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Original article

Ultrastructural immunogold labeling of lipid-laden enterocytes from patients with genetic malabsorption syndromes

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Summary – Intestinal biopsies from patients having genetic disorders of lipoprotein assembly and secretion, such as abetalipoproteinemia (ABL) or Anderson's disease (AD), contain large amounts of lipids which are accumulated in the enterocytes. Determination of the intracellular sites in which the lipids accumulate and to which apolipoproteins the lipids are bound would help to identify the defects in these diseases and further elucidate the mechanisms by which lipoprotein assembly and secretion occur normally. Ultrastructural immunogold labeling, however, is hampered by the poor preservation of the lipids accumulated in the enterocytes of these patients. We have used routine electron microscopy (fixation and ultra-thin sectioning) along with three methods for immunogold labeling of lipid-laden enterocytes: ultrathin cryosectioning, low temperature freeze substitution with embedding in Lowicryl K4M, and ultra-low temperature freeze substitution with embedding in Lowicryl HM20, to establish a protocol for investigating the intestinal tissue from these patients. Ultracryosectioning, while preserving the overall morphology of the lipid laden enterocytes, did not preserve the lipid content and the immunogold labeling of apolipoprotein B (ApoB) appeared dislocated. Freeze substitution and low temperature embedding in Lowicryl K4M, in contrast, appeared to better preserve the lipid and lipoprotein structures; however, the antigenicity of both apoAI and apoB appeared to be lost and no specific labeling could be obtained. Freeze substitution and embedding in Lowicryl HM20 best preserved the lipid and lipoprotein structures while maintaining apoprotein antigenicity. In conclusion, immunogold labeling of apolipoproteins on lipid structures in the lipid-laden enterocytes of patients with ABL and AD is best obtained by freeze substitution and embedding in Lowicryl HM20.

Anderson's disease / abetalipoproteinemia / freeze-substitution / low temperature embedding / cryo-ultramicrotomy / protein A-gold / immunogold labeling / lipoprotein assembly / malabsorption

Introduction

In man, apolipoprotein B (apo B) containing lipoproteins play important roles in lipid transport and metabolism [26]. They have also been implicated in the pathogenesis of cardiovascular disease which is one of the major causes of mortality in western countries [47]. Although much is known about the structure and physiology of these particles, the mechanisms of assembly and secretion of the apo B containing lipoproteins in the intestine and the liver are less well understood. The initial assembly of apo B with lipids apparently occurs cotranslationally in the rough endoplasmic reticulum [1, 10, 11, 18]. Nascent lipoproteins are then transported to the Golgi apparatus and subsequently to the intestinal intercellular spaces at the basolateral side of the enterocyte and to the space of Disse in hepatocytes. It is unknown, however, exactly how lipids and apo B are brought together to form a lipoprotein particle. It is clear that, at least, one other factor, microsomal triglyceride transfer protein (MTP), is involved in the assembly of lipoproteins [24, 38 , 46].

To learn more about these mechanisms, we have chosen to study two inherited disorders: abetalipoproteinemia (ABL) and Anderson's disease (AD) [2, 13–15, 20, 24, 34, 36, 39, 46]. These diseases are genetic defects of the assembly and the secretion of apo B lipoproteins, not related to the apo B gene. They are characterized by a marked accumulation of lipids alone (ABL) or lipids associated with large vesicles containing numerous lipoprotein-like structures (AD). Identification of the subcellular structures where these lipid or lipoprotein-like structures are stored may help to identify the factors involved in these defects (apolipoproteins or other factors) and lead to a better understanding both of these diseases and of lipoprotein assembly and secretion in normal individuals.

Immunolabeling at the ultrastructural level is a powerful technique for studying directly the *in situ* localization and the intracellular pathways of proteins in cells. As compared to immunofluorescence methods [17], ferritin-labeled antibodies [40] provide the advantage of being visible at the electron microscopic level, however, the disadvantage of this technique is an impaired penetration of the labeled antibodies into the tissues. More recently, enzyme labeled antibodies [4, 28] have become widely used for the localization of cellular components in investigative and diagnostic pathology as well as for research [5, 29].

When immunoelectron microscopy following peroxidase labeling of apolipoproteins was used to study the enterocytes from patients having ABL or AD, lipid and lipoprotein structures were poorly preserved and the interpretation of the labeling was very difficult (Bouma *et al*, unpublished results). Visualization of the particular subcellular structures to which apo B or other proteins were bound was not possible. This difficulty was due to the marked intra-cytoplasmic accumulation of lipids and lipoprotein-like structures in both the enterocytes and hepatocytes from patients with ABL and AD, resulting from the inability of these cells to export fat as lipoprotein particles.

The development of the protein A-gold immunochemical labeling technique associated with improved embedding procedures has further extended the investigation of protein secretion at the ultrastructural level [7, 8, 31]. Several proteins have been localized with high resolution and specificity in particular subcellular compartments, confirming the data obtained by autoradiography and cell fractionation concerning the secretory pathway [30]. Because of their small size, gold particles provide accurate and easy identification of these compartments and the intensity of the labeling can be quantified simply by counting the number of gold particles. Double labeling with gold particles of different sizes can demonstrate two different antigenic sites on the same tissue section [6]. These different techniques have been used to study lipoprotein biosynthesis and secretion in the rat, chicken and in man [1, 11–13, 16, 19–21, 23, 25, 27, 37]. Unfortunately, immunolabeling at the ultrastructural level of the intestinal biopsies of patients with ABL and AD is greatly hampered by the marked accumulation of lipids.

Ultrathin cryosectioning [43] and freeze substitution followed by low temperature embedding in Lowicryl [3, 32, 33, 45] associated with protein A-gold labeling may avoid some of the problems due to the intracellular lipid accumulation [45]. Levy *et al* [27] and Dixon *et al* [19] showed the feasibility of using Lowicryl K4M embedding for studying normal human intestinal and chicken liver ultrastructure, respectively. Dullaart *et al* [20] used ultra-thin cryosectioning to study the ultrastructure of intestinal fragments from a patient with abetalipoproteinemia. These interesting data suggested that it might be possible to preserve the antigenicity of the proteins as well as the ultrastructure of lipids and lipoproteins in the lipid-laden enterocytes from patients with abetalipoproteinemia and Anderson's disease. We studied several approaches to fixing and embedding intestinal biopsies from normal individuals as well as from patients with ABL or AD. After optimizing the experimental conditions, we found that good preservation of the subcellular architecture could be obtained while maintaining the antigenicity of the apolipoprotein when the tissue was pre-fixed in paraformaldehyde with or without glutaraldehyde and then freeze-substituted at –90°C and embedded in Lowicryl HM20 at –50°C. The same protocol worked well with biopsies from normal individuals and should prove useful for the intracellular localization of apoprotein antigens.

Materials and methods

Human intestinal biopsies

Human duodeno-jejunal fragments were obtained by fiber optic endoscopy, from patients diagnosed as having abetalipoproteinemia or Anderson's disease after the nature of the study and its possible consequences were explained and after informed consent was obtained. Normal biopsies were a part of those taken from normo-lipidemic individuals for diagnostic purposes, after obtaining informed consent. All biopsies were obtained in the fasting state (12–15 h). The procedures for obtaining biopsies and the experimental methods employed in their analysis have been approved by INSERM (Institut National de la Santé et de la Recherche Médicale) and the Bioethics Committee of the Bichat Hospital (Paris, France), as part of a biomedical project (Projet de Recherche Biomedicale no 94002) for the study of hereditary disorders of malabsorption and lipoprotein assembly and secretion. Biopsies were treated according to four protocols. The first, fixation and ultrathin sectioning, was used for routine staining prior to electron microscopy. The other three, fixation and cryo-ultramicrotomy, freeze-substitution and low temperature embedding in Lowicryl K4M, and freeze-substitution and ultra-low temperature embedding in Lowicryl HM20, were used prior to immunogold labeling.

Routine electron microscopy

Fixation and ultrathin sectioning

Intestinal fragments (2–3 mm^3) were fixed immediately by immersion in 2.5% glutaraldehyde (TAAB, Aldermaston, UK) buffered with 0.1 M sodium phosphate (pH 7.4) for 2 h at 4°C. After post fixation in 1% osmium tetroxide for 1 h at 4°C, the fragments were dehydrated with ethanol at 4°C (70% ethanol overnight; 95% ethanol for 20 min; 100% ethanol, twice for 20 min; and then once for 30 min), then immersed in two baths of pure propylene oxide at 4°C (for 20 min and then for 30 min) and then immersed in a 1:1 mixture of propylene oxide and Epon (TAAB, Aldermaston, UK) followed by embedding in Epon and polymerization at 60°C for 48 h. Ultrathin sections, prepared with an LKB Ultramicrotome III 8802A (Bromma, Sweden), were counterstained for 10 min with 1% aqueous uranyl acetate and for 10 min with Reynold's lead citrate. They were observed with a Jeol 1010 electron microscope (Jeol, Croissy-sur-Seine, France).

Immuno electron microscopy

Fixation and cryo-ultramicrotomy

Intestinal fragments (about 2–3 mm^3) were fixed immediately by immersion in either 2% paraformaldehyde (freshly prepared from Sigma, St Louis, USA) alone or in a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde both in 0.1 M sodium phosphate (pH 7.4) for 2 h at room temperature. In both cases, after fixation, fragments were stored in a 2% paraformaldehyde solution, at 4°C, until further processing. In our experience, tissues could be stored up to 6 months without any apparent modification of the immunolabeling or the lipid preservation. Ultrathin cryosectioning of the fixed biopsy fragments was performed with modifications of techniques previously described [22, 43, 44]. The fixed tissue fragments were cryoprotected by infusion under stirring, with 2.3 M sucrose in 0.1 M phosphate buffer (pH 7.4) for 30 min at 20°C, then placed on copperblocks and frozen in liquid nitrogen. Ultrathin cryosections were prepared using an FC4D ultracryotome (Reichert, Vienna, Austria) and then processed for immunogold-staining.

Freeze-substitution and low temperature embedding in Lowicryl K4M

Low temperature embedding in Lowicryl K4M was performed, with some modifications, as previously described [3, 32, 33]. Briefly, intestinal biopsies were fixed with 2% paraformaldehyde or with a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer as described above. They were infused under stirring for 30 min in 2.3 M sucrose buffered with 0.1 M sodium phosphate (pH 7.4) at room temperature and then frozen in liquid nitrogen. Fixed tissues were then dehydrated in a graded ethanol series, during which the temperature was lowered stepwise to –35°C (30% ethanol, 0°C, 30 min; 50% ethanol, –20°C, 60 min; 70% ethanol, –35°C, 60 min; 80% ethanol, –35°C, 60 min; 90% ethanol, –35°C, 60 min; 100% ethanol, –35°C, 2 h). Infiltration with Lowicryl K4M and embedding in beam capsules was done at –35°C (Lowicryl:ethanol 1:1, 60 min; Lowicryl:ethanol 2:1, 60 min; Lowicryl 100%, 60 min, Lowicryl 100%, overnight). The material was embedded in pure Lowicryl K4M in beam capsules and polymerization was induced by UV light at –40°C for 48 h and then completed by 48 h of incubation at room temperature. Ultrathin sections were prepared using an LKB Ultramicrotome III 8802A and were then processed for immunolabeling.

Freeze-substitution and ultra-low temperature embedding in Lowicryl HM20

Intestinal biopsies were first fixed with 2% paraformaldehyde or with a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer as described above. Fixed intestinal biopsies were then infused under stirring for 30 min in 2.3 M sucrose buffered with 0.1 M sodium phosphate (pH 7.4) at room temperature and then frozen in liquid nitrogen. They were then dehydrated in methanol at –90°C for at least 48 h in a CS auto, cryo-substitution apparatus (Reichert). The embedding was performed with increasing concentrations of Lowicryl HM20 in methanol (Lowicryl:methanol 1:1, 1 h; twice; Lowicryl:methanol 2:1, 1 h; twice; pure Lowicryl for 1 h, then 16 h and finally for 1 h) by raising the temperature from –90°C to –50°C at a rate of 4°C per hour. Samples were transferred, inside the Reichert CS-auto, to an embedding mould filled with pure Lowicryl HM20 and polymerization was induced by indirect UV light at –50°C for 48 h and was completed by incubation at room temperature for 2 more days [45]. Ultrathin sections were prepared using an LKB Ultramicrotome III 8802A and were then processed for immunolabeling.

Immunogold labeling of ultrathin cryosections and ultrathin Lowicryl sections

The frozen ultrathin cryosections and the ultrathin Lowicryl sections were transferred to carbon coated collodion grids and immunogold labeled by modifications of previously described techniques [22, 31]. We investigated various durations (15 min to 1 h) and various concentrations of bovine serum albumin (BSA) (1 to 5%) and gelatin (1 to 4%) for the preincubation period at room temperature in order to determine that the optimal conditions were to preincubate thawed cryosections and Lowicryl sections at room temperature for 1 h in a solution of 1% BSA and 4% gelatin dissolved in phosphate buffered saline, pH 7.4 (buffer A). This incubation was necessary to reduce the non-specific immunolabeling that occurred, particularly in the lipid-laden cells of the patients. Buffer A was present throughout the treatment of the ultrathin sections (including incubations with the antibodies and with protein A gold as well as during washing steps). Sections were then incubated at room temperature for 1 h with a polyclonal (rabbit anti apoB anti-serum diluted 1/100) or monoclonal antibody (murine anti-apoB diluted 1/50 or 1/100; murine anti-apoAI diluted 1/50 or 1/100) specific for the apolipoprotein diluted in buffer A. Polyclonal rabbit antibodies against apoB were prepared by A Mazure (Institut National de la Recherche Agronomique, Laboratoire des maladies métaboliques, Clermont-Ferrand) and by S Salmon (INSERM Unit 312, Museum National d'Histoire Naturelle, Paris, France). The murine monoclonal anti-apoB antibodies were from M Ayrault-Jarrier [35] and the murine monoclonal anti-apo A_I was from Daiichi (Tokyo, Japon). Both the rabbit polyclonal antibodies against apoB gave identical labeling of the cellular structures, which was better than that obtained with the murine monoclonal antibodies, and they were, therefore, used for this study. After incubation with the first antibody for 1 h at room temperature, the sections were washed with buffer A (three times for 10 min) and then incubated for 1 h with protein A complexed to 10 nm colloidal gold (1/400), as previously described [9, 41]. After washing with distilled water, the sections were stained with a saturated aqueous solution of uranyl acetate (10 min) and with Reynold's lead citrate (10 min). They were examined at 80 kV with a Jeol 1010 electron microscope. Non-specific labeling was evaluated by omitting the specific antiserum and incubating the sections with pre-immune serum or with protein A gold reagent alone.

Results and discussion

Routine electron microscopy

Intestinal tissues from patients with abetalipoproteinemia were characterized by a marked accumulation of large lipid vacuoles ranging in size from 200 nm to 8 μm (fig 1a). The Golgi apparatus appeared flat, devoid of lipid or lipoprotein-like structures (fig 1a, inset). Intestinal tissue from patients with Anderson's disease also contained marked accumulation of lipids (fig 1b). Some of the lipid however, appeared to be lipoprotein-like structures and were observed within the Golgi apparatus (fig 1b, inset). The size of these lipoprotein-like structures varied from 18 nm to 1 μm and they resembled lipoproteins observed in the Golgi apparatus of enterocytes from normal individuals having a dense rim around the edge of the particle. In both groups of patients, the lipid droplets exhibited the typical electron dense aspect of lipid material, due to the fixation by osmium tetroxide. These results are consistent with current concepts that abetalipoproteinemia is primarily a problem of lipoprotein assembly whereas Anderson's disease would appear to be a problem related to the secretion of already formed lipoprotein particles [26]. It should be noted that adequate vizualisation of the lipid and lipoprotein-structures in these fat laden tissues depends upon dehydration at 4°C. Dehydration at 20°C results in increased lipid extraction, leaving holes in the tissue (results not shown).

Immuno electron microscopy

Ultracryosectioning

Ultrathin cryosectioning of intestinal tissues from normal fasted control subjects gave good preservation of the subcellular structures as has been previously noted [22]. After immunogold labeling with anti apoB antiserum, chylomicron-like structures decorated by gold particles could be observed in the intercellular spaces (fig 2a). No gold particles, however, were detected in the endoplasmic reticulum or in the Golgi apparatus.

Ultrathin cryosectioning of intestinal tissues from patients with ABL or AD also gave adequate preservation of the overall structural morphology of the enterocytes, however, the content of the lipid rich structures (both the lipid droplets and the lipoprotein-like particles) that accumulated in the cells of the patients frequently disappeared, leaving a collapsed appearance. Immunogold labeling with anti-apoB antiserum gave numerous gold particles scattered on the exceptionally remaining lipid structures. The immunolabeling of both abetalipoproteinemia and Anderson's disease enterocytes appeared dislocated with many of the gold particles no longer overlaying the lipid structures (fig 2b).

Freeze substitution with Lowicryl K4M

Freeze substitution with Lowicryl K4M better preserved the lipid and lipoprotein-like structures, particularly in the intestinal tissue from the patients with Anderson's disease. Chylomicron-like structures were visible in large vesicles and they resembled the images of normal tissues [15] that had been prepared for routine electron microscopy by fixation with glutaraldehyde and post-fixation with osmium tetroxide followed by post-embedding in epoxy resin. However, the protein antigenicity apparently was lost during the Lowicryl K4M freeze substitution procedure since no specific immunogold labeling was observed with anti-apo B antiserum (results not shown). These results are in contrast with those obtained by Dürer *et al* [21] who observed gold labeling of structures in the rough

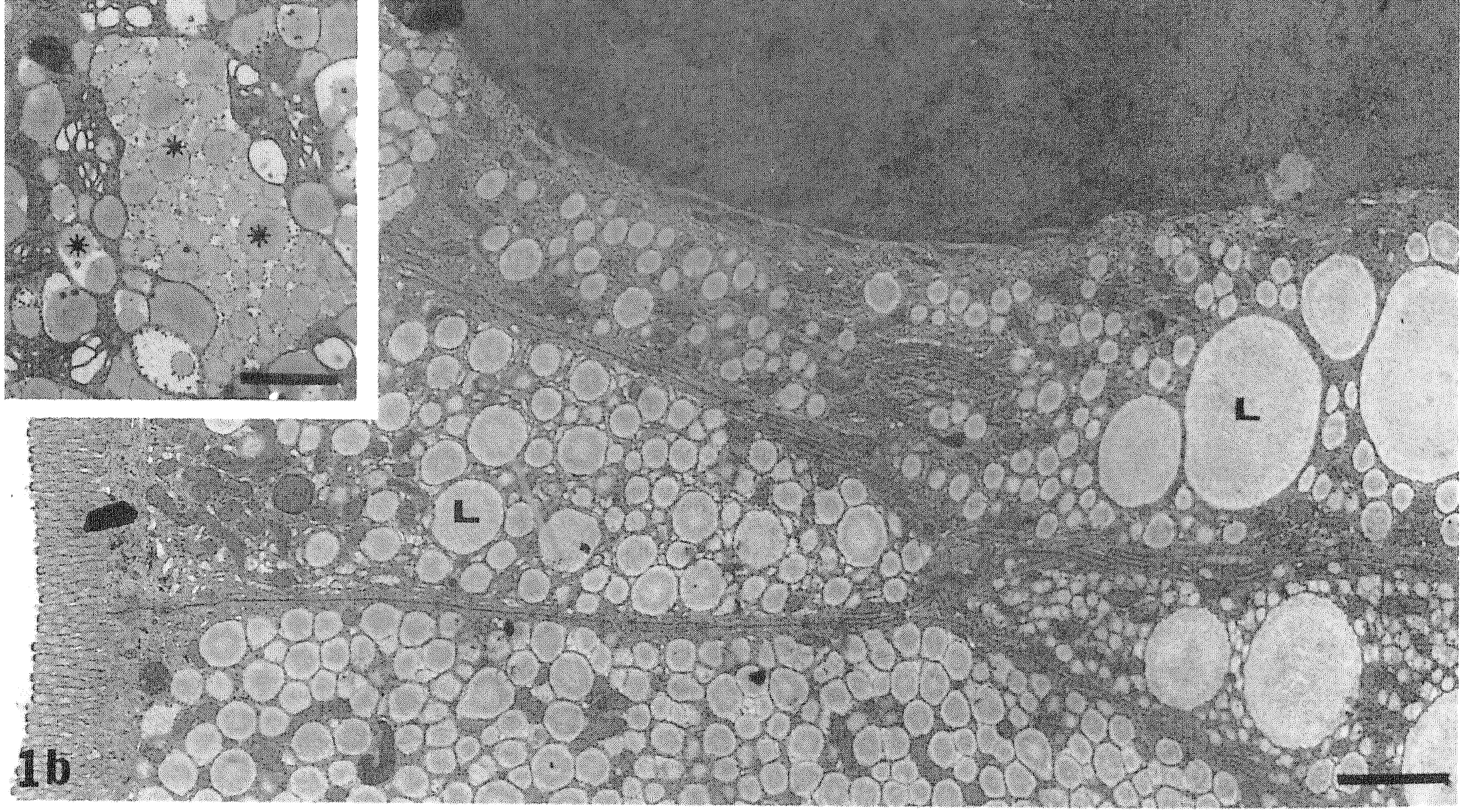
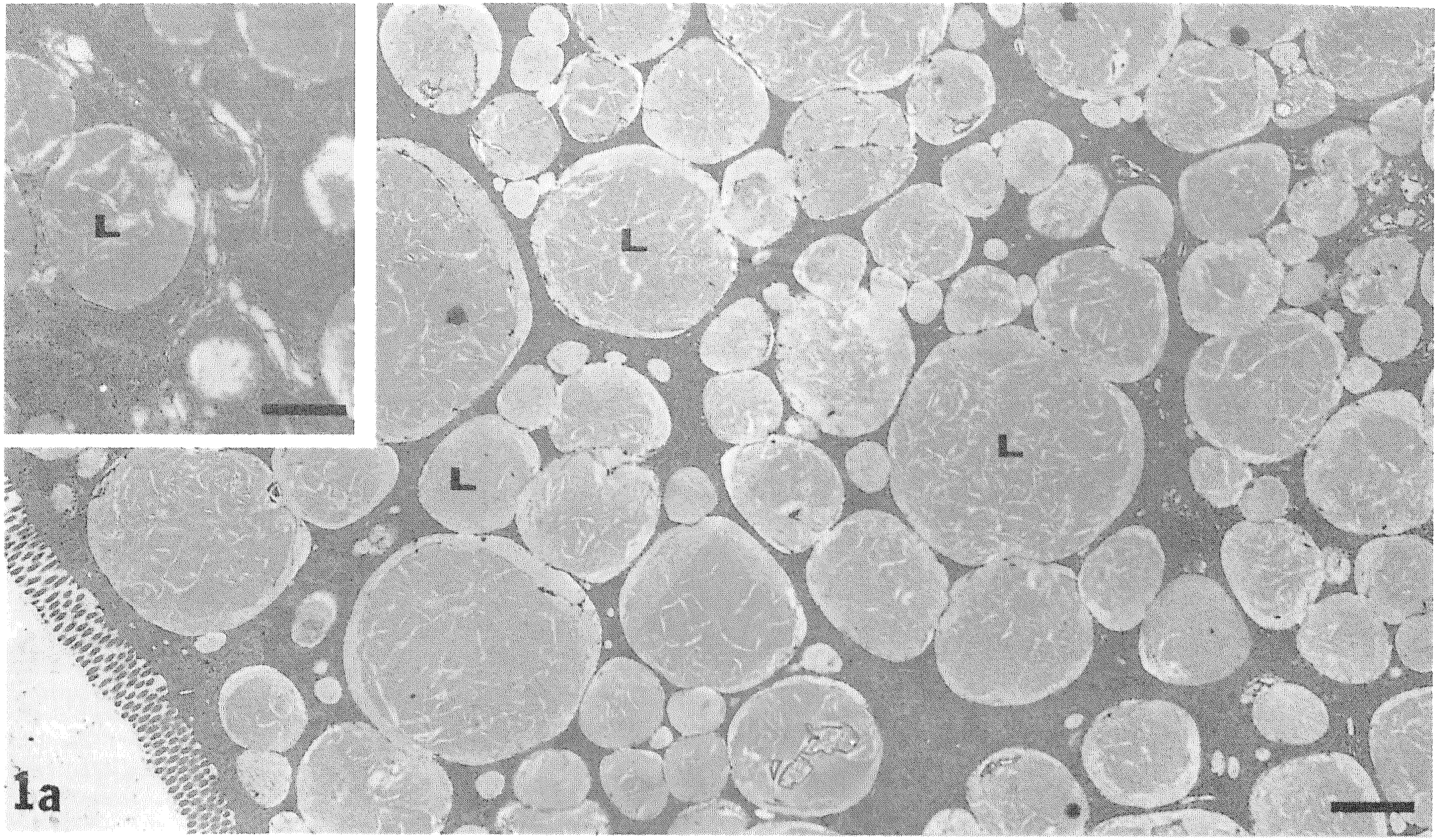


Fig 1. Electron micrographs of ultrathin sections of glutaraldehyde and osmium tetroxide-fixed intestinal tissues from a patient with abetalipoproteinemia (**a**) and a patient with Anderson's disease (**b**) showing the marked lipid engorgement of the enterocytes that occurs in these diseases (L). In enterocytes from the patient with abetalipoproteinemia, the Golgi apparatus is flat and devoid of lipoprotein-like structures (**a**, inset). In enterocytes from patients with Anderson's disease, the Golgi apparatus is engorged with lipoprotein-like structures (**b**, inset, stars). (**a**, × 5 000, inset × 10 000; **b**, × 7 500, inset × 5 400). Bars: **a**, 2 μm/inset, 1 μm; **b**, 2 μm/inset, 2 μm.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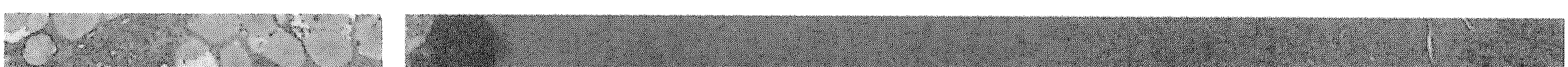

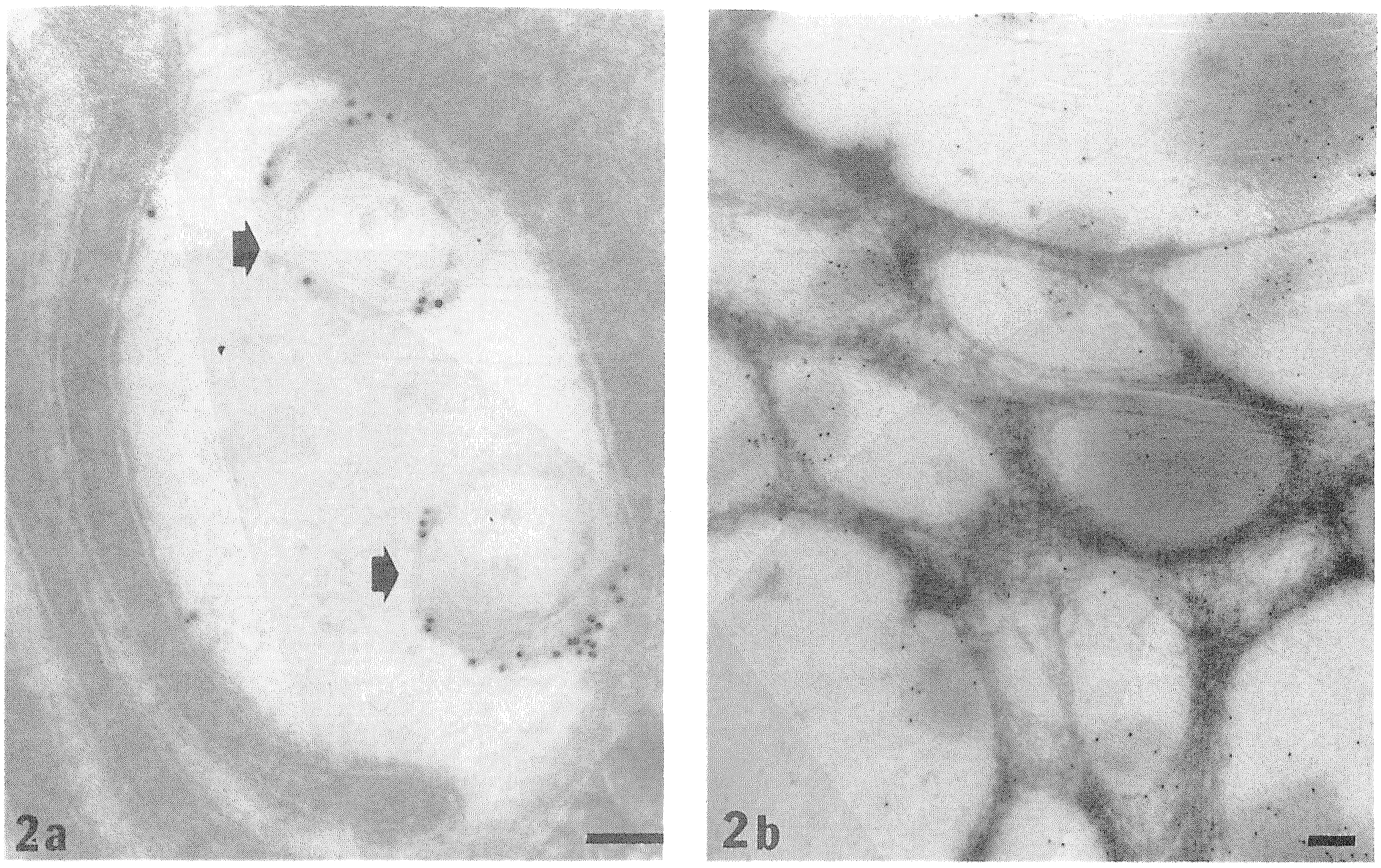

Fig 2. Electron micrographs of ultrathin cryosections of intestinal tissue. In normal enterocytes (**a**), there is good preservation of the ultrastructure with occasional immunogold labeling of apo B on chylomicron-like structures in the intercellular space (arrow). In enterocytes from a patient with Anderson's disease (**b**), there is poor preservation of lipid structures and dislocation of the immunogold labeling of apo B (**a** × 92 000; **b** × 34 000). Bars: **a**, 0.1 μm; **b**, 0.2 μm.

endoplasmic reticulum and in the Golgi apparatus of fetal rat hepatocytes after embedding in Lowicryl K4M. Despite some differences in the fixation procedures used in that and in the present study, we believe that the discrepancy observed is due rather to the differences in the nature of the specimens (fetal *versus* adult and rat *versus* human).

Freeze substitution with Lowicryl HM20

Lowicryl HM20 is known to provide improved embedding qualities compared to other resins as exemplified by less rupturing and smoother sectioning of the resin, and by an increased contrast [45]. Freeze substitution (–90°C) followed by embedding at ultra-low temperature (–50°C) in Lowicryl HM20 also has the advantage of minimizing lipid extraction. Vangenderen et al [45] reported that lipid-rich structures, such as lamellar bodies in lung alveolar type II cells, were very well preserved using this technique. We obtained comparable results using the biopsies from the patients with abetalipoproteinemia and Anderson's disease. The lipids and the lipoprotein structures were thus better vizualised. Intestinal tissue from patients with abetalipoproteinemia contained large lipid droplets located throughout the enterocyte (fig 3a). The Golgi apparatus, however, appeared flat and devoid of lipoprotein-like structures (fig 3b). Both the large and small lipid structures in enterocytes in the intestinal biopsies from patients with Anderson's disease were well preserved (data not shown). Further, lipoprotein-like structures could also be detected in the Golgi apparatus. The ultrastructure of the enterocytes in intestinal biopsies from the normal control subjects was similarly well preserved, and lipoproteins could be observed in the intercellular spaces (data not shown).

Immunogold labeling

When immunogold labeling was performed, gold particles were found to decorate the lipid and/or lipoprotein-like structures in the enterocytes of patients with Anderson's disease and abetalipoproteinemia (fig 4). Apolipoproteins AI (fig 4a) and B (fig 4b) were readily identified. Lipid-structures with numerous gold particles and to a lesser extent the Golgi apparatus-derived vesicles were distinctly labeled. The rough endoplasmic reticulum, which was dilated in these patients, was slightly labeled. On the brush border, as well as on some goblet cells, some labeling was present but to a much lesser extent than that observed in the rough endoplasmic reticulum and the Golgi apparatus. No gold particles were detected in the other organelles, such as the nucleus and the mitochondria (fig 4b). The non-specific absorption of the various reagents to the tissue sections was evaluated by incubation with either colloidal gold particles or protein A-gold particles alone, or with non-immune serum followed by protein A-gold. No immunogold labeling was observed under these conditions (data not shown).

The results of this study clearly demonstrate that the technique of freeze substitution followed by embedding in Lowicryl HM20 preserves both the antigenicity of apolipoprotein B and apolipoprotein AI as well as the ultrastructure of the fat-laden enterocytes. One of the main advantages of freeze substitution, in combination with ultra-low temperature embedding in Lowicryl HM20, is that lipid extraction appears to be minimized. The fixation of the samples with aldehydes and their freezing in liquid nitrogen after cryoprotection limits the formation of ice crystals and leads to good ultrastructural preservation. Additionally, lipid-rich structures such as lipid vacuoles, in patients with abetalipoproteinemia or the chylomicron-like structures in patients with Anderson's disease, are very well preserved in contrast to the results obtained with ultra-thin cryosectioning of the same tissue.

The results obtained with ultra-low temperature embedding in Lowicryl HM20 showed marked improvement in comparison to the results obtained with pre-embedding immunoperoxidase labeling. The latter technique has not permitted, in lipid-rich tissues, the identification of the particular subcellular structures to which apo B is bound. Dissolution of lipid structures probably results from the dehydration and the subsequent heating necessary for the polymerization and the embedding in the epoxy resin. The lipid-rich content of the lipid vacuoles or the lipoprotein-like structures disappears almost completely following these procedures. Finally, the peroxidase immunostaining results in a thin layer of precipitated reaction products which leads to poor visualization of the antigenic determinants (Bouma *et al*, unpublished results). In contrast, the use of colloidal gold particles as immuno-cytochemical markers for electron microscopy is useful for the intracellular localization of proteins [7, 8, 31]. Indeed, when ultrastructural preservation of the cellular organelles is achieved, the structures that are labeled can be determined with precision.

Although Lowicryl K4M has been recommended for embedding tissues destined to be immunogold labeled and has been used to localize apoB in normal human intestine, fetal rat and chicken hepatocytes [19, 21, 27], we prefer the hydrophilic resin Lowicryl HM20 for the embedding of lipid-filled enterocytes. Lowicryl HM20 maintains the antigenicity of the protein for immunogold labeling and provides better embedding qualities. The lower temperature of the substitution and the embedding procedures appears to be important for the preservation of lipid structures. Stein and Stein [42] have noted that less lipid extraction occurs when alcohol dehydratation is performed at low temperature.

In conclusion, the protocol described here allows the immunogold labeling of proteins (apolipoproteins or other factors) present in fat laden enterocytes. It offers us a powerful tool to investigate more readily lipid-laden tissues to further understand the biosynthesis, intracellular processing and secretion of lipoproteins in man.

Acknowledgments

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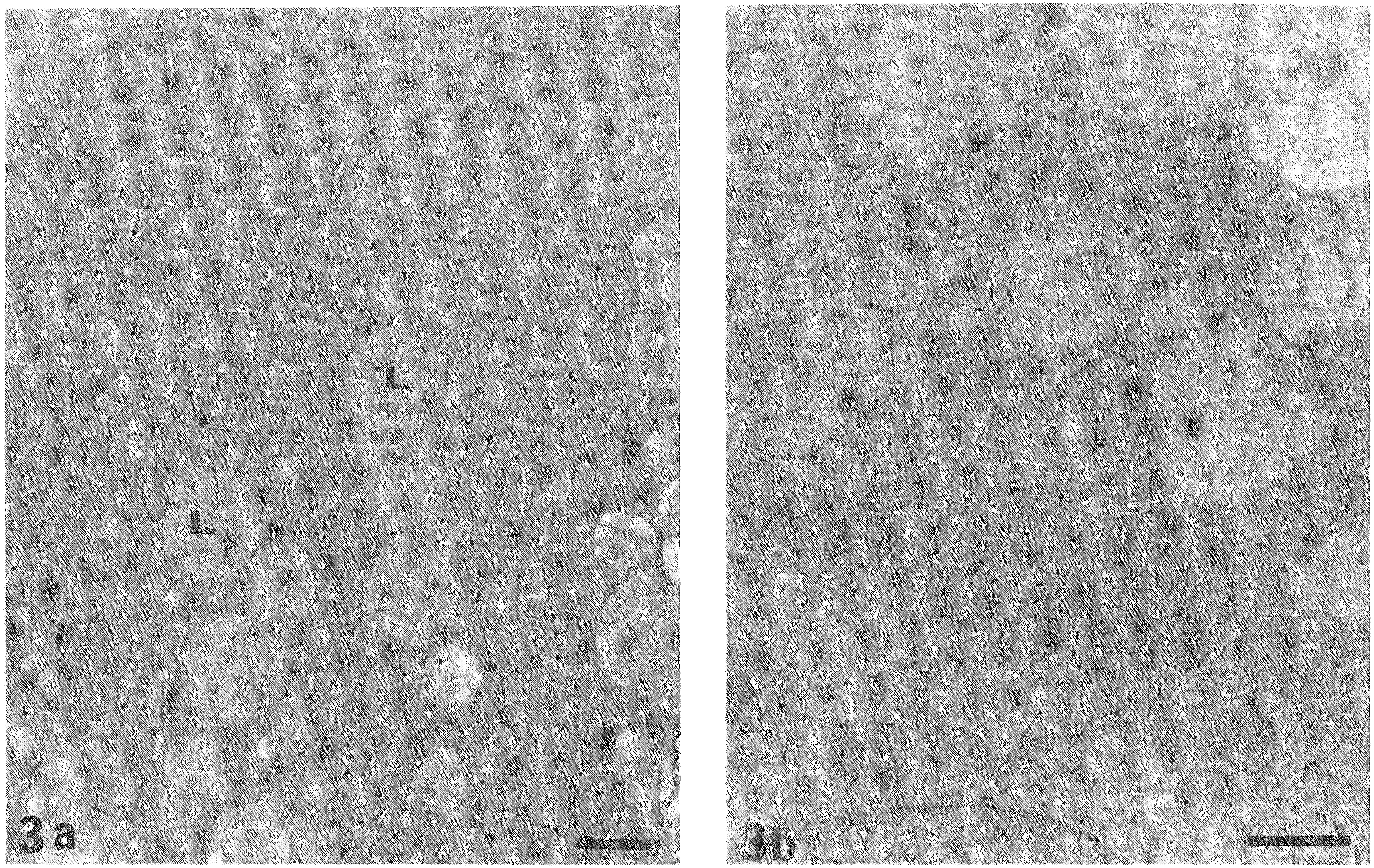

Fig 3. Electron micrographs of ultrathin sections of Lowicryl HM20 embedded human intestinal biopsy tissue from a patient with abetalipoproteinemia. The overall morphology of the enterocytes and the lipid structures are preserved (L). The Golgi apparatus is flat and devoid of lipid particles (**a**, × 9 450; **b**, × 26 000). Bars: **a**, 1 μm; **b**, 0.5 μm.

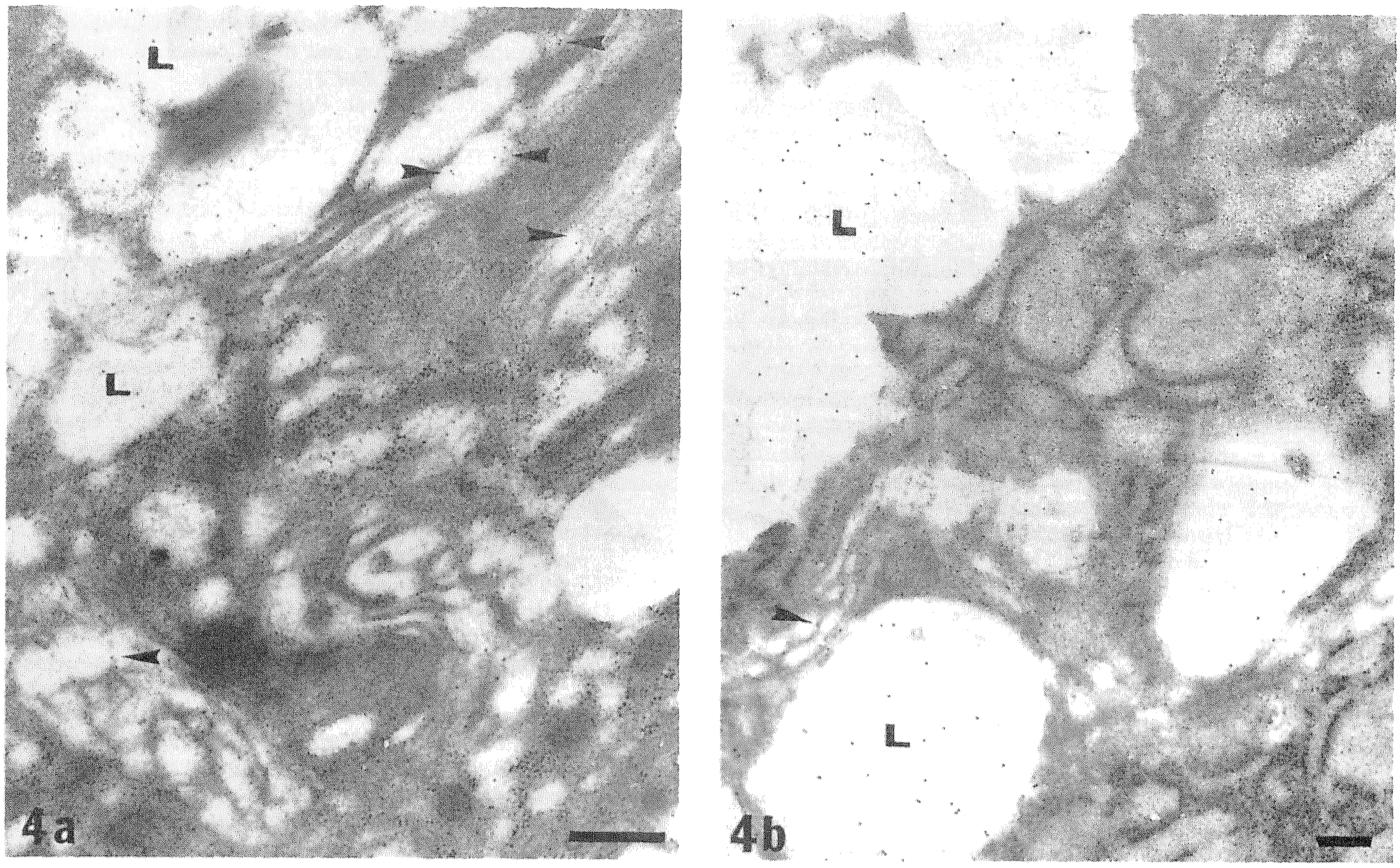

Fig 4. Immunogold labeling of apo AI (**a**) and apo B (**b**) on ultrathin sections of intestinal biopsies from a patient with Anderson's disease (**a**) and from a patient with abetalipoproteinemia (**b**) embedded in Lowicryl HM20. Gold particles are present on lipid structures (L) and on the Golgi apparatus to a lesser extent (arrows) **a**, × 24 320; **b**, × 35 000). Bars: **a**, 0.5 μm; **b**, 0.2 μm.

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