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# Ultrastructural lipid and glycoconjugate cytochemistry of membranous lipodystrophy (Nasu-Hakola disease)

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**Summary.** In order to assess the lipid and glycoconjugate characteristics of membranous lipodystrophy, a 29-year-old male with this disease was studied using an ultrastructural cytochemical approach. The specific membranocystic lesions of the disease are composed of cystic spaces and the lining membranes. The membranes were observed to have a two-layered character: microtubular structures in the layer adjacent to the spaces and a central amorphous zone. Lipid staining and the lipase digestion test revealed triglycerides localized not only in the cystic spaces but also in the microtubular structures. Lectin histochemical examintion of carbohydrate components demonstrated that *Maclura pomifera* agglutinin bound strongly to the membranes, while *Griffonia simplicifolia* I, *G. simplicifolia* II, *Concanavalia ensiformis* and *Triticum vulgaris* agglutinin reacted weakly. Our results indicate the presence of triglycerides and carbohydrates with mainly α-D-galactose residues in the distinctive membranocystic lesions, in particular in the microtubular structures.

**Key words:** Membranous lipodystrophy – Nasu-Hakola disease – Lipid and glyconconjugate – Cytochemistry – Electron microscopy

## Introduction

Membranous lipodystrophy is a relatively newly established clinical entity first analysed clinicopathologically by Nasu et al. in 1973. Detailed neuropsychiatric and genetic studies on familial cases were first described by Hakola (1972), who named this disease "lipomembranous polycystic osteodysplasia". The two cases reported independently from Finland and Japan later proved to be of the same type, which is now also known as "Nasu-Hakola disease" (Hanawa et al. 1981, Matsushita et al. 1981; Amano et al. 1987; Kitajima et al. 1988, 1989). Currently, this disease is recognized as being hereditary and is chararacterized by polycystic changes in many bones with a variety of central nervous system changes due to disturbance of lipid metabolism by an unknown mechanism (Harada 1975; Wood 1978; Ohtani et al. 1979; Bird et al. 1983; Hakola and Partanen 1983; Machinami 1983; Sageshima et al. 1987; Kitajima et al. 1989). Although there is much information on its morphological basis, the lipid and glycoconjugate cytochemical characteristics have not been clarified (Suganuma et al. 1987; Kitajima et al. 1988).

The purpose of this study was to clarify the lipid and glycoconjugate characteristics of a case of membranous lipodystrophy, with particular emphasis on their ultrastructural localization within the membranocystic lesions of the bone marrow.

## Materials and methods

A 29-year-old male was admitted first to the hospital in 1987 for bilateral heel pain. Radiological examination revealed bilateral po-

**Table 1.** Carbohydrate specificities and binding reactions of lectins used

| Lectin group | Lectin | Carbohydrate specificity | Binding |
|---|---|---|---|
| D-Gal | GS-I | α-D-Gal > α-D-GalNAc | + |
| | PNA | β-D-Gal(1–3)-D-GalNAc | – |
| D-GlcNAc | WGA | $[\beta(1\text{–}4)\text{-D-GlcNAc}]_2$ | + |
| | GS-II | D-GlcNAc | + |
| (Sialic acid) | LFA | NANA | – |
| D-GalNAc | SBA | α-D-GalNAc > β-D-GalNAc | – |
| | DBA | α-D-GalNAc | – |
| | MPA | α-D-Gal(1–3)-D-GalNA<br>α-D-GalNAc > α-D-Gal | ++ |
| D-Man | Con A | α-D-Man > α-D-Glc | + |
| L-Fuc | UEA-I | α-L-Fuc | – |

Gal, Galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NANA, *N*-acetylneuraminic acid (sialic acid); Man, mannose; Glc, glucose; Fuc, fucose
Binding reaction: ++, strong; +, weak; –, negative

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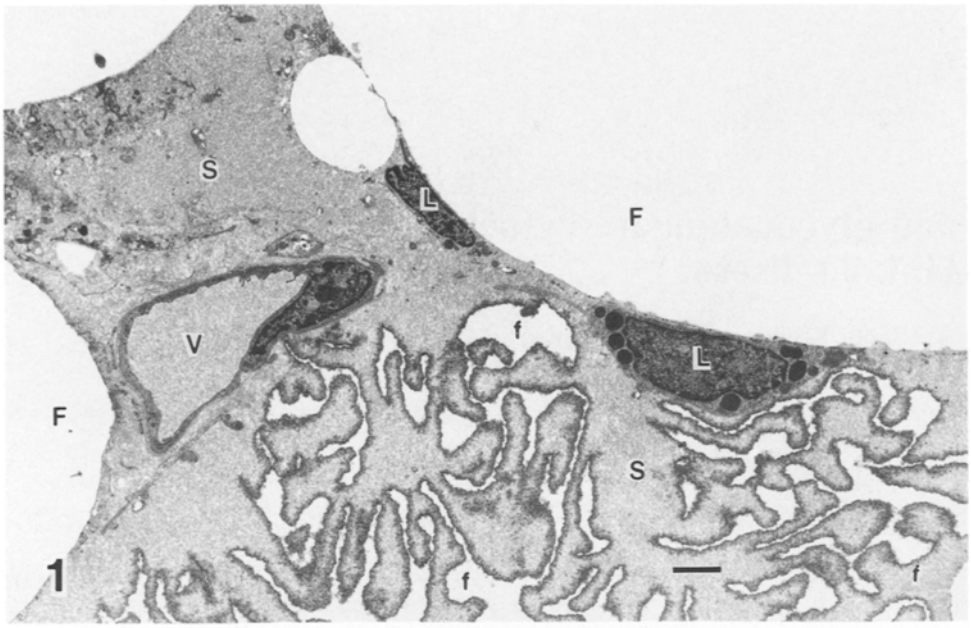


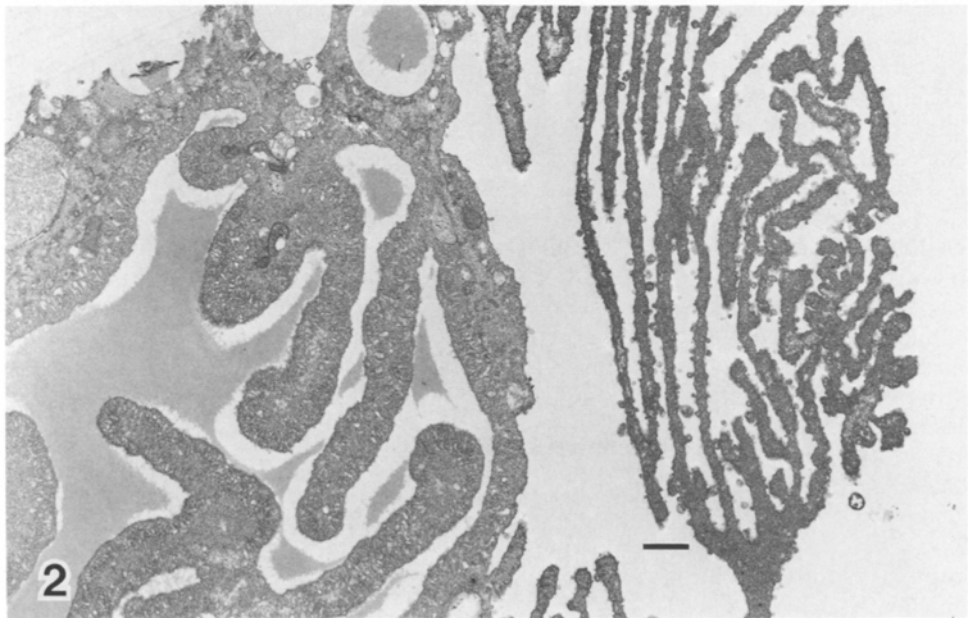




**Fig. 1.** Low magnification electron micrograph of a membranocystic lesion. The large cystic spaces (*F*) and the undulating narrow spaces (*f*) are surrounded by membrane-like septa (*S*) in which lipoblasts (*L*) and blood vessel (*V*) are observed. *Bar* = 2 μm. ×4000

**Fig. 2.** Electron micrograph of another area of a membrano-cystic lesion. Note the variously shaped membrane structures and the marked development of microtubules. *Bar* = 1 μm. ×8000

lyostotic cystic lesions in the calcaneus, talus and femur. He underwent curettage and an iliac bone graft for his painful talar bone. Histological examination of the material obtained from the lesion showed membranocystic changes characteristic of membranous lipodystrophy. About 1 year later, he was readmitted to the hospital for left knee pain persisting for 2 months. Curettage with hydroxyapatite grafting was performed for a lesion in his left distal femur. The specimens examined in the present study were obtained from the marrow cavity of this bone at the time of operation. Specimens revealed the same changes as those from the talus at the first operation with the appearance of membranocystic change in adipose tissues in the bone marrow.

The specimens were cut into small blocks were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4° C for 2 h and then washed with the same buffer containing 0.25 M sucrose at 4° C for 16 h. Tannic acid (1%) was added to this fixative to enhance the contrast of the specimens when necessary. They were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer at 4° C for 1 h, followed by dehydration in a graded series of ethanols and embedding in Quetol 812 (Nisshin EM, Tokyo, Japan). Thick sections cut at 1 μm were stained with toluidine blue to allow selection of representative areas from which to prepare ultra-thin sections for electron miroscopic observation. The sections were cut with an LKB-8800 ultratome and contrasted with uranyl acetate and lead citrate. They were examined under a JEOL 1200-EX electron microscope.

For identification of lipids, the *p*-phenylenediamine (PPD, Nacalai Tesque, Kyoto, Japan) stain and the digestion test with pancreatic lipase (Sigma, St. Louis, Mo, USA) were used in this study. These were performed according to techniques described by Boshier et al. (1984) and Adams et al. (1966), respectively. For PPD staining, specimens were fixed in 2.5% glutaraldehyde in 0.07 M cacodylate buffer (pH 7.4) for 6 h at 4° C, washed overnight in the same buffer at 4° C, followed by post-fixation in 1% osmium tetroxide for 3 h at 4° C, staining in 1% PPD in 70% ethanol, dehydration, and then embedding in Quetol 812 using standard procedures. For lipase digestion, small blocks of tissues were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4° C for 1 h. Sections cut at about 50 μm thickness with a model G Vibratome (Oxford Instruments, San Mateo, Cal., USA), were incubated for 30 min at room temperature in the following medium: 50 mg pancreatic lipase; 10 ml of 2%

calcium chloride; 15 ml of 0.2 M Tris buffer at pH 7.0; and 25 ml distilled water. The sections were then washed with distilled water, followed by treatment with 1% lead nitrate for 20 min. They washed again with distilled water, post-fixed for 18 h in 1% osmium tetroxide, dehydrated in a graded series of ethanols, and embedded in Quetol 812. The sections were processed for electron microscopy as detailed above without further staining.

For investigation of glycoconjugates, lectin staining was performed on specimens according to the method of Roth (1983). Briefly, specimens were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4° C for 1 h, embedded in Lowicryl K4M (Ladd Research Industries, Burlington, Vermont, USA) and polymerized under UV irradiation at room temperature for 12 h. Ultra-thin sections mounted on nickel grids were floated on a drop of 1% bovine serum albumin (BSA)-added phosphate buffered saline (PBS) for 10 min, and transferred to a drop of biotinylated lectin-containing solution (25 μg/ml of 0.1% BSA-PBS) for 1 h. They were washed in PBS and then incubated in streptavidin colloidal gold complex (15 nm in dimeter, E-Y Laboratories, San Mateo, CA, USA) diluted in PBS to adequate concentrations for 1 h, followed by washing with PBS and then distilled water. Control sections were treated with specific binding sugars for each lectin, prior to their treatments. They were then contrasted only with uranyl acetate and examined under an electron microscope. The lectins used in the present lectin histochemistry and their abbreviations and carbohydrate specificities are summarized in Table 1. All were purchased from E-Y Laboratories.

## Results

On low power electron microscopy many cystic spaces of various sizes and shapes are observed. Some of the cysts are filled with amorphous materials and others are empty, probably due to loss during dehydration and embedding procedures. In the membrane-like septa, lipoblasts are observed surrounding each large cyst, no such cell components lining the slit-like narrow spaces (Fig. 1). Membrane thickness varies greatly from area to area and the sizes and shapes of cystic spaces are also very irregular (Fig. 2). At high magnification, the membranous structures are seen to consist of two layers, at least in the thicker areas. The outer layers facing the cystic spaces demonstrate complicated microtubular or microvesicular structures arranged perpendicularly to the surfaces and are evident as electron-dense areas at low magnification (Fig. 3). The inner layer appears to be amorphous without organelle-like structures, except for rarely observed small amounts of collagen fibrils and debris from degenerated fibroblastic or histiocytic cells (Fig. 4). Blood vessels are occasionally evident in the outer layer, most of them appearing normal (Fig. 1). There is continuous transition and no clear border between the two layers.

In sections treated with lipase, the amorphous material within the cysts is digested and the lead precipitates formed as the final reaction products after hydrolysis of triglycerides are found mainly within the cystic spaces (Fig. 5). Triglyceride in the cystic spaces is confirmed by the results of PPD staining. The density of the amorphous material included is strongly enhanced and the variety of membranocystic spaces is observed more clearly (Fig. 6). It is of special interest that materials stained with PPD can be demonstrated not only in the cystic spaces but also in the outer layers of the membranous structures. Higher magnification appraisal clearly reveals PPD-positive material as scatterings or gatherings of many spots within the microtubular or microvesicular elements (Fig. 7). Thus the existence of triglycerides in both the microtubules of the membranes and the cystic spaces is indicated.

In lectin histochemistry the membranous structures are strongly labelled with *Maclura pomifera* agglutinin (MPA)-colloidal gold conjugate, which is considered to recognize α-D-galactose, and weakly labelled with *Griffonia simplicifolia* I (GS-I), *G. simplicifolia* II (GS-II)

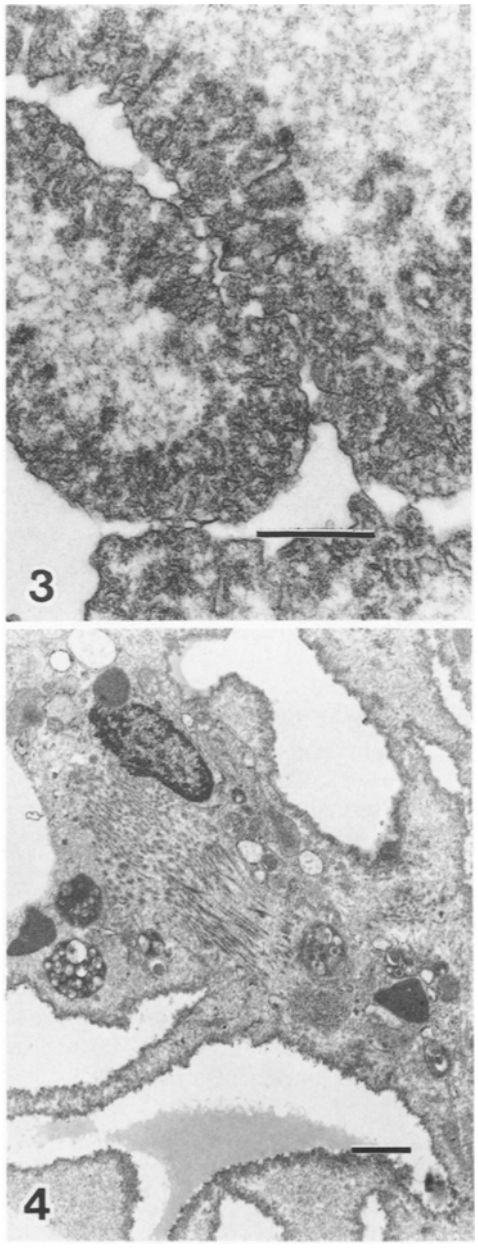



**Fig. 3.** High magnification electron micrograph of a membranocystic lesion. The microtubular structures arranged perpendicularly to the surfaces lining the cysts and the amorphous areas in the inner layer are clearly visible. *Bar* = 0.5 μm. × 40000

**Fig. 4.** Electron micrograph of a membrane. Note the degenerated cell, dispersed cell debris and scattered collagen fibrils. *Bar* = 1 μm. × 10000

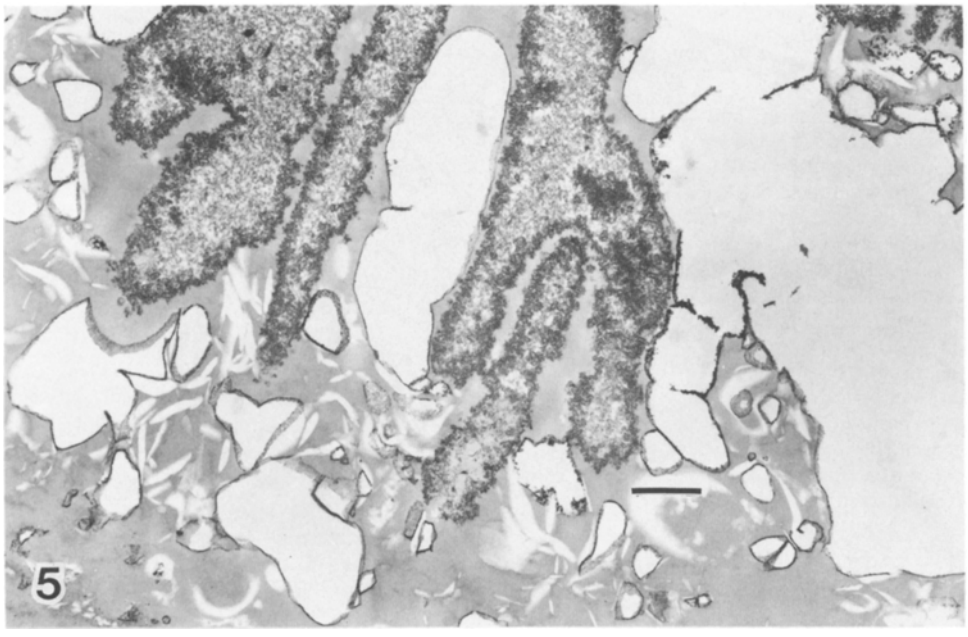



**Fig. 5.** Electron micrograph of a lipase-digested specimen. Reaction products are found mainly in the cystic spaces. *Bar* = 1 μm. ×12000

*Concanavalia ensiformis* (Con A) and *Triticum vulgaris* agglutinin (WGA), which recognize α-D-galactose, *N*-acetyl-glucosamine, α-D-mannose and *N*-acetyl-glucosamine, respectively (Fig. 8). The particles conjugated with these lectins are observed mainly in the outer layers of the membranous structures. The other lectins used in this study, however, did not bind to any site. Mature lipid droplets, the materials in the cystic spaces and other cytoplasmic structures were not labelled with any of the lectin-colloidal gold conjugates. In the control sections pretreated with specific binding sugars for each lectin and the sections made of specimens from normal adipose tissue, no postitive labelling was observed. The results for lectin cytochemistry are summarized in Table 1.

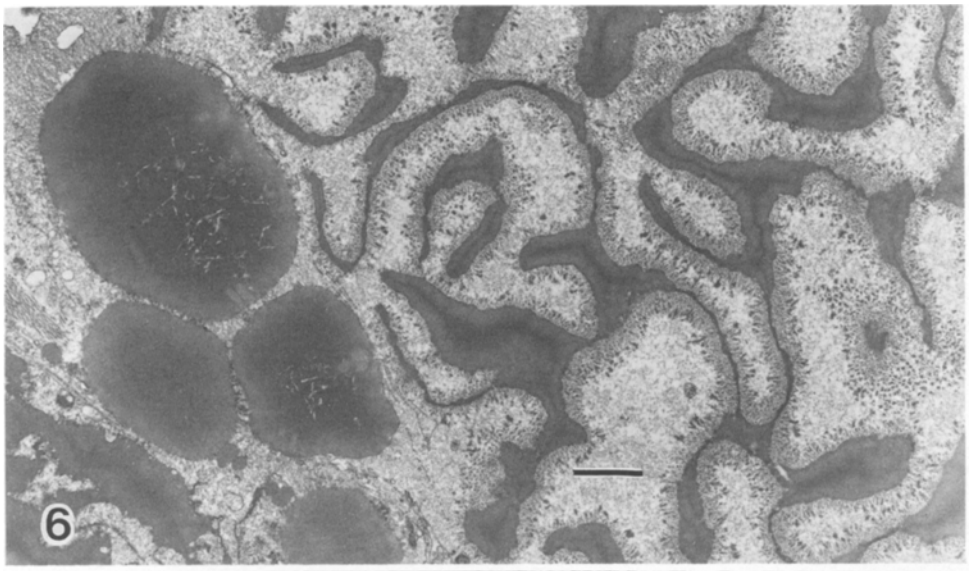
## Discussion

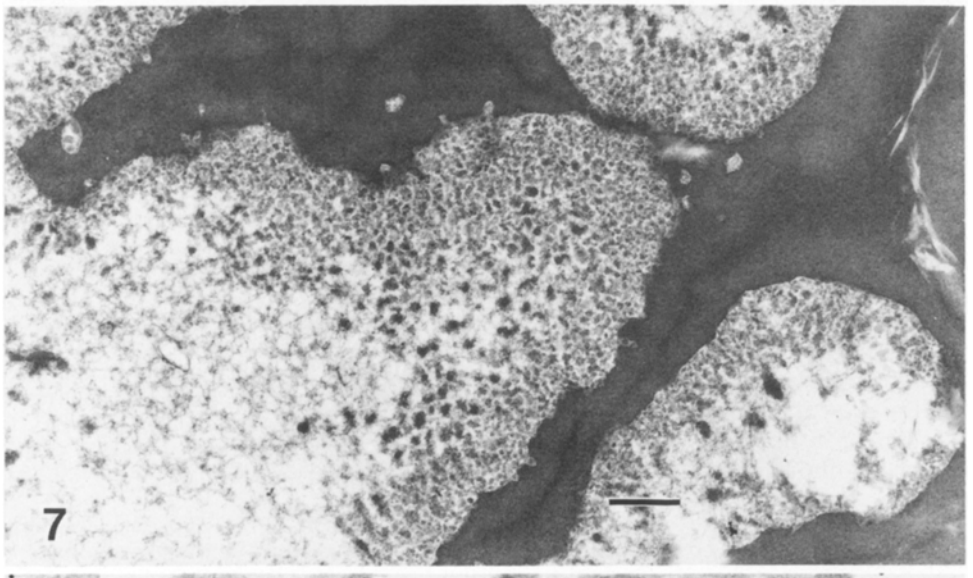
The ultrastructural characteristics revealed in the present study were similar to those previously reported in the literature in membranous lipodystrophy (Yagishita et al. 1976; Akai et al. 1977; Wood 1978; Sageshima et al. 1987; Suganuma et al. 1987). In summary, the membranocystic lesions were composed of two distinct components: the cystic spaces and the surrounding membranes, which in turn demonstrated two layers, one at the outer surfaces, characterized by microtubular structures, and the second in the centre composing amorphous areas. Many authors have mentioned microtubular or vesicular elements whithin the membrane as one of the characteristic findings of this disease (Wood 1978; Ikeda et al. 1984; Machinami 1984; Suganuma et al. 1987) with various terms being proposed: minute tubular structures (Nasu et al. 1973), peripheral microvilli (Wood 1978), or complicated tubulosaccular structures (Sageshima et al. 1987). Presumably the tubular, saccular or vesicular appearance depends on the plane of section.

Although many clinicopathological studies have clarified the morphological features, the aetiology of the disease remains controversial (Kitajima et al. 1989). Since debate continues regarding the roles played by lipid and glycoconjugate metabolism, we performed the present lipid and lectin cytochemical study.

In the lipid-stained specimens, the most striking observation was the enhanced density PPD-positive material in the membranocystic lesions. This, indicating the presence of triglycerides in the cystic spaces, is generally in agreement with the findings of other authors (Nasu et al. 1973; Tokunaga et al. 1981; Kitajima et al. 1989) and was also confirmed by the pancreatic lipase digestion test in the present study. The new cytochemical demonstration of PPD-positive triglycerides in the microtubular structures strongly suggests the possibility that the surface layer containing these elements bears some direct relationship to lipid deposit or storage in the cystic spaces. It has been reported that the identity of lipid droplets in electron micrographs depends on their stabilization during fixation, the specificity of the stain used for their visualization and their retention during dehydration and embedding procedures. Since the PPD stain method described by Boshier et al. (1984) is considered to be optimal for lipid staining at the ultrastructural level, it was applied to the cytochemical demonstration of triglycerides in the present study. The results are therefore unlikely to be artefactual and, furthermore, are also consistent with the findings of the biochemical studies reported by Nasu et al. (1973) and Kitajima et al. (1989). Further studies are necessary to determine the exact relationship between triglycerides in the microtubular structures and those within the membranocystic spaces.

Investigation of glycoconjugate cytochemistry revealed MPA, which specifically recognizes α-D-galactose residues, binds strongly to the membranes in the membranocystic lesions. GS-I, GS-II, Con A and WGA dem-



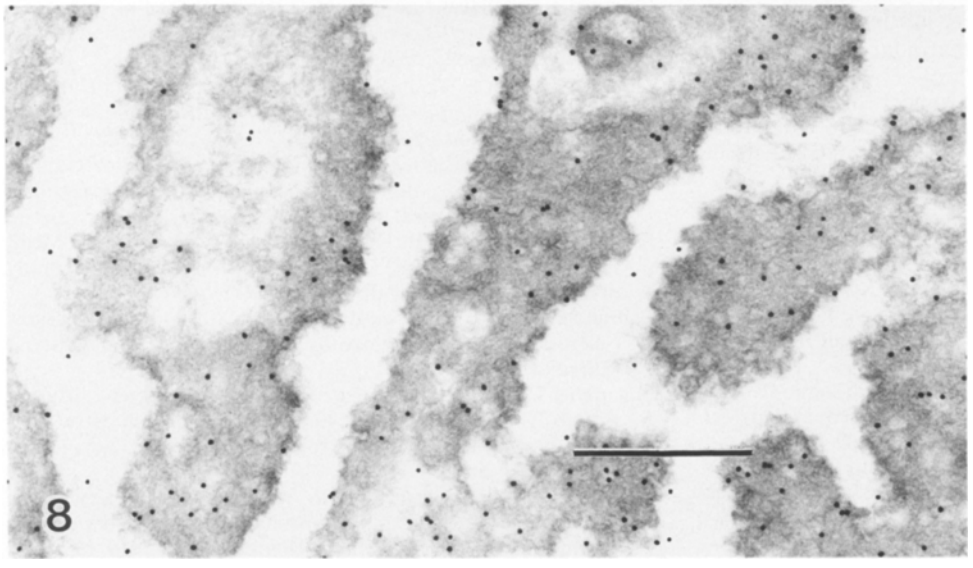




**Fig. 6.** Low magnification electron micrograph of a *p*-phenylenediamine (PPD)-stained specimen. PPD-positive material is demonstrated both in the cystic spaces and the microtubule-containing layer of the membrane. *Bar* = 2 μm. × 6000

**Fig. 7.** High magnification electron micrograph of a PPD-stained specimen. Note PPD-positive material in the microtubular structures. *Bar* = 0.5 μm. × 24000

**Fig. 8.** Electron micrograph of a lectin-stained preparation. The membranous structures are labelled with *Maclura pomifera* agglutinin-colloidal gold conjugate. *Bar* = 0.5 μm. × 60000

onstrated weak binding in the same area and the other lectins used in the present study were negative. These results were consistent with the findings reported by Kitajima et al. (1988), indicating that the membranocystic lesions, and especially the microtubular structures, contain glycolipids or glycoproteins with α-D-galactose residues at the terminal ends of the carbohydrate chains (Bausch and Poretz 1977). As far as we know, there has been only one previous study (Kitajima et al. 1988), which succeeded in demonstrating the precise localization of α-D-galactose residues in the membranocystic lesions at the electron microscopic level. The findings for lectin cytochemistry in membranous lipodystrophy documented in the present report, taken together with the evidence of Kitajima et al. (1988, 1989), indicate distinctive characteristics for this disease, whose significance requires elucidation.

Several hypotheses have been advanced for the pathogenesis of membranous lipodystrophy. Jarvi (1970) and Sourander (1970) considered that secondary oedema and nutritional disturbances can lead to fat necrosis in bone marrow and myelin degeneration of the white matter of the brain. Recently, Machinami (1984) reported three cases with similar membranocystic lesions in subcutaneous fatty tissue together with ischaemic necrosis of the legs. They concluded that the changes were caused by ischaemia due to arteriosclerotic obstruction. Nasu et al. (1973) and many other Japanese authors suggest the possibility of a primary defect in lipid metabolism. Matsushita et al. (1981) and Amano et al. (1987), in independent studies, have both stressed the presence of degeneration in the nervous system of patients with this disease. However, since the patient studied in the present investigation had no neurological clinical signs or symptoms, this does not seem to be a necessary precondition.

In conclusion, while of controversial pathogenesis, membranous lipodystrophy would appear to be a disease entity with unique characteristics. Further research should facilitate a more complete understanding and may present a new approach to treatment.

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