gone through a period of doubt. This always happens when new tools and methodologies become available.

For some years pathologists, analyzing merits and defects, limits and possibilities, have begun to regard SEM as a promising tool in biomedical research (1, 19, 20, 21, 22, 24, 25).

Today, morphological investigation requires a constant and wide correlation between all available technologies, among which is SEM. This complete morphological approach, along with the biochemical one, is essential in clinical research.

In the present paper, we would like to point out some fields of particular current clinical interest in which we have accumulated, over the years, personal experience as scanning electron microscopists. These fields are the cardio-vascular, gastrointestinal and ophthalmological ones. Moreover, studies of blood cells are also considered in this review. Our experience is still limited in this area, however, we firmly believe in its potential development and clinical significance.

Finally, we will present and discuss tissue processing methods already in use as well as the new ones. They appear important in order to gain more precise correlations and better information from small human specimens.

Cardiology

Since the early days of SEM, the heart has not received particular attention. This most likely depends on the fact that the myocardium is not naturally accessible to SEM observation. More or less sophisticated methods of processing, in particular fracturing, are required to allow a suitable surface examination. Furthermore, SEM

is often unable to resolve the fine surface details due to its resolution limits. For these reasons, there are, in literature, various attempts to assess the most suitable tissue processing conditions for the study of normal and diseased myocardium specimens (8, 9, 27, 28, 47, 48, 99, 100, 103, 104, 147, 148). However, it is our opinion that preparative techniques are not yet optimal. Thus, the SEM contribution to myocardium pathology is still limited, both in animal models (6, 7, 149), and in humans (136, 139, 152).

As regards the physiopathology of myocardium ischemia, several problems of capital importance are still unsolved: identification of subcellular mechanisms which subtend myocardial ischemia and in particular of those leading to irreversible myocardial cell injury; fine investigation on the effects of reoxygenation after ischemic injury, as it can produce on the sensitized myocytes explosive lesions by means of free radical production (some cases of "sudden death" commonly observed in the Coronary Unit Division); evaluation of those drugs which are claimed to prevent or minimize these undesirable pathological changes.

Our specific experience is still limited to the "in vitro" perfused rat heart. In this model the myocardial structural changes induced either by short rates of ischemia (84) or by standard procedures of reperfusion (unpublished observations) as well as the structural maintenance induced by drugs linked to the myocardial cell metabolism can be sufficiently well studied. Our results appear to be encouraging and possibly useful for clinical application. Many pathological features, not sufficiently defined at the light microscopic level, could be disclosed by means of different SEM processing techniques associated with TEM. Conventional SEM methods showed changes in fibre sizing and in the capillary network as well as interstitial oedema. Waving fibres (Fig. 1), focal necrosis and sarcolemmal folding (Fig. 2) due to fiber contraction were clearly documented. The simple fracturing technique results were in substantial accordance with those from more conventional TEM (37): extensive loss of myofilaments (Fig. 3), subsarcolemmal accumulation of mitochondria (Fig. 4), distortion or loss of T-tubules. This consistent pattern of lesions was significantly prevented by the administration of L-Carnitine (Fig. 5) thus suggesting a promising clinical application. An accurate characterization of heart failure may be possible only when all clinical, biochemical and morphological data are considered. SEM, however, should be considered a reliable technique to complete the information

Fig. 1. "In vitro" perfused rat heart. Ischemic myocardium. Some waving myocardial cells undergoing necrosis are shown. Bar = 10 μm.

Fig. 2. "In vitro" perfused rat heart. Effects of reperfusion after ischemic injury. Focally hypercontracted myocardial cells with sarcolemmal folds (arrows) projecting into the extracellular space. c = capillary. Bar = 10 μm.

Fig. 3. "In vitro" perfused rat heart. Effects of reperfusion after ischemic injury. A cryofractured myocyte shows varying degrees of myofilament disruption (arrows).
s = residual sarcomeres. Bar = 2 μm.

Fig. 4. "In vitro" perfused rat heart. Effects of reperfusion after ischemic injury. Subsarcolemmal accumulation of mitochondria (m), varying in size, in area partially devoid of myofilaments. Bar = 10 μm.

Fig. 5. "In vitro" perfused rat heart. Effects of reperfusion on ischemic heart after L-Carnitine treatment. The myocytes (arrows) seem to retain their normal appearance. Bar = 10 μm.

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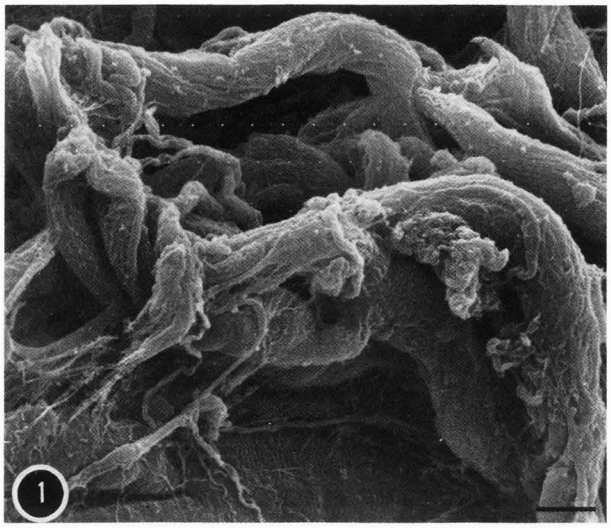
obtained from the other sources.

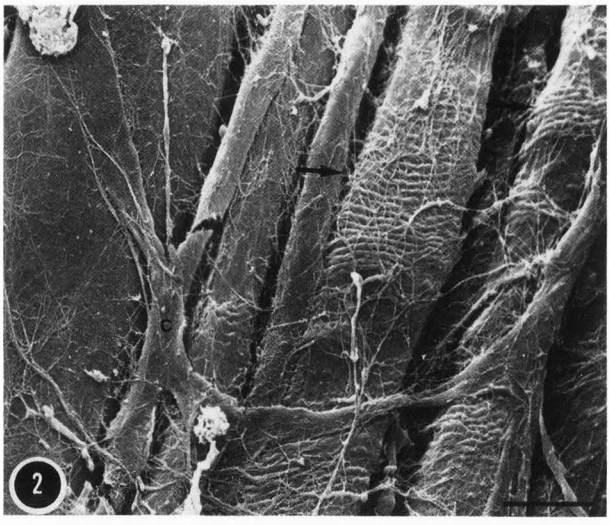
In the future, better results can be expected with the use of fracturing techniques associated to osmic maceration as proposed by Yoshikane et al. (170, 171). In particular macerated specimens viewed in high resolution SEM, can provide new and important information on the muscle cell membrane components, namely sarcolemma and related surface vesicles, sarcoplasmatic reticulum, T-tubules, as well as on the junctional systems (intercalated discs). The potentiality of these high resolution SEM techniques for the study of the membranous apparatus in ischemic related heart diseases should also be considered.

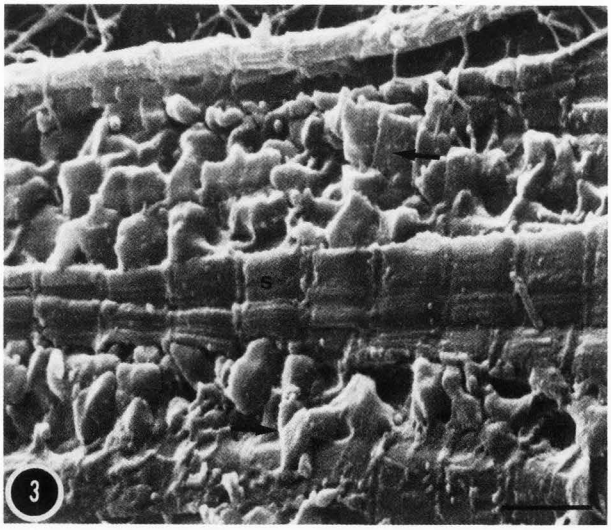
Atherosclerosis

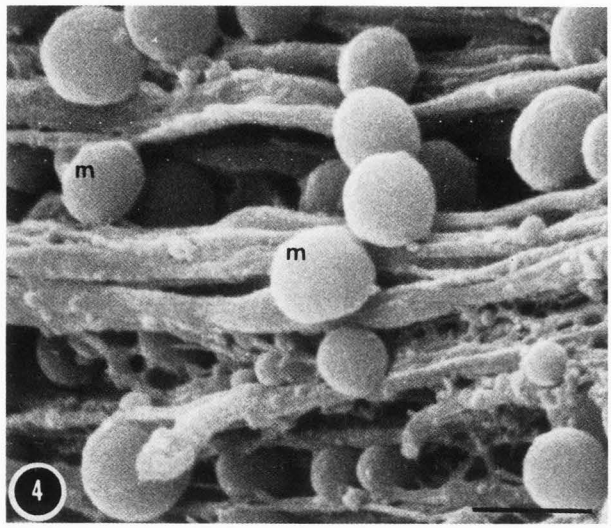
For many years we have studied atherosclerosis in man. The human model presents, of course, many difficulties and limitations. Anyway, it is well known that there are great differences in the evolution of animal and human atherosclerotic lesions, thus extrapolations from experimental models, so frequently used, are undoubtedly aleatory (81).

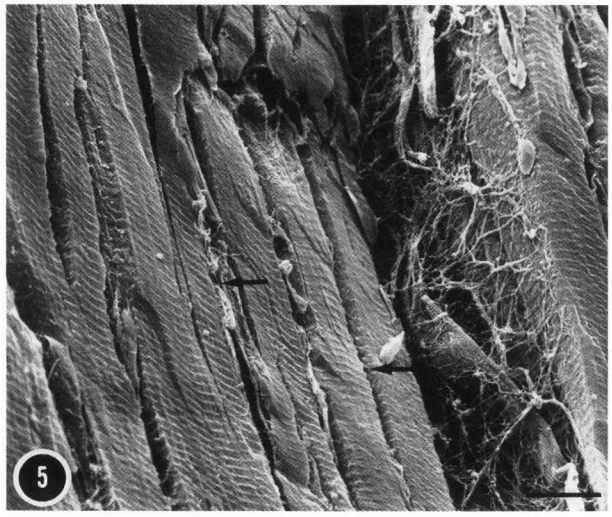
Relatively few satisfactory morphological studies have been carried out on atherosclerosis in man (14, 45, 46, 53, 66, 72, 77, 96, 144, 161).











Some studies have been performed on material obtained at autopsy. However, the poor preservation of the specimens has made an accurate investigation of the lesions very difficult. On the other hand, Ross and coworkers (124) made a systematic analysis of the atheromatous fibrous plaques obtained from occluded femoral arteries during by-pass surgery. These lesions were typically fibroproliferative and contained numerous senescent smooth muscle cells mixed with a varying number of macrophages. Studies like this are undoubtedly of great importance. However, they cannot supply useful information on the early stages of the disease. Thus integrated studies of the "prelesional events" occurring in animals appear to be indispensable (92, 140).

Experimentally induced atherosclerosis can be obtained by means of different procedures. It was proposed a dietary-induced hypercholesterolemia (67, 70, 76, 109, 126), an ischemic or toxic injury (5, 52, 74, 106, 107) to endothelium and a mechanical removal of the inner vessel wall (26, 90, 118, 133). This latter condition is difficult to correlate with what most likely happens in humans, so we feel it is not methodologically proper. However, it allows us to obtain some information about the pathophysiological responses of the vascular tissue and in particular the interactions between normal resident vascular cells and circulating blood elements. On the contrary, the metabolic models appear to be more stimulating. Scanning electron microscopy has provided an additional measure of the vascular tissue response in these experimental conditions. Faggiotto et al. (34) described the early changes in the aorta and iliac arteries wall leading to fatty streak formation. In general, these lesions appear as focal elevations on the vascular lumen due to the intimal accumulation of numerous foam cells. Later, the fatty streak conversion to fibrous plaque was observed at the same anatomical sites (35). Loss of endothelial continuity and therefore platelet adherence to exposed subendothelial matrix was detected only at this stage. This feature seemed to the authors a critical step for the subsequent proliferation of resident smooth muscle cells. However, the evidence for conversion of fatty streaks into fibrous plaques remains a controversial issue (101) and other authors (132, 151, 154, 155) maintain that fatty streaks in swine abdominal aorta develop from small intimal smooth muscle cell masses which are most likely present at birth. Moreover, SEM helped to settle the old question of the endothelium injury (121, 122, 123, 125, 126). Most of the observations revealed that the endothelium was retained as a confluent layer over the developing lesion in different experimental models (30, 54, 150). In the light of few but relevant contributions (68, 69, 88, 109, 117, 164) the presence of a functionally altered endothelium not yet detectable at morphological level should be taken into consideration. As for platelets, their role in the development and complications of atherosclerosis has been recently reviewed by Packham and Mustard (110), and SEM contributed satisfactorily to these studies (87). New views and hypotheses arose from the observation of focal infiltrates of circulating mononuclear cells over a confluent endothelium. The fundamental importance of monocytes in early atherosclerosis was originally suggested by Leary (86) and successively by Duff (33). Today, it has been emphasized by

Fig. 6. Human carotid artery. Atheromatous plaque. A fibrous plaque occluding the artery lumen (arrows). Bar = 50 μm.

Fig. 7. Human carotid artery. Atheromatous plaque. Many newly formed vessels (arrowheads) as well as an area of intramural hemorrhage (arrows) are detected in the wall thickness. Bar = 50 μm.

Fig.8. Human carotid artery. Atheromatous plaque. SEM discloses an unexpected intraluminal thrombus. Bar = 100 μm.

Fig. 9. Human carotid artery. Atheromatous plaque. A patch of calcification in the wall thickness as revealed by BSE (+) mode. Bar = 100 μm.

Fig. 10. Human carotid artery. Atheromatous plaque. Lipid deposition (arrows) along the plaque periphery. Digitonin-osmium tetroxide reaction detected by BSE (+) mode. Bar = 100 μm.

Fig. 11. Human carotid artery. Atheromatous plaque. Detection of fragmented and ballooned elastic fibers (arrows). Tannic acid-osmium tetroxide reaction revealed by BSE (-) mode. Bar = 10 μm.

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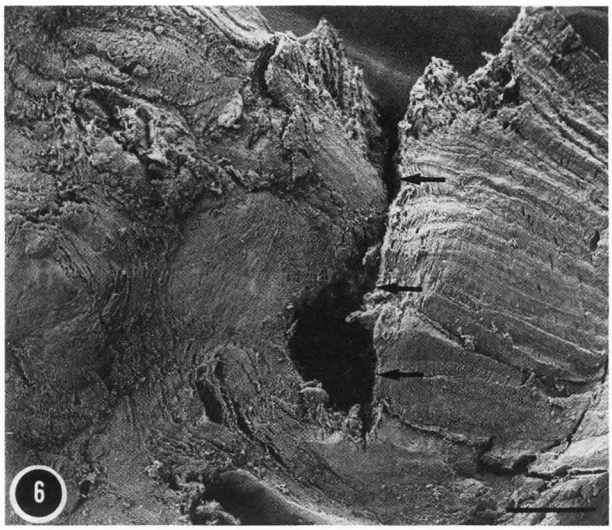
numerous authors (49, 50, 51, 71, 129, 131, 163). Focal adherence of monocytes to the endothelium, followed by migration into the intima and by progressive loading with lipids, seems to be the sequence leading to fatty streak formation Thus, a solid bridge between atherosclerosis and inflammation has been built (91). Finally, the overall tridimensional architecture of the elastic components has been disclosed by Wasano et al. (162). Remodelling of the elastic laminae in early atherosclerosis has been finely described. It was assumed to be a protective mechanism intended to prevent successive lesions (105, 169).

Our observations have been performed in particular on human atheromatous plaques removed from the internal carotid artery during surgery (82, 83). Most of these lesions were fibroproliferative with significant stenoses (> 70%). Plaques appeared more than twice the original wall thickness (Fig. 6) and showed evidence of complication such as thrombosis, calcification as well as intramural hemorrhage. Hemorrhage was frequent in plaques associated with focal neurologic deficits. In these patients, cryofractured specimens revealed numerous newly formed vessels along the plaque thickness (Fig. 7). SEM proved to be particularly useful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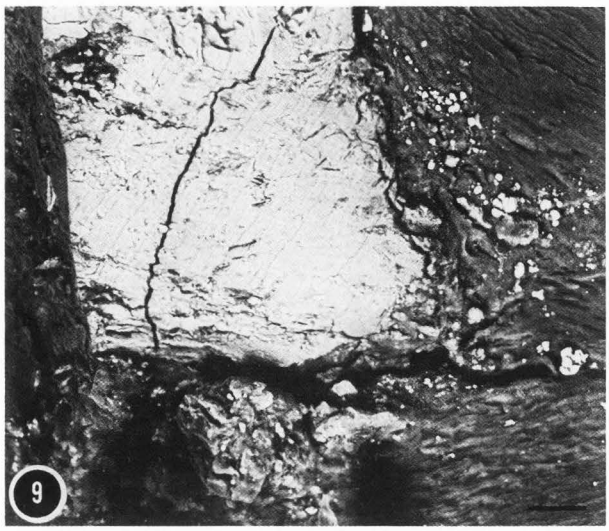
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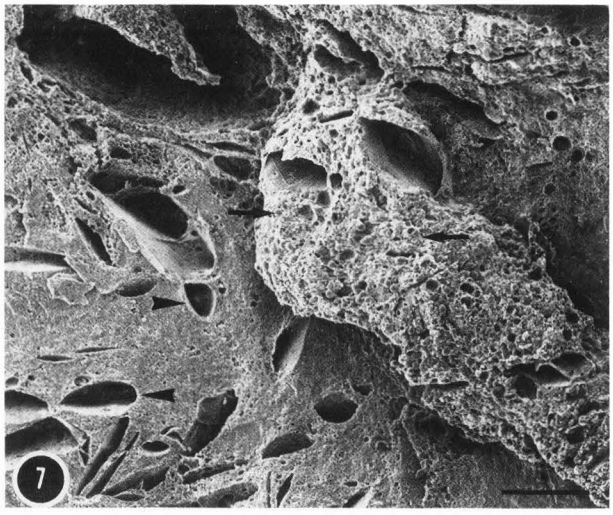
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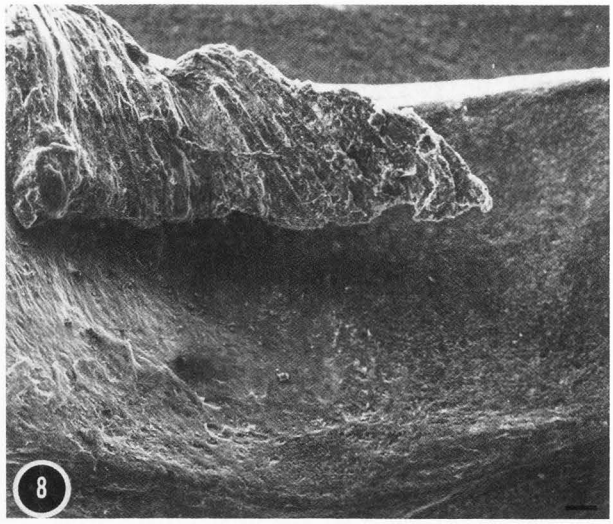
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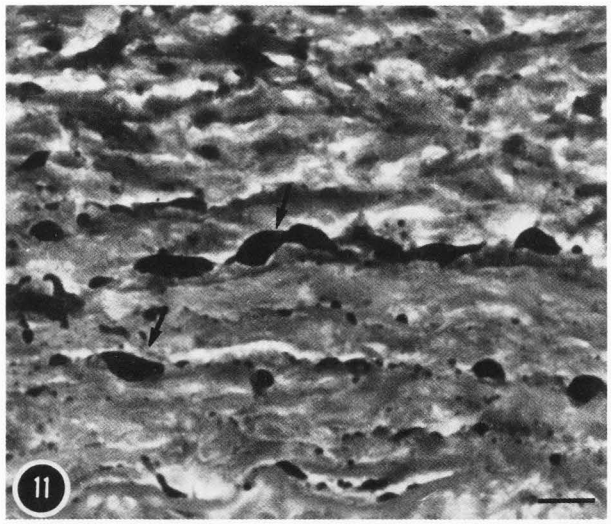
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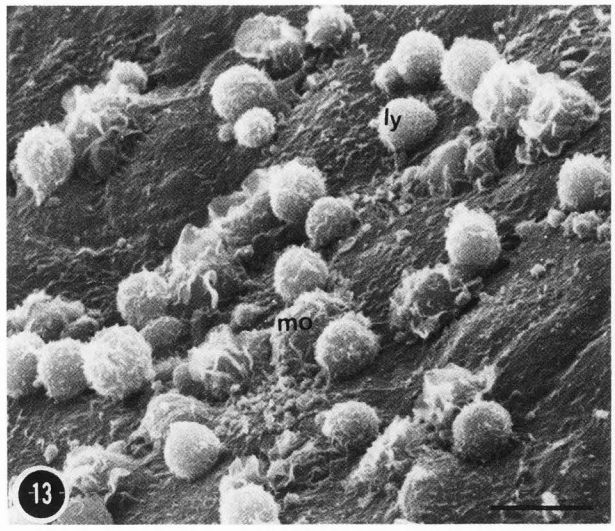


Fig. 12. Human carotid artery. Apparently unaffected area. Endothelial cells display an uneven arrangement as well as differences in size and shape. Bar = 20 μm.

Fig. 13. Human carotid artery. Apparently unaffected area. Lymphocytes (ly) and monocytes (mo) focally adhere to the endothelium. Bar = 10 μm.

to detect floating thrombi (Fig. 8) and surface ulcerations (64) which had not resulted at angiography. These features were easily related to transient ischemic attacks in patients without gross ulcerations. Foci of calcification, confirmed by X-ray microanalytical techniques, appeared as irregular patches on the wall thickness (Fig. 9). Cytochemical techniques using digitonin associated to controlled osmication showed a wide spectrum of lipid deposition increasing with plaque thickness. Lipids were located at the plaque periphery (Fig. 10) and in the central necrotic core which also contained numerous cholesterol crystals. The surface of the plaque lacked endothelial coverage. Its luminal side consisted of amorphous material mixed with collagen fibrils as well as atheromatous debris. The inner plaque ultrastructure showed a typical organoid pattern. The plaque was, in fact, composed of a highly cellular peripheral zone delimitating a deep necrotic core. The former zone contained numerous layers of smooth muscle cells separated by abundant extracellular matrix (fibroproliferative zone). The smooth muscle cells varied quite a lot in appearance. Some of them were typical contractile smooth muscle cells, while others expressed a synthetic phenotype. Furthermore, some of the smooth muscle cells were filled with lipid droplets. The synthetic smooth muscle cells seemed to be responsible for the abnormal synthesis of the extracellular matrix components: fragmented elastic fibers (Fig. 11) containing entrapped lipid droplets, collagen fibers with a flower-like appearance, proteoglican filaments which appeared thicker than normal. The central core of the plaque was less cellular. Necrosis, lipid infiltration and calcification were prevalent features. These data as a whole suggest that there seems to be no possibility of regression when the plaque reaches a similar pattern in its natural evolution. Defining the exact moment in which the atheromatous lesions in man are no longer susceptible to regression seems to be of fundamental value. With this aim, today, we are focussing our attention on the apparently unaffected areas next to human carotid plaques. Our results are preliminary but already suggest some new ideas (85). These areas are covered by an endothelial lining showing an uneven orientation as well as differences in cellular shape and size (Fig. 12). In many patients, a focal sticking of mononuclear cells (monocytes and lymphocytes) to the endothelium was observed (Fig. 13). Adhesion of these cells occurred both on endothelial cells and on exposed subendothelial matrix where single endothelial cells were degenerating. In some instances, the endothelial cells retracted to such an extent that the underlying lipid-

laden macrophages were exposed to the blood flow. This feature seemed to facilitate platelet adherence and subsequent aggregation. The same specimens recovered for TEM showed advanced smooth muscle cell proliferative lesions, besides an unusual lymphocyte infiltration just below the endothelial sheet. Monocyte-macrophages were also present. These observations suggest that inflammation may be frequently associated to florid atheromatous lesions. It may represent an immune response to some modified components of the atheromatous arterial wall. Moreover, most of the modulated smooth muscle cells appeared to take on new phagocytic properties as demonstrated by the presence of acid lipase positive vacuoles in their cytoplasm. Smooth muscle cells looked like substitute macrophages in the scavenger role. However, this new digestive capacity seems most likely defective and probably due to anomalous lysosomal activity.

Gastroenterology

The potential role of SEM in clinical investigation of the gastrointestinal tract has been widely assessed. At present, SEM seems to be one of the best morphological techniques to finely investigate the mucosal surface changes of the human digestive system. Sensitive applications in various diseases have been described by numerous authors (2, 36, 73, 93, 130, 137, 138, 145). SEM was of value in defining different small bowel disorders associated with chronic diarrhea in childhood (114). Siew claimed SEM to be an aid in diagnosing clindamycin-associated pseudomembranous colitis (135). Promising results on quantitative differences in the cellular microridge pattern among normal, dysplastic and inflamed esophageal mucosa have been reported by Goran and coworkers (55).

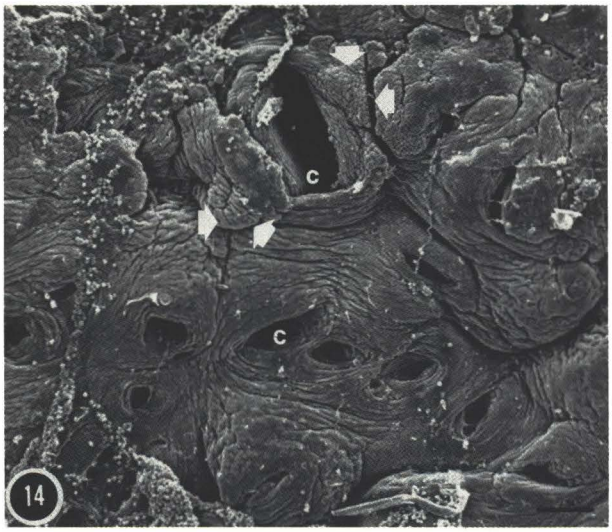
Moreover, the importance of correlative microscopy in recovering SEM bulky specimens for LM and TEM, and of LM paraffin blocks for SEM has been outlined by Carr et al. (23). The significance of such a combined approach in the assessment of human digestive mucosa has been recently stressed and improved by our group (16). A full characterization of both surface morphology of gastric mucous cells and their mucin secretion was achieved by using as cytochemical probes, the lectins, on paraffin blocks reprocessed for SEM and on histological sections viewed in BSE mode.

In our personal view, gastrointestinal diseases with primitive or secondary changes of surface mucosa find an accurate characterization at SEM level. Some meaningful examples will therefore be shown. As for coeliac disease, SEM shows a higher sensitivity with respect to LM. Different appearances of flat small intestine mucosa in untreated patients were easily recognized by Bonvicini et al. (15) (Fig. 14). These results were in accordance with those by Poley (114). These features seem to reflect different stages of severity in the onset of the disease. SEM can be used to assess the early mucosal response to gluten-free diet. Moreover, it allows a fine evaluation of the "maturity" of the repairing mucosa, by identifying the intermediate patterns of recovering (cerebriform, transitional, villous) which are not detectable at LM examination (Fig. 15).

SEM may usefully integrate the endoscopic information obtained from patients affected either by ulcerative or Chron's colitis (15, 119, 134). The surface morphology of mucosa, close to the acute ulcers, appears subverted in the ulcerative colitis (Fig. 16) and relatively unchanged in Chron's disease (Fig. 17). Differences in the appearance and in the number of goblet cells (decreased in ulcerative colitis and increased in Chron's disease) can be detected. This information facilitates the differential diagnosis between the two pathological entities which are usually difficult to separate. Some infectious agents are readily recognized by SEM. Giardia Lambdia (Fig. 18) as well as Candida Albicans (Fig. 19) duodenal colonization have been clearly documented (61). These observations may be of particular value due to the increased incidence of the opportunistic infections in immunodepressed patients.

Gastric and duodenal lesions related to the oral administration of non-steroidal anti-inflammatory drugs have been widely investigated by us. Minimal mucosal changes, mainly located at some distance from the usual macroscopic lesions (hyperaemia, erosions and/or ulcerations), were observed at SEM even when endoscopy was completely normal (172) (Fig. 20). The positive influence of histamine H_2-receptor antagonists in preventing the onset of such lesions was successively evidenced (Fig.21)(173).

In peptic diseases, SEM seems to be a valuable tool in the follow-up of ulcer healing in patients submitted to therapy. At times, causes of recurrence may be detectable. Spiral bacteria bearing a polar flagellum and located at the edges of the gastric cells are easily observed (Fig. 22). These bacteria look like the Campylobacter Piloridis which seem to be involved in the pathogenesis of gastric inflammatory diseases as well as peptic ulcer (94, 116). It has been claimed that, in some cases, drugs with bactericidal properties seem to prevent ulcer relapse. At gastric level, a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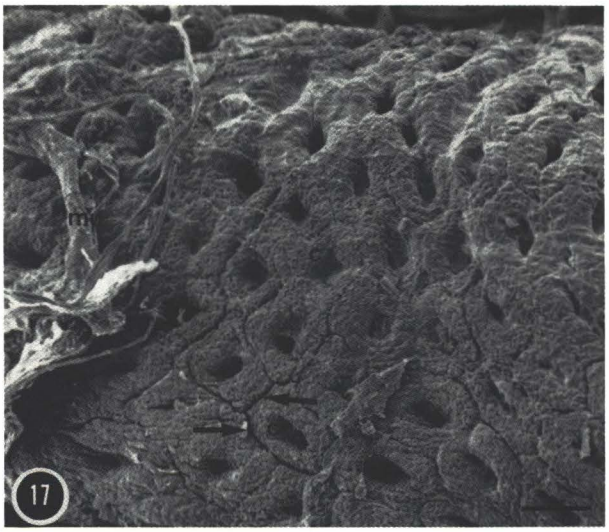
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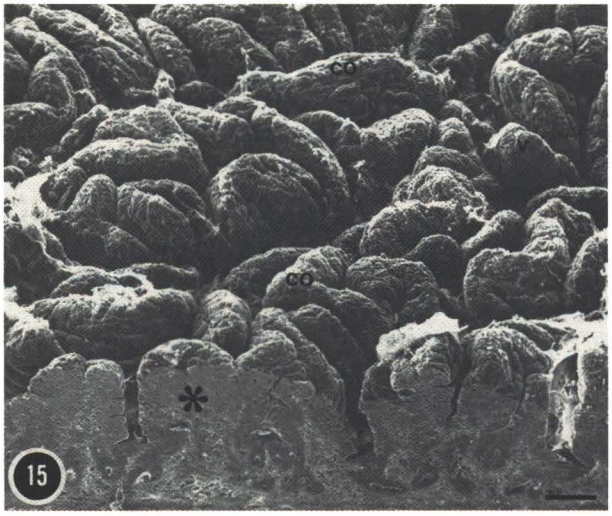
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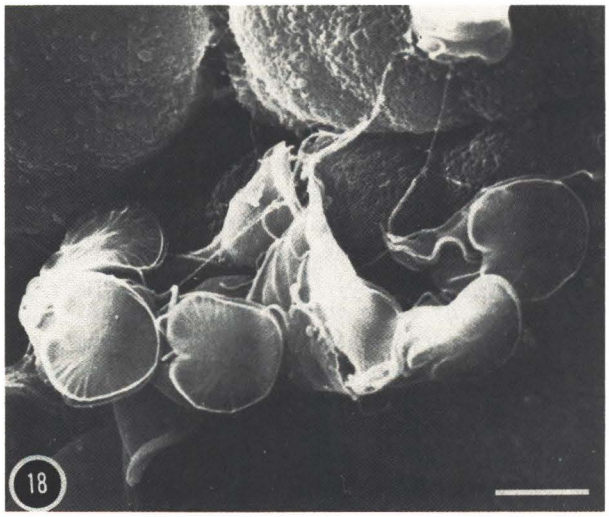
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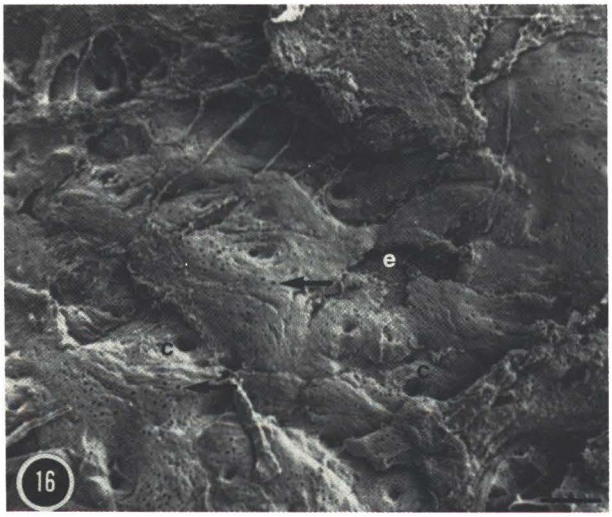
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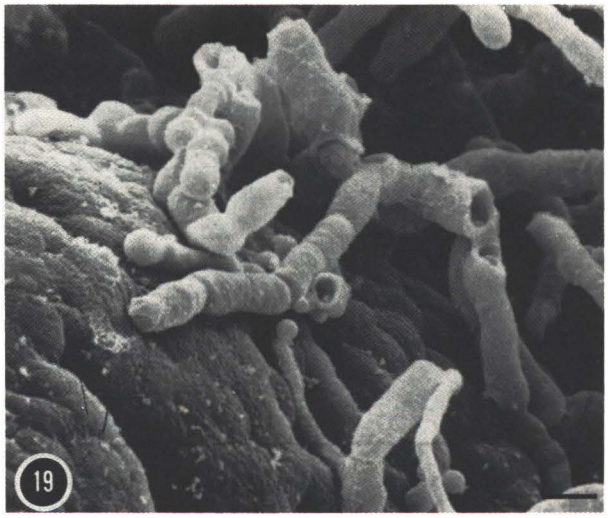
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Fig. 14. Small intestine mucosa of untreated coeliac disease: flat surface with regenerating (arrows) epithelial areas. c = crypts. Bar = 50μm.

Fig. 15. Small intestine mucosa of treated coeliac disease. Reprocessed paraffin block: the transected edge (*) shows the light microscopy appearance of "partial villous atrophy"; the mucosal surface shows a "transitional" pattern. co = convolutions; v = villi. Bar = 100μm.

Fig. 16. Ulcerative colitis: subverted mucosal surface architecture. c = crypt openings; arrows = goblet cells; e = surface erosion. Bar = 50 μm.

Fig. 17. Chron's colitis: preserved mucosal surface architecture. c = crypt openings; arrows = crypt units; mu = mucus. Bar = 50 μm.

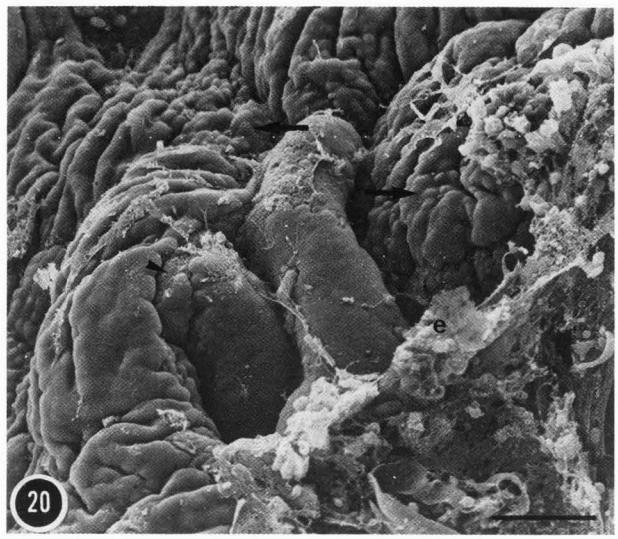
Fig. 18. Giardia trophozoites on small intestine mucosal surface. Bar = 5 μm.

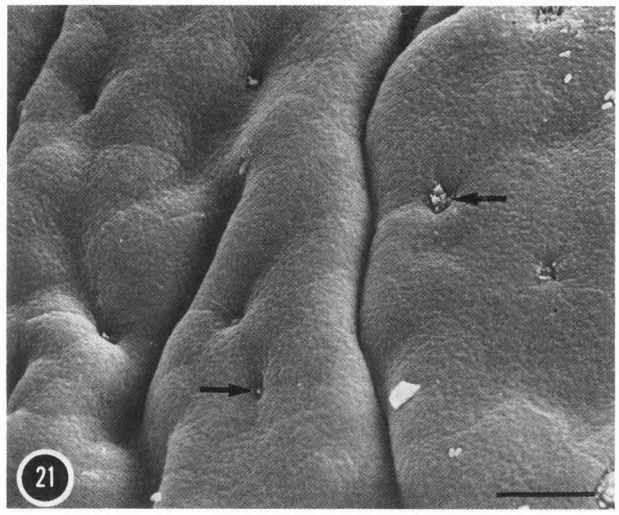
Fig. 19. Hyphae of Candida Albicans on small intestine mucosal surface. Bar = 5 μm.

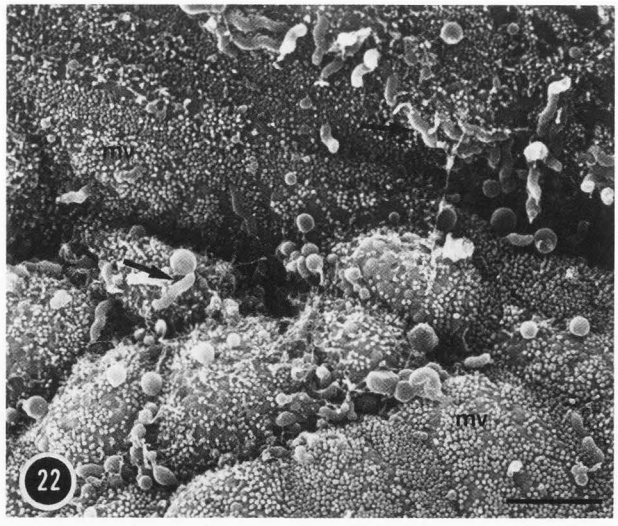
Fig. 20. Duodenal mucosa after the administration of non-steroidal anti-inflammatory drugs (FANS). Minimal changes: dome-shaped cells (arrows); disepithelialization (arrowhead); e = exfoliated cells and debris. Bar = 50 μm.

Fig. 21. Duodenal mucosa after the administration of FANS associated to a histamine H_2-receptor antagonist. Detail of normal enterocytic surface. The fuzzy coat covers the microvilli. arrows = goblet cells. Bar = 10 μm.

Fig. 22. Spiral bacteria on gastric mucosal surface (arrows). mv = microvilli. Bar = 5 μm.







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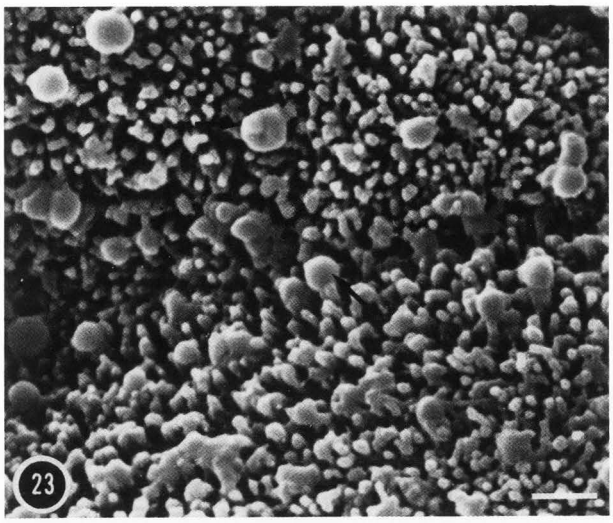
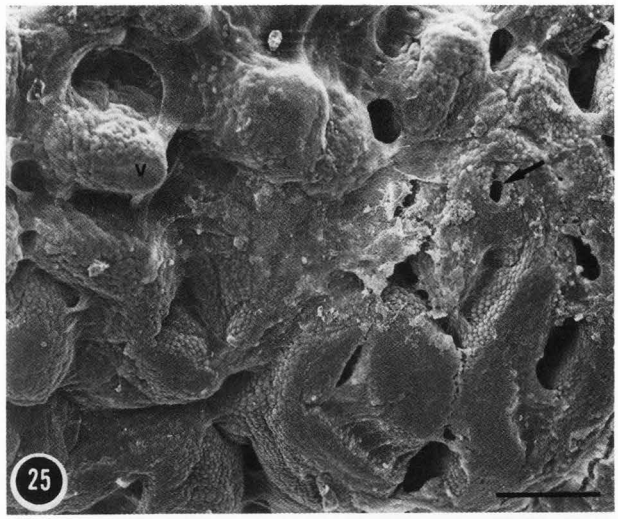
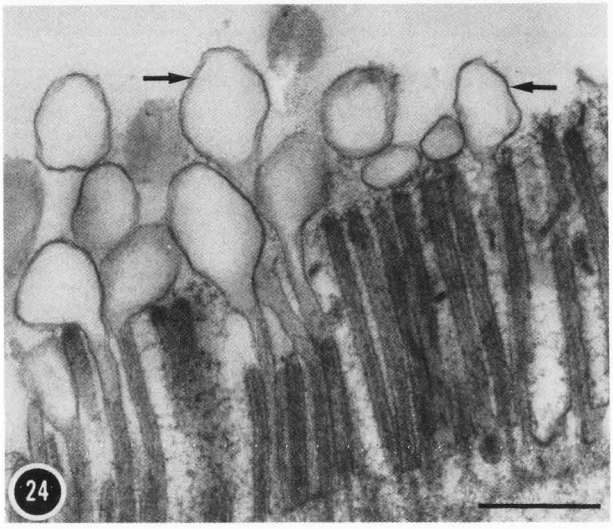
morpho-functional correlation with regard to the mucous secreting cells revealed the prevalence of "resting cells" at the edge of the ulcer as well as some changes in the patterns of lectin labeling (16). Similar modifications in the intracellular mucous composition could be responsible for failure in gastric protection. As for peptic disease expressed at duodenal bulb, SEM reveals enterocyte apical surface membrane changes both at the edge of the ulcer and at some distance from it. These membrane alterations, called blebs, are the earliest detectable lesions and may be considered predictive morphological signs of relapsing (Figs. 23, 24).







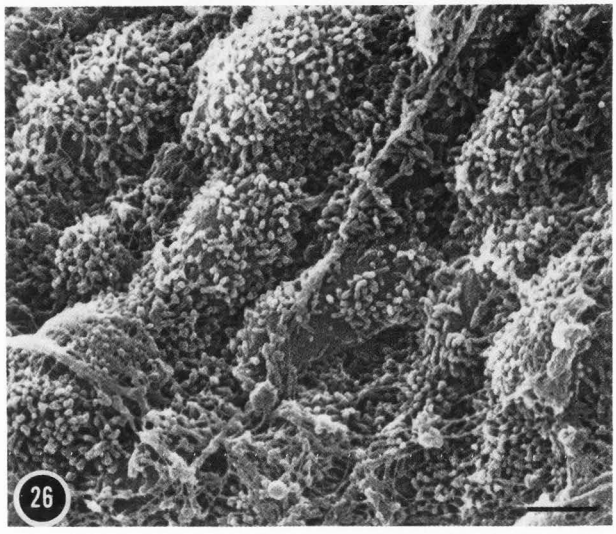


Fig. 23. Duodenal mucosa in peptic disease. Blebs (arrows) of the "brush border" microvilli. Bar = 1 μm.

Fig. 24. Blebs (arrows) of the duodenal enterocyte microvilli viewed at TEM. Bar = 0.5 μm.

Fig. 25. Intestinal metaplasia. v = stumpy villi; arrow = gastric foveola. Bar = 100 μm.

Fig. 26. Detail of Fig. 25. Cells with intermediate surface aspects between gastric and intestinal type. Bar = 2 μm.

Intestinal metaplasia is a common feature of gastric inflammatory conditions. SEM can appreciate its real surface extension. Moreover it can contribute to the assessment of the maturity of the epithelium on the basis of the microvillous pattern. By SEM, different types of mucosa are observed: i) a mucosa provided with stumpy villi lined by cells displaying an intermediate aspect between gastric and intestinal cells (Figs. 25, 26); ii) a mucosa with villous structures showing intestinal cells; iii) a flat mucosa which was also covered by cells with surface appearance of enterocytes. All these patterns were defined as "complete intestinal metaplasia" at LM level. Moreover, mucin characterization by means of lectins revealed mucous changes in all kinds of metaplasia with respect to normal small intestine mucosa. Thus, a better characterization of gastric intestinal metaplasia appears to be indispensable in the identification of lesion prone to malignant transformation.

Ophthalmology

Surface microscopy is of particular interest in ophthalmology, since the eye, as a hollow apparatus, has numerous inner surfaces. Nevertheless, few review papers have appeared in the past on important applications of scanning electron microscopy in the ophthalmological science (62, 63, 65, 79). Recently two authors (17, 78) reviewed the SEM contribution to the study of, respectively, the retina and the sutures of the lens.

Our own personal experience concerns the investigation of the anterior surface of the eyeglobe, i.e., the cornea and the conjunctiva. These two tissues can be examined by the clinician directly in vivo with the aid of a slit lamp, which provides 3-D information at 8, 10 x magnification. In this respect SEM appears to be a very interesting tool, as it enhances the images obtained with biomicroscopy, which are very familiar to the clinician, at ultrastructural level.

We have described in detail the relationship between cell surface morphology and the developmental stage in human corneal epithelium (156). The corneal anterior surface appears constituted by a mosaic of polygonal cells. These cells, depending on their brightness, are classified in dark, medium light and light cells (Fig. 27). According to our observations, the light cells are the younger cells, with a great quantity of surface microprojections. The dark cells appear to have few knobs or are completely smooth and represent the older, exfoliating cells. The medium light or intermediate cells are considered to be in a transitional stage. These results demonstrate that SEM provides a semiquantitative analysis on the tissue exfoliation-rate.

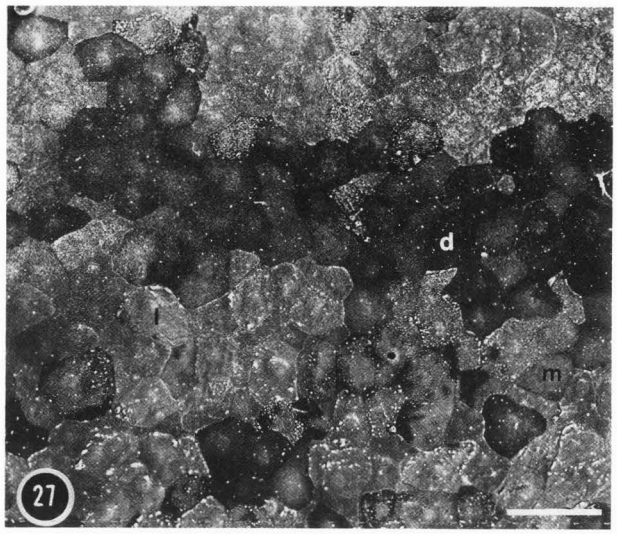
Our studies were performed on specimens obtained by scraping-off in vivo and confirmed the results of previous investigations carried out on buttons from cadavers (108, 113, 120). This suggests that the post mortem degenerative changes are relatively few in corneal cells. This finding is of particular value in respect to the problems related to the source of tissue for corneal grafts.

The different zones of human conjunctiva have been described in detail by SEM (29, 56, 60). The surface morphology of this tissue comprises epithelial cells covered with microvilli and mucus producing (goblet) cell orifices (Fig. 28). The SEM confirmed the higher density of the goblet cells on the nasal side of the lower tarsus (157) as already reported by Kessing (75) by using more conventional LM techniques. On the contrary, the cell orifices appear reduced in Keratoconjunctivitis sicca (KCS) (Fig.29). Anyway, one cannot exclude the presence of innermost goblet cells, whose orifices have not yet reached the surface.

In certain areas of KCS specimens, the microprojections seem to be lacking; the TEM analysis of the re-embedded biopsies recovered after SEM observation (Fig. 30) demonstrated that the microvilli were only obscured by overlying layers of mucus, but they were already present. This result confirms that in KCS the failure of tear film stability is not due to the absence of microvilli. On the other hand, our recent cytochemical studies (158) suggest that an alteration in the composition of mucus produced by goblet cells could be responsible for the unstable preocular film.

As to microvilli, SEM readily provides information on their arrangement. Greiner (59) introduced the concept of the 'Second Mucous System' (SMS) in human conjunctiva by correlating the microvillar rearrangement in tufted structures as seen at SEM, with the presence of mucus producing subsurface vesicles in non-goblet epithelial cells (Fig. 31). This feature was observed in asymptomatic contact lens wearers (58) and patients suffering from Giant Papillary Conjunctivitis (GPC) (57). Our own SEM observations in adenovirus follicular conjunctivitis and hay fever conjunctivitis (157) found an increase in activity of this SMS.

Moreover, SEM significantly contributed to the study of contact lens surfaces (38, 39, 40, 41, 97) by correlating at ultrastructural level the images obtained in LM and interference microscopy. In this respect, it is important to establish the exact nature of the deposits which accumulate day after day on the lens. It is believed that these deposits denaturate and this leads to tissue involvement (165). Organic material deposited as a matted coating on the lens surface (Fig. 32), exfoliated (corneal and/or conjunctival) cells (Fig. 33), microorganisms, inorganic crystals, defects or ruptures of the lens surface itself as a cause of wear are very easily detected by SEM. Obviously, SEM morphology does not provide a full characterization of the substances on the lens surfaces. As a consequence, it is necessary to perform studies in SEM, TEM, X-ray microanalysis and immunocytochemistry on the same lens (160) to achieve complementary information and to exactly correlate the morphology of the deposits with their composition.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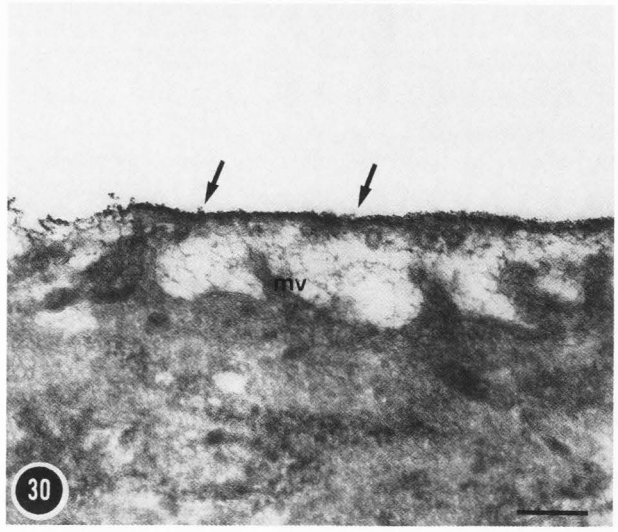
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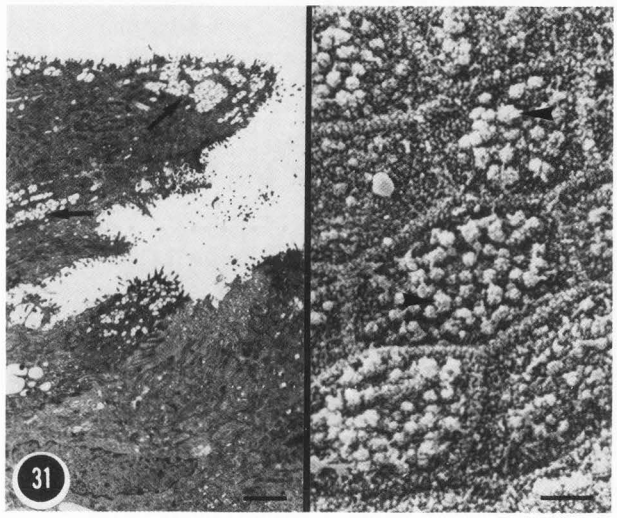
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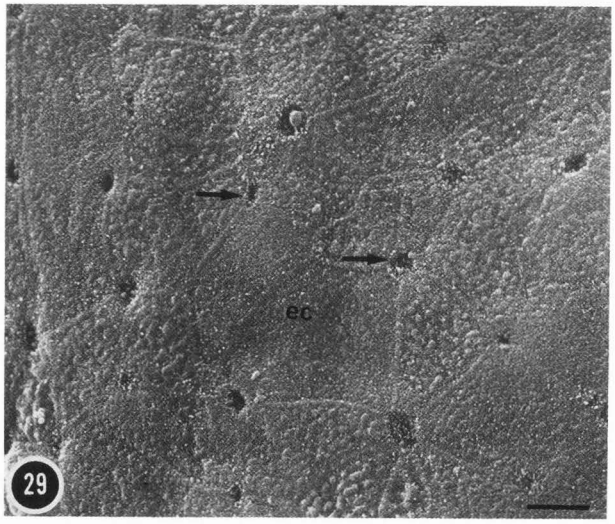
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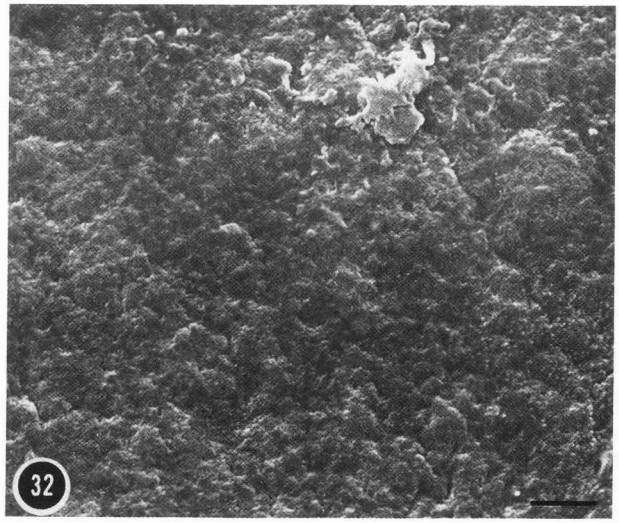
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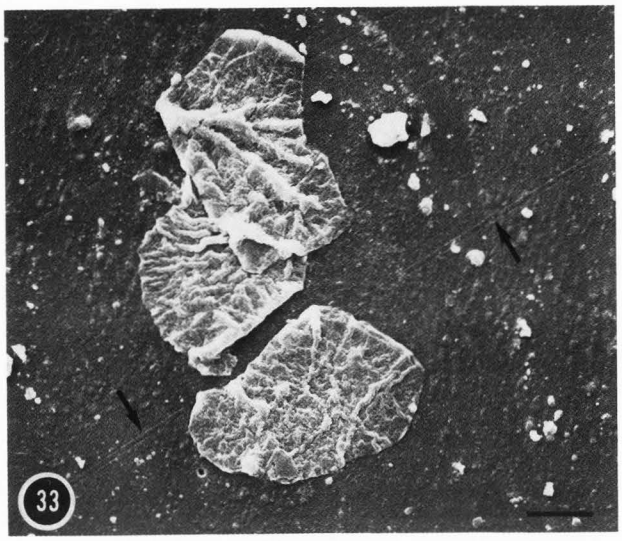


Fig. 27. Normal human corneal epithelium. Light (l), medium light (m) and dark (d) cells are clearly evidenced by SEM. Bar = 50 μm.

Fig. 28. Normal human conjunctival epithelium. Epithelial cells with the regular pattern of microvilli (mv) and goblet cell orifices (arrows) are shown. Arrowhead = mucus extruding from a goblet cell. Bar = 5 μm.

Fig. 29. Human conjunctival epithelium. Keratoconjunctivitis sicca. Empty goblet cell orifices (arrows). Some epithelial cells (ec) appear to be lacking of microvilli. Bar = 5 μm.

Fig. 30. Human conjunctival epithelium. Keratoconjunctivitis sicca. Recovered specimen. The gold layer (arrows), sputtered for the previous SEM observation, obscures the underlying microvilli (mv). Bar = 200 nm.

Fig. 31. Normal human conjunctiva. Subsurface epithelial vesicles belonging to the "second mucous system" (arrows) are shown on the left in TEM. The corresponding surface appearance is shown on the right in SEM; microvilli are aggregated in small clusters cemented by mucus (arrowheads). TEM Bar = 2 μm. SEM Bar = 2 μm.

Fig. 32. Soft contact lens. Mucus material deposited as smooth matted coating on the surface. Bar = 5 μm.

Fig. 33. Soft contact lens. Exfoliated cells are deposited on the surface. arrows = polish marks. Bar = 10 μm.

Hematology

Scanning electron microscopy investigation has, from the beginning, played an important role in the study of blood cells. Early studies carried out in 1968 and 1972 using air-dried cells concluded that SEM was uneffective in differentiating the white series cells which showed minimal differences in the surface morphology. It was only with the introduction of the critical point drying that the complex architecture of the leucocytes was evidenced, even if the cellular differentiation based only on the morphology cannot be sustained because the arrangement of the microvilli may vary with: temperature, cell cycle, intercellular contacts, antigenic maturity or stimulation, variations, even small, in the preparative methods (166).

Bessis's (13) early studies have lead to a new tridimensional classification of red blood cell (RBC) disorders based on alterations in size, shape and surface topography. RBC alterations are evidenced as follows: a) thalassaemic syndromes; b) hereditary abnormalities (acanthocytosis, elliptocytosis or stomatocytosis) are differentiated at SEM by the crenated RBC. Polliack (115) states: "There is no doubt that in respect to RBC pathology, SEM contributes a vivid three-dimensional image of the cell deformities .... and has led to a better understanding of RBC disorders".

The study of the white series is more complex. At the beginning the aim was to differentiate the elements by assessing the pattern of the microvilli, microridges, blebs; the presence, however, of microprojections could be indicative of maturity and activation rather than of origin. Thus, in spite of various attempts, observation at SEM cannot differentiate the various types of leukemia (115), other than the hairy cell subtype.

The necessity to correlate the surface appearance with the internal features of the cytoplasm in order to better characterize the cells was later emphasized. Albrecht et al. (3) applied specific cytochemical reactions (esterase, peroxidase, toluidine blue for the azurophylic granules, tantalum as marker for phagocytosis) detected with BEI and X-ray microanalysis associated to surface SEI. Soligo et al. (141, 142) used the same method; they applied other cytochemical reactions (alkaline and acid phosphatase, OsO_4-DAB). This method does not differentiate the various lymphocyte subpopulations but is useful in the identification of normal and leukemic cells. The most recent step in the

characterization of blood cells associates internal cytochemistry to surface immunocytochemistry (31, 32, 143) detected in BEI or with a signal mixing device. Immuno-scanning electron microscopy is important as it "adds the immunological dimension to ultrastructure and is potentially useful as a sensitive aid in attempting to classify leukemic cells" (43, 44).

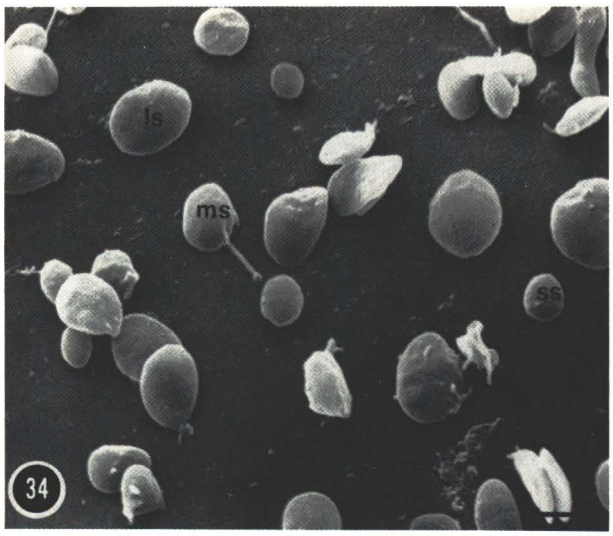
The same attempts have been made in the study of platelet physiologic and pathologic processes (4). Colloidal gold-conjugated anti-fibrinogen antibodies were visualized on the platelet surface by means of SEM. Stereo HVEM, moreover, contributed to simultaneously detect the pattern of distribution of these receptors and the internal cytoskeleton. Knowledge concerning the cytoskeletal events which occur during adhesion and which lead to the release of granule constituents was widely deepened in these years (12, 89, 98). The various stages of platelet adhesion and spreading have been described in correlative SEM/TEM studies (167) in order to better understand the involvement of these cells in many pathological disorders. It was thus possible to correlate shape changes with functional state (42).

In our specific experience we studied patients affected by type IIa heterozygous hypercholesterolemia, a frequent condition in which atherosclerosis develops early. SEM was used to estimate the platelet size and shape changes. These parameters, in fact, are considered to be related to platelet activity (153). Platelets from hypercholesterolemic patients displayed only minimal shape changes, i.e., emission of long, slender pseudopods projecting from the cell periphery. As to the size changes, a statistically significant decrease in the mean platelet diameter, due to an increased frequency of small platelets, has been detected (Figs. 34, 35). This finding suggests that hypercholesterolemia could directly affect the platelet production from megakaryocytes at bone marrow level (85, 95).

Correlative Microscopy

In our laboratory, correlative studies are routinely performed on human specimens. There are several reasons which support a similar choice. Sometimes it becomes a real necessity. It is the case of human conjunctival biopsies which are so small that very often it is impossible to divide them into small fragments (for SEM/TEM/ICC and others). In other circumstances, it represents a practical advantage:

. to gain all possible information in the same specimen;



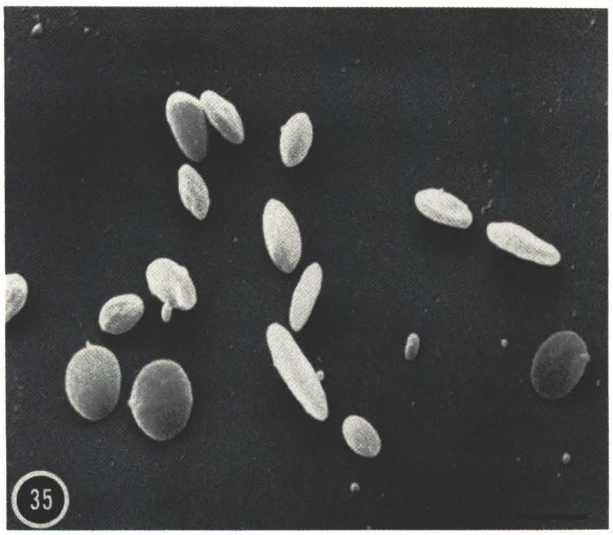
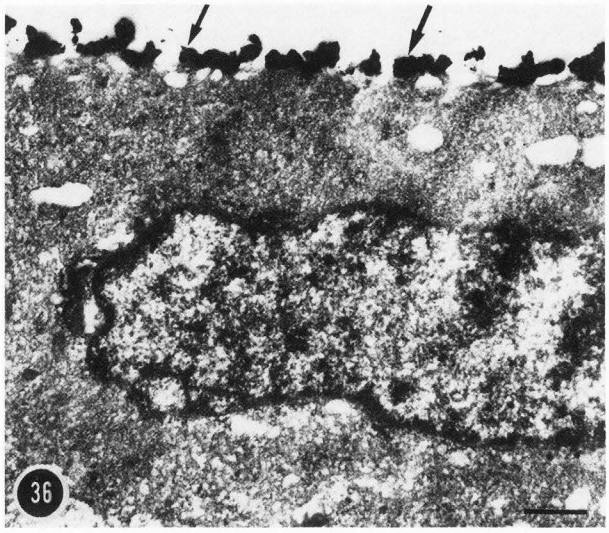


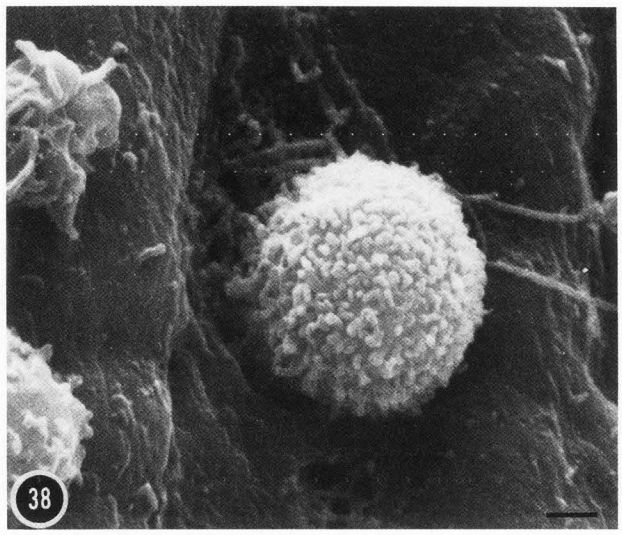
Fig. 34. Circulating platelets from a normal subject. SEM view of resting discoid platelets. Large (ls), medium (ms) and small (ss) sized platelets are recognized. Bar = 2 μm.

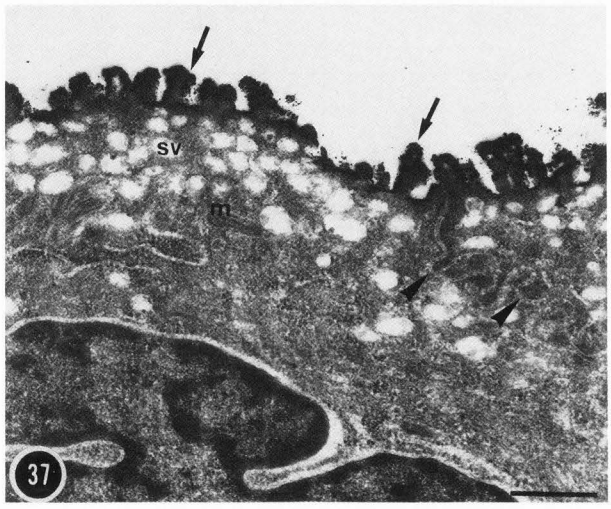
Fig. 35. Circulating platelets from a type IIa hypercholesterolemic patient. SEM shows an increased number of small discoid platelets. Bar = 2 μm.

. to perform retrospective studies on human material stocked in histological archives;
. to confirm and extend successively different information;
. to better define points useful for diagnostic purposes.

Thus, we have improved and optimized some methods particularly useful for intermicroscopic correlations:







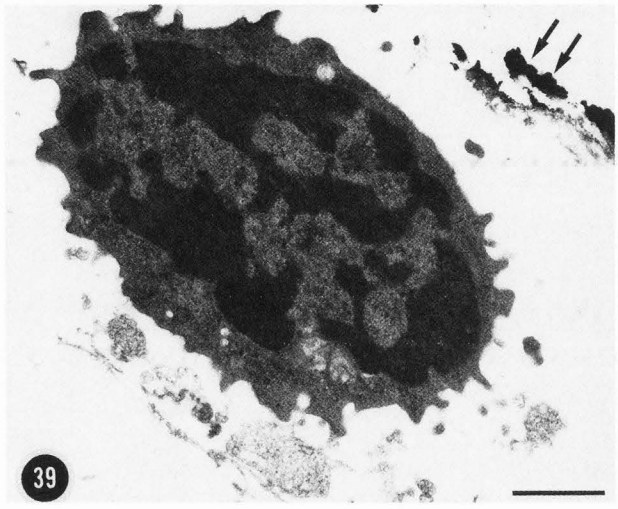


Fig. 36. Normal human conjunctival epithelium. Specimen recovered from SEM with no rehydration step before embedding in the resin. The cytoplasm appears packed, the ultrastructural morphology of the organelles is very poor. Arrows: gold layer sputtered. Bar = 0.5 μm.

Fig. 37. Normal human conjunctival epithelium. Specimen recovered from SEM with the rehydration step before embedding. Mitochondria (m), subsurface vesicles (sv), intercellular edges (arrowheads) are well recognizable. Arrows: gold layer sputtered. Bar = 0.5 μm.

Fig. 38. Human atheromatous carotid artery. SEM view of a lymphocyte on the endothelium. Bar = 1 μm.

Fig. 39. Human atheromatous carotid artery. Recovered specimen. A good TEM morphology is achieved by applying the rehydration step before embedding. Arrows = gold layer sputtered. Bar = 1 μm.

SEM►LM/TEM

This is the most common procedure which permits us to gain ultrastructural information on the same tissue sample (10, 18, 102, 112, 168). In this case, the major limitation consists of the often poor morphology of the recovered material as seen in TEM. This is mainly related to a shrinkage caused by the CPD step applied for previous observation at SEM.

For this reason, we support the value of

partial rehydration of the specimen before embedding in the plastic resin (159). Briefly, the specimen is removed from the stub, set in propylene oxide, rehydrated through a descending series of ethanol and allowed to rest in 0.1 M cacodylate buffer for 10 min. Therefore, the biopsies are conventionally dehydrated and embedded in Epon. This procedure was found to allow a better infiltration of the resin and a partial recovering of the miniaturization of the cytoplasmatic organelles due to CPD. We have applied this new schedule in the study of human conjunctival biopsies (Figs. 36, 37) and of human atheromatous plaques (Figs. 38, 39).

LM►SEM

The possibility of recovering paraffin blocks for SEM observation has been proposed for many years (11, 21, 23, 146). This fact is useful as it permits retrospective studies on a large amount of biopsies stocked in the histological archives. In addition, it provides a finer analysis of the morphology of the transected edge in relation to the surface features of the same specimen. In some instances this additional step can deepen the previous LM analysis. A clear example of its usefulness is in the study of the pattern of the jejunal mucosa at the beginning of a gluten-free diet in coeliac patients. In fact, while in LM the mucosa appears quite flat the SEM of the recovered specimen shows surface mucosal changes related to an initial repairing stage (Figs. 40, 41). In addition, the recovered material may still exhibit a certain degree of antigenicity. This is demonstrated by the detection of WGA glycosidic receptors on the apical membrane of the enterocytes as well as the goblet cells (16) (Fig. 42).

Semithin Sections

A further example of the importance of correlation in the clinical application of SEM is the use of semithin sections, originally developed as a bridge between conventional histological techniques and TEM. Impressive patterns of staining have been achieved on semithin sections by improvement methods of tissue embedding and staining (80). Thus, familiar LM images with high definition are provided to pathologists.

Additional techniques of tissue processing have allowed us to survey, in sequence, the same field of semithin section first by LM and later by SEM (111). Cytochemical and immunocytochemical procedures were successfully applied. Moreover, suitable parameters of observation were standardized (127, 128). Overall, SEM of semithin sections yelds SE images showing a resolution in the range between the light microscope and the transmission electron microscope and a contrast similar to that of the LM image. Used with

Fig. 40. Small intestine mucosa of a coeliac patient. Reprocessed paraffin block. (*) = transected edge. Bar = 100 μm.

Fig. 41. Small intestine mucosa of coeliac patient after 1 month of gluten-free diet. The transected edge (*) is similar, but the treated mucosa shows initial repairing in respect to Fig. 40. Bar = 100 μm.

Fig. 42. Small intestine mucosa. Reprocessed paraffin block incubated with WGA-colloidal gold - silver enhanced. The reaction products are visible on the brush border and goblet cells. BSE (+) mode. Bar = 50 μm.

Fig. 43. Human rete testis. Light microscopic image. Clusters of neoplastic cells (arrows) are embedded in a fibrous stroma displaying a prominent inflammatory infiltrate (arrowheads). Bar = 10 μm.

Fig. 44. Human rete testis. SE(-) image of the same semithin section of Fig. 43. An intercellular crypt, lined by numerous microvilli, is readily recognized. Arrows: intercellular junctions. Bar = 2 μm.

Fig. 45. Human rete testis. Apart from resolution, TEM provides similar information to those obtained by SEM. ic = intercellular crypt; arrow = desmosome; arrowhead = tight junction. Bar = 10 μm.

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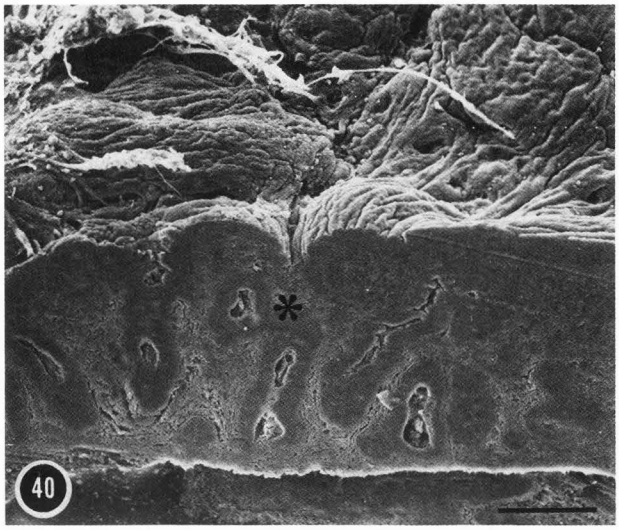
appropriate caution, the SEM may lead to the identification of some sufficiently reliable morphological features useful for diagnostic purposes. At LM these would remain incompletely defined. For instance, inter/intracellular crypts (Figs. 43, 44, 45), bundles of electron-dense tonofibrils, and sparse neurosecretory granules can be easily disclosed in tumors.

Finally, SEM of semithin sections enables correlative X-ray microanalytical studies on the same section thus providing an additional analytical dimension. This has proven to be useful, in many clinical cases, in order to identify both exogenous (i.e., lead, gold) and endogenous (i.e., iron) substances.

Conclusion

In conclusion, our opinion based on long experience is that SEM plays an important role in clinical research. Some general advantages are well established, i.e., the possibility of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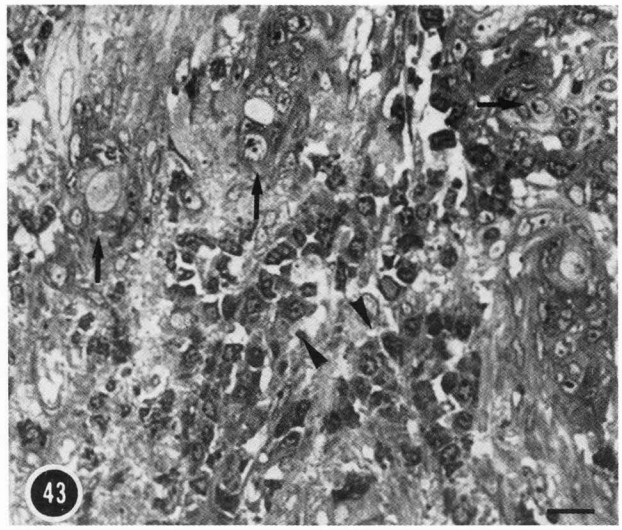
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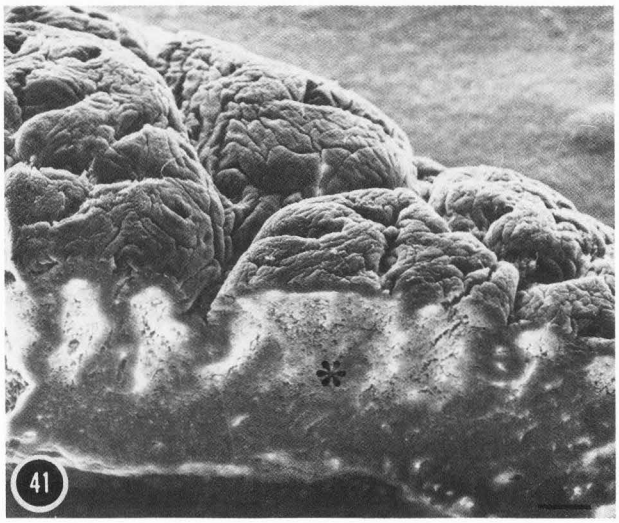
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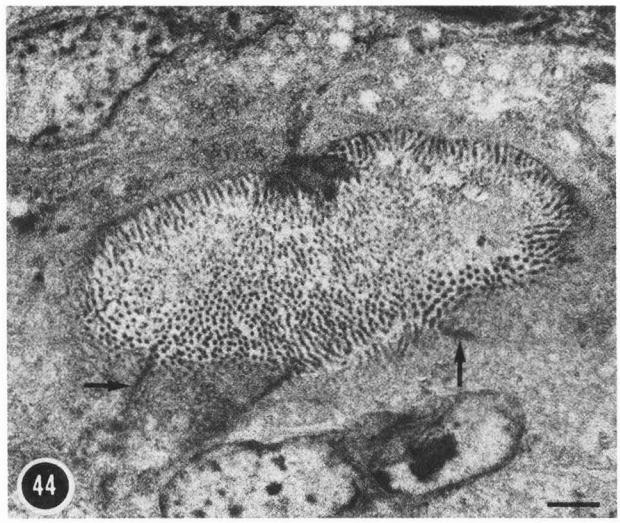
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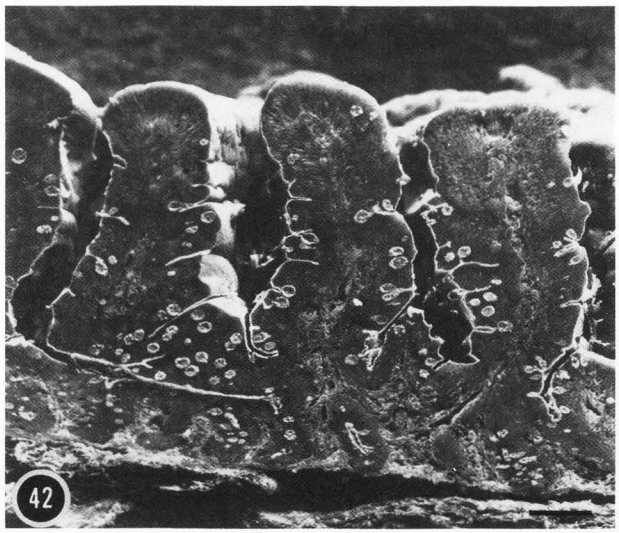
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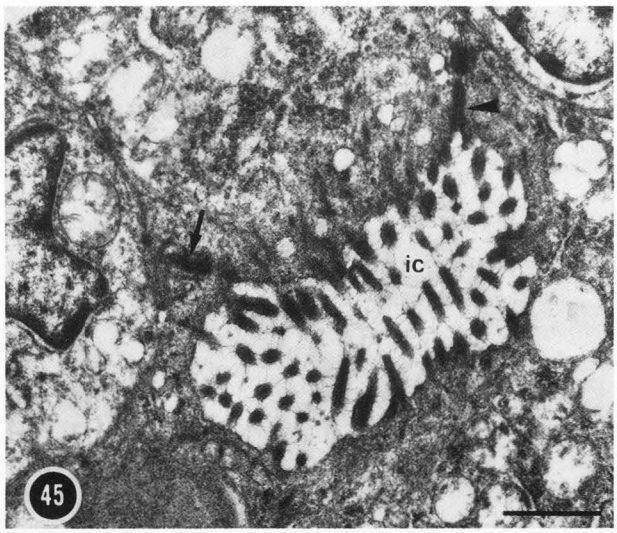
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surveying relatively large specimens at ultrastructural level thus avoiding time-consuming reconstruction methods. In addition, there are situations, such as those reported in this paper, in which the information obtained through SEM tridimensional images evidences aspects which did not result from routine diagnostic investigation for example endoscopy and angiography.

Some conditions, however, appear indispensable:

. continuous and simultaneous correlation between SEM and other methods and instruments for morphological investigation;
. the sample preparative methods are still far from being optimal.

The processing techniques for human material will have to undergo, in the years to come, sophisticated improvements. This will enhance the performance of instruments and also SEM. These prospects are today only partially predictable.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.