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Ultrastructural demonstration of *Maclura pomifera* agglutinin binding sites in the membranocystic lesions of membranous lipodystrophy (Nasu-Hakola disease)

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Summary. This paper reports three cases of membranous lipodystrophy (Nasu-Hakola disease) in two families and studies the carbohydrate components of membranocystic lesions in all three cases, using twelve kinds of lectins labelled by horseradish peroxidase (HRP). *Maclura pomifera* agglutinin (MPA), which specifically binds α-D-galactose residues, strongly stained typical membranocystic lesions, whereas the other lectins did not. However, *Helix pomatia* agglutinin (HPA), which specifically binds to N-acetyl-D-galactosamine (GalNAc), stained the membranes of degenerated adipose cells. These were thought to appear during the initial or early stage of the membranocystic lesions. This suggests that a change of carbohydrate residues occurs during the formation of the membranocystic lesions. We also investigated the lectin binding sites at the ultrastructural level using MPA-HRP colloidal gold (CG) conjugate. In the well developed membrane, CG particles were arranged regularly along the minute tubular structures. On the other hand, there were a few irregularly spaced CG particles on the thinner membranes and also on the membranes of the degenerating adipose cells. No CG particles labelled the cell membranes of normal adipose cells. The presence of α-D-galactose residues in the membranocystic lesions is demonstrated for the first time at the electron microscopic level.

Key words: Membranous lipodystrophy (Nasu-Hakola disease) – Membranocystic lesion – Lectin histochemistry – α-D-galactose – MPA-HPR colloidal gold

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Introduction

Membranous lipodystrophy (Nasu-Hakola disease) is a new clinical entity and characterized by peculiar arabesque profiles, involving various adipose tissues systematically, and accompanied by leukodystrophy of the brain. Familial cases were first reported in Finland by Hakola (1972) and given descriptive the name of "progressive dementia with lipomembranous polycystic osteodysplasia". The first autopsy case was reported in Japan by Nasu et al. (1973) and named "membranous lipodystrophy". Since then this disease has been known as "Nasu-Hakola disease". Approximately 60 cases have been reported in Japan (Inoue et al. 1975; Yagishita et al. 1976; Akai et al. 1977; Tanaka 1980), 10 cases in Finland (Hakola 1972; Laasonen 1975) and 5 cases in USA (Wood 1978; Bird 1983).

Although the pathogenesis of this disease has not been clarified, we thought that histochemical studies of the membranocystic lesions might shed more light on their characterization. Fujiwara (1979) suggested that the membranocystic lesions in this disease might be stable complexes containing carbohydrates, lipids, phospholipids and proteins. Histochemical studies using marker-labelled lectins are reported to provide more precise information of carbohydrate components, but to date, there is only one lectin histochemical study of the membranocystic lesions in a case of membranous lipodystrophy (Suganuma et al. 1987).

The aim of this study is to provide more precise information on the carbohydrate components and detailed structure of the membranocystic lesions in three cases of membranous lipodystrophy, using marker-labelled lectins at the light and electron microscopic levels.

Materials and methods

Case 1 was a 39-year-old male hospitalized because of seizures. Gait disturbance and disorientation appeared when he was 32. Urinary disturbance developed at the age of 36. From the age of 38, convulsive seizures were frequently noted. His height on admission was 158 cm and he weighed 38 kg. No scoliosis or trunk deformity were found. All extremities showed joint contracture. He could not speak or move easily and was completely incontinent. Deep tendon reflexes were markedly hyperactive and there were bilateral toe extensor signs.

Case 2 was a 36-year-old female, the younger sister of case 1, admitted because of dementia. She was apparently healthy till 27 years of age when forgetfullness set in. This gradually progressed, and personality changes including a tendency to laugh at trivial thing were also noted. Gait disturbance, which was progressive occurred around the age of 34. Physical examination on admission revealed moderate obesity with a height of 153 cm and a weight of 58 kg. She did not have joint contractures but was otherwise similar to case 1.

Case 3 was a 34-year-old male, admitted because of urinary incontinence. He was apparently healthy till 30 years of age when forgetfullness was noted, after which he developed personality and emotional changes. Spastic gait was noted at the age of 32 and dementia progressed. He was 158 cm high and weighed 55 kg on admission. All extremities showed joint contracture. He was completely incontinent and deep tendon reflexes were markedly hyperactive with bilateral toe extensor signs.

In all three cases there were no significant results from haemogram, hepatic and renal function tests, nor from biochemical investigations of cholesterols, triglycerides, lipoproteins and lipid peroxides in the serum. Hormonal function tests also revealed no abnormalities in pituitary, thyroid, parathyroid and adrenal glands. Computed tomography (CT) of all three cases revealed severe cortical atrophy with calcification of basal ganglia (Fig. 1a). Magnetic resonance imaging (MRI) of the brain in case 3 showed severe atrophy of the frontal lobe, which spared the occipital lobe and cerebellum. Electroencephalography (EEG) demonstrated a moderate amount of 2 Hz delta activity with occasional sharp and slow wave complexes in all three cases. Roentgenograms showed cystic transparencies in the hands and the right femur of case 1 (Fig. 1b), both the calcaneous bones of case 2, and the right femur of case 3.

Biopsy material from the cystic transparencies in the bones and subcutaneous adipose tissues of all cases were obtained. Uninvolved subcutaneous adipose tissues from age-matched patients of osmidrosis axillae and uninvolved bone marrow adipose tissues from a patient with sea blue histiocytosis were used as histochemical controls. Specimens were fixed in a half strength of Karnovsky's fixative and divided into three portions. One portion was embedded in paraffin for light microscopy and another portion in Epon 812 for electron microscopy and lectin histochemistry. The remaining portion was preserved in a half strength of Karnovsky's fixative for the study of lectin-colloidal gold (CG) conjugate histochemistry.

On light microscopy the paraffin sections of bone marrow and subcutaneous adipose tissues in all cases were stained with haematoxylin and eosin (H&E), periodic acid Schiff (PAS), Luxol fast blue (LFB), alcian blue (pH 2.5), Sudan black B (SBB), oil red O (ORO), Nile blue, toluidine blue, Congo red and Ziehl-Neelsen (Table 1).

For electron microscopy specimens embedded in Epon 812 were cut with a Porter-Blum MT 2B ultramicrotome and then stained for 5 min with a saturated aqueous solution of uranyl acetate and for another 5 min with a lead citrate solution. The specimens were then examined under a JEOL-100B electron microscope.

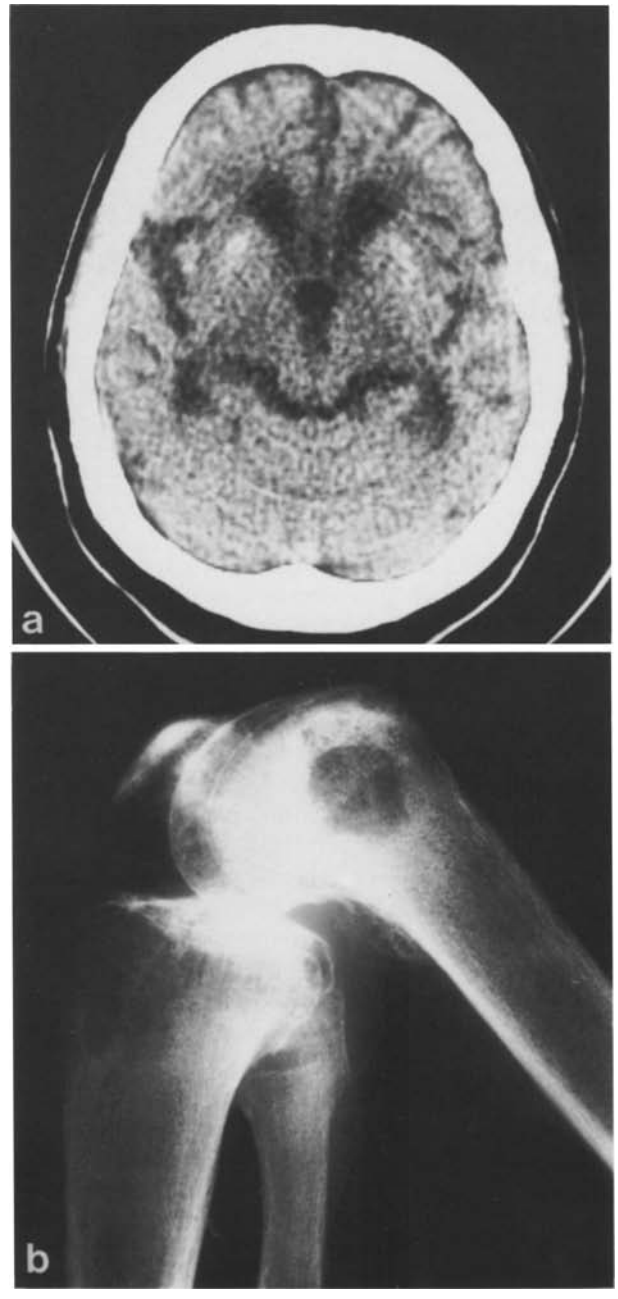

Fig. 1. (**a**) Head CT findings of case 2. Cortical atrophy with calcification in the basal ganglia. (**b**) Bone radiography in the right femur of case 1 revealed cystic transparency

In order to perform lectin staining for light microscopy, Epon embedded thick sections were treated for 5 min with 2.0 g KOH in a mixture of 10 ml pure methanol and 5 ml propylene oxide in order to remove the epoxy resin, according to the method of Maxwell (1978). After rinsing in 50% methanol and distilled water, sections were dipped in 0.3% hydrogen peroxide in pure methanol for 20 min to inhibit the activity of endogeneous peroxidase, and then rinsed in 1% bovine serum albumin

Table 1. Histochemical findings of membranocystic lesions

Stains		Case 1		Case 2		Case 3	
		BM	SA	BM	SA	BM	SA
Periodic acid Schiff	(PAS)	+++	+++	+++	+++	+++	+++
Luxol fast blue	(LFB)	++	++	++	++	++	++
Alcian blue (pH 2.5)		++	+	++	+	++	+
Oil red O	(ORO)	+++	+++	+++	+++	+++	+++
Sudan black B	(SBB)	+++	+++	+++	+++	+++	+++
Nile blue		++	++	++	++	++	++
Toluidine blue		+	+	+	+	+	+
Congo red		−	−	−	−	−	−
Ziehl-Neelsen		−	−	−	−	−	−

Extent of the staining: +++ strong reaction; ++ moderate reaction; + weak reaction; − negative; BM = Bone marrow; SA = Subcutaneous adipose tissue

Table 2. Lectin histochemistry of membranocystic lesions in Nasu-Hakola disease

Lectin	Source	Haptenic sugar	Case 1		Case 2		Case 3		Controls*	
			BM	SA	BM	SA	BM	SA	BM	SA
Con A	Canavalia ensiformis	D-mannose	++	+	++	+	++	+	−	−
MPA	Maclura pomifera	α-D-galactose	+++	+++	+++	+++	+++	+++	−	−
PNA	Arachis hypogaea	β-D-galactose	+	+	+	+	+	+	−	−
RCA-I	Ricinus communis I	β-D-galactose	−	−	−	−	−	−	−	−
SBA	Glycine max	GalNAc	−	−	−	−	−	−	−	−
HPA	Helix pomatia	GalNAc	−	−	−	−	−	−	−	−
DBA	Dolichos biflorus	GalNAc	−	−	−	−	−	−	−	−
GS-I	Griffonia simplicifolia I	α-D-galactose	+	+	+	+	+	+	−	−
GS-II	Griffonia simplicifolia II	GlcNAc	−	−	−	−	−	−	−	−
WGA	Triticum vulgaris	GlcNAc	−	−	−	−	−	−	−	−
LFA	Limax flavus	NeuNAc	−	−	−	−	−	−	−	−
UEA-I	Ulex europeus I	L-fucose	−	−	−	−	−	−	−	−

* Histochemical controls: three adult subjects with uninvolved adipose tissues. Extent of the staining: +++ strong reaction, ++ moderate reaction, + weak reaction, − negative GalNAc: N-acetyl-galactosamine, GlcNAc: N-acetyl-glucosamine, NeuNAc: N-acetyl-neuraminic acid (sialic acid) BM: bone marrow, SA: subcutaneous adipose tissue

in phosphate-buffered saline (PBS) (pH 7.4) for 10 min. Sections were incubated in 30 μg/ml horseradish peroxidase (HRP)-labeled lectins for 45 min. The twelve kinds of lectins used in this study are shown in Table 2 and all were purchased from E-Y Laboratories (San Mateo, CA, USA). After further rinsing in PBS, the sections were incubated in the diaminobenzidine (DAB) hydrogen-peroxidase medium (Graham and Karnovsky 1966). All these procedures were performed at room temperature. As a control, the HRP-labeled lectins were preincubated with their specific binding sugars (Table 2) and then applied onto sections.

For lectin staining for electron microscopy the specimens of all three cases were fixed in a half strength of Karnovsky's fixative. For Lowicryl K4M embedding, the specimens were washed with PBS for 24 h, and then dehydrated in a graded ethanol series at lower temperatures, down to −35° C. After infiltration with Lowicryl K4M at −35° C for 24 h, UV polymerization was done at −35° C for 24 h according to the procedure of Carlemalm et al. (1982). Ultrathin sections were cut with a Porter-Blum MT 2B ultramicrotome and mounted on nickel grids having a carbon coated formvar film. HRP-labeled MPA was obtained from E-Y Laboratories (San Mateo, CA, USA). CG particles (8 nm and 15 nm) were prepared using the alternative citrate-tannic acid procedure (Slot and Geuze 1981) and the trisodium citrate reduction method (Frens 1973) respectively. The pH of CG solution was adjusted to pH 7.8 with 0.1 M K_2CO_3. Since MPA-HRP and CG conjugate is more stable than MPA and CG conjugate, the conjugation of MPA-HRP and CG was performed according to the method of Roth (1983). The ultrathin sections were floated on a drop of PBS for 10 min, transfered to a drop of the MPA-HRP-CG complex solution (10 μg of MPA-HRP/ml), and then incubated for 30 min. After incubation, the sections were washed in PBS and then in distilled water. They were then stained with saturated aqueous solution of uranyl acetate for 5 min and with lead acetate solution for a further 1 min, and examined with a JEOL 100B electron microscope. As a control, the MPA-HRP-CG complex was incubated for 30 min with 0.5 M α-D-galactose (Sigma/St. Louis, Mo, USA), which is a specific binding sugar for MPA, prior to its application to the sections.

Results

In the bone marrows of all three cases, using light microscopy, we found several undulating mem-

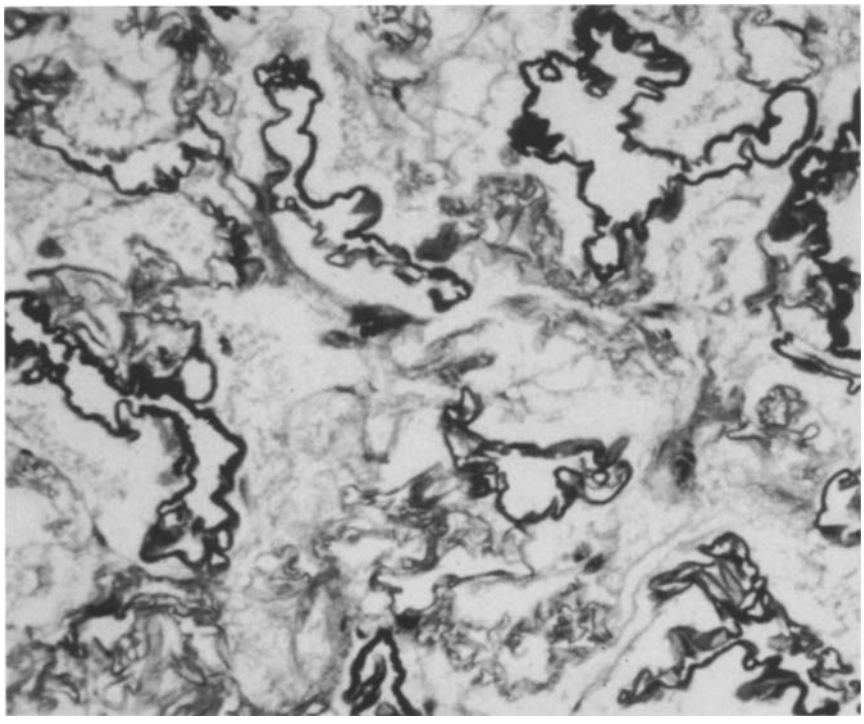

Fig. 2. Several undulating membranocystic lesions which formed arabesque profiles and reactive to PAS. Bone marrow of case 1. (PAS, ×360)

branocystic lesions, similar to those of membranous lipodystrophy (Nasu et al. 1973, Akai et al. 1977, Fujiwara 1979). Although these membranocystic lesions were also found in subcutaneous adipose tissues, there were not as many as in the bone marrow. They were strongly stained with ORO, SBB and PAS (Fig. 2), moderately with LFB and Alcian blue and weakly with toluidine blue. There was no staining with Congo red and Ziel-Neelsen. The conventional histochemical findings of the membranocystic lesions are summarized in Table 1.

On electron microscopy we found various types of membranocystic lesions in the bone marrow and subcutaneous adipose tissue of all cases. Well developed membranocystic lesions in the bone marrow were composed of undulating membranes (Fig. 3a). Higher magnification revealed that the membranes of these lesions were composed of numerous minute tubular structures, arranged perpendicularly to the inner surface (Fig. 3b). Another type of membranocystic lesion, consisting of thinner membranes without tubular structures was also observed in the same lesions (Fig. 3c). However, there were several degenerating adipose cells adjacent to the membranocystic lesion. The basement lamina of these dying cells had usually disappeared. In addition, electron dense substances were occasionally observed at the lipid-cytoplasmic interface of the degenerating adipose cell (Fig. 3d).

In light microscopic lectin histochemistry the Epon-embedded thick sections showed foci of markedly undulating membranocystic lesions with arabesque profiles. Specimens of all three cases were studied with the twelve kinds of HRP labelled lectins. MPA, which specifically recognizes α-D-galactose residues (Baush et al. 1977), stained all of the typical membranocystic lesions markedly and strongly (+++) (Fig. 4a). However, membranes of both degenerated and normal adipose cells were not stained with MPA-HRP. Concanavalin A (Con A), which recognizes D-mannose and peanut agglutinin (PNA), which recognizes β-D-galactose and *Griffonia simplicifolia* I (GS I), which recognizes α-D-galactose sequence, moderately (++) or weakly (+) stained the the typical membranocystic lesions. However, the other lectin conjugates failed to stain the membranocystic lesions.

HPA, which specifically binds to N-acetyl-D-galactose (GalNAc) (Hammerström et al. 1969) did not label the typical membranocystic lesions but stained the membranes of degenerated adipose cells moderately and also stained the plasma membranes of some bone marrow cells (Fig. 4b). In addition, the staining of uninvolved adipose tissues used in this study was virtually negative. These lectin histochemical results are summarized in Table 2. The specificity of the lectin staining observed was substantiated by the results of the control experiment using appropriate monosaccharides.

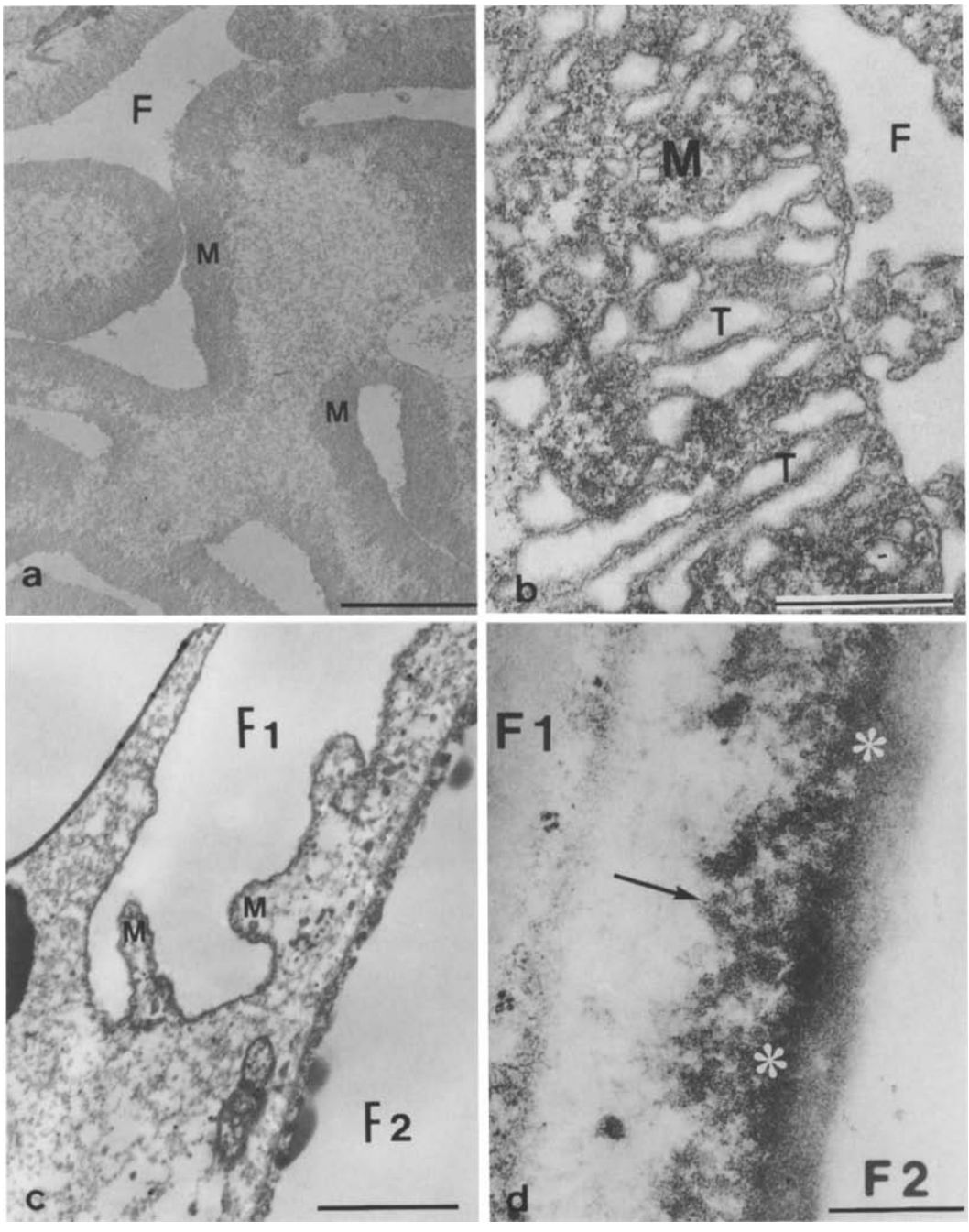

Fig. 3. (**a**) Ultrastracture findings of typical membranocystic lesions in the bone marrow of case 2. (**b**) Higher magnification of Fig. 3a; well developed membranocystic lesions were composed of numerous minute tubular structures arranged perpendicularly to the inner surface. (**c**) Ultrastructural findings of the other type of membranocystic lesions, which consisted of undulating thinner membranes without tubular structures in the same lesions of Fig. 3a. (**d**) Electron dense substances (*arrow*) were observed at the lipid-cytoplasmic interface (*asterisks*) of the degenerated adipose cells of case 3. The basement lamina notably disappeared. Magnifications: (**a**) ×18000, (**b**) ×86000, (**c**) ×36000, (**d**) ×74000; bar: (**a**)=1 μm, (**b**)=0.2 μm, (**c**)=0.5 μm, (**d**)=0.2 μm; F, F1, F2=lipid of a fat cell; M=membranocystic lesions; T=tubular structure)

Electron microscopic cytochemistry with MPA-HRP-CG conjugate showed that the well developed membranocystic lesions in all cases were intensely labeled with this lectin-CG conjugate (Fig. 5a). Numerous CG particles were observed regularly along the membranes of the minute tubular structures of these lesions under high magnification (Fig. 5b).

The undulating thinner membranes without tubular structures were also stained with MPA-HRP-

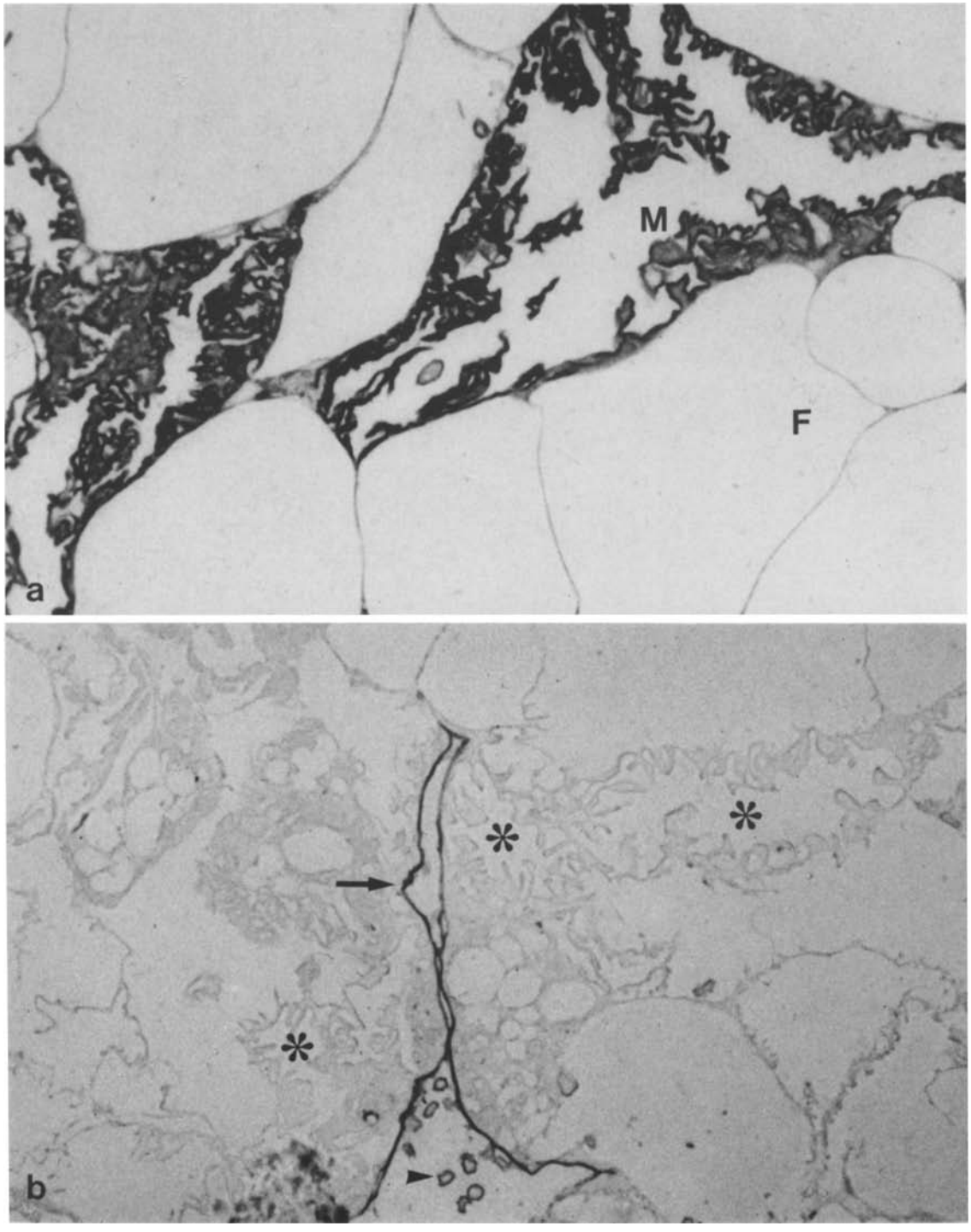

Fig. 4. (**a**) Light microscopic lectin histochemistry of case 1. MPA, which specifically recognizes α-D-galactose residues, definitely stained the whole of the typical membranocystic lesions. (MPA-HRP, ×720: F = lipid in a fat cell, M = membranocystic lesions). (**b**) HPA, which specifically binds to GalNAc did not label the typical membranocystic lesions of case 1 (*asterisks*) but stained the membranes of degenerated adipose cells (*arrow*) and plasma membranes of some bone marrow cells (*arrowhead*). (HPA-HRP, ×620)

CG conjugate. CG particles were observed along the outer face of the membranes (Fig. 5c). However, the labeling of CG particles in the thinner membranes of the degenerated adipose cells, was less intense than that of the typical membranocystic lesions (Fig. 5d). The cell membranes of normal adipose cells were not labelled with this conjugate.

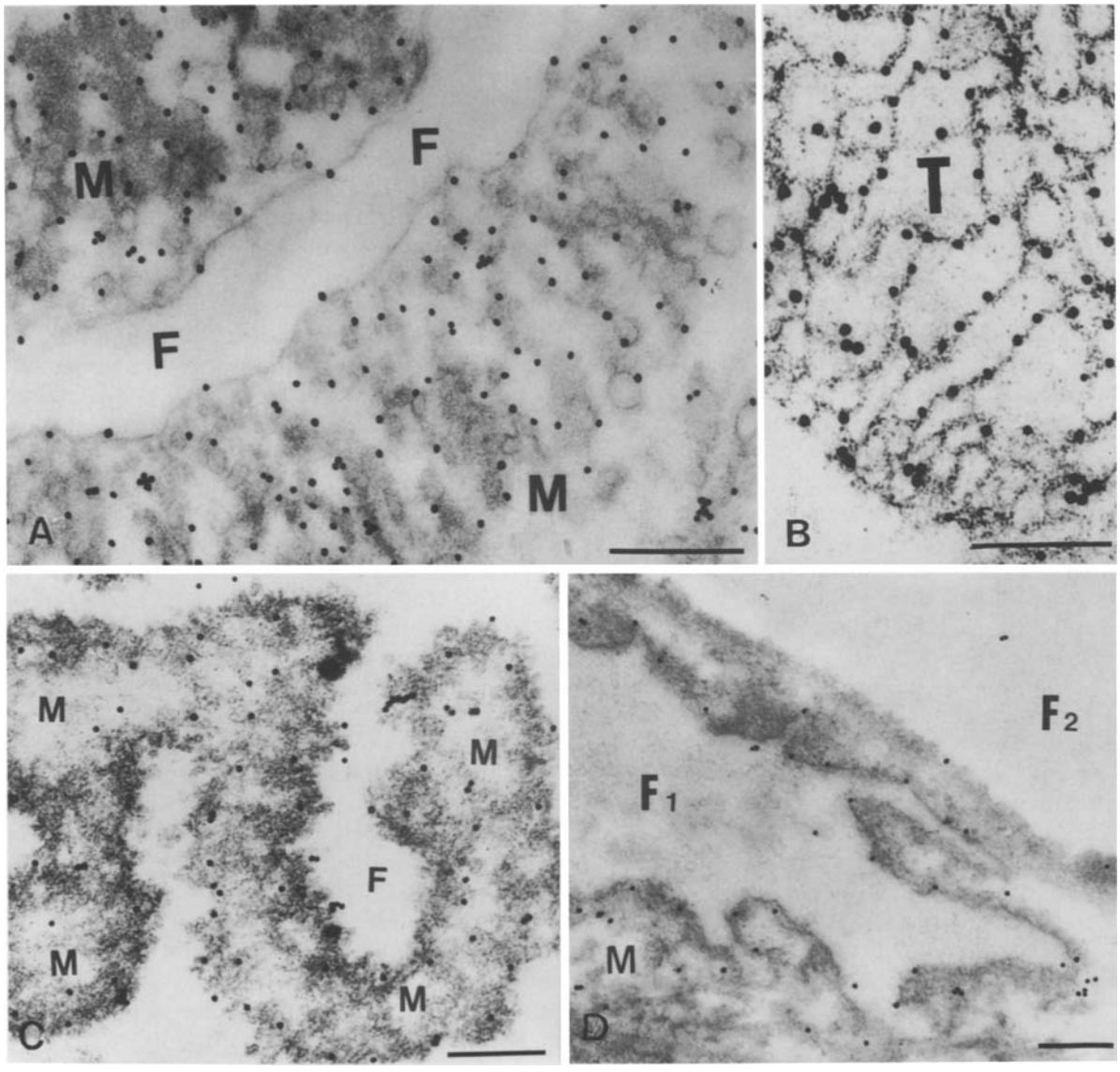

Fig. 5. (**A**) Electron microscopic cytochemistry with MPA-HRP-CG conjugate of case 1. Well developed membranocystic lesions were intensely labelled with lectin-CG conjugate. (**B**) Numerous CG particles were observed along the membrane of the minute tubular structures in typical membranocystic lesions of case 1. (**C**) The undulating thinner membranes without tubular structures were also stained with MPA-HRP-CG conjugate and conjugates were observed along the outer face of the membranes. (**D**) The labeling of conjugates in the degenerated adipose cells, was less intense than that of the typical membranocystic lesions. Colloidal gold particle size: (**A**, **C**, **D**) = 15 nm, (**B**) = 8 nm; Magnification: (**A**) ×42000, (**B**) ×92000, (**C**) ×40000, (**D**) ×46000; *bar:* (**A**) = 0.5 μm (**B**) = 0.2 μm (**C**) = 0.4 μm (**D**) = 0.2 μm; F, F1, F2 = lipid in a fat cell, M = membranocystic lesions, T = tubular structure

Discussion

We have presented findings from three patients in two families, who showed progressive presenile dementia and spastic paraplegia. Roentgenography of their bones revealed polycystic transparency, indicating membranocystic lesions. The clinical features and histopathological findings of these cases were compatible with membranous lipodystrophy (Nasu-Hakola disease).

Although many investigators have tried to analyze lipid metabolism of the bone marrow and brain of this disease (Nasu et al. 1973, Ohtani et al. 1979, Tokunaga et al. 1981) and to clarify the pathological aspects (Nasu et al. 1973, Harada 1975, Tanaka et al. 1980), the pathogenesis of this disease is not clearly known. Investigation of the characteristic membranocystic lesions in the affected adipose tissues might shed more light on this disease.

Electron microscopic study revealed that there were several types of membranocystic lesions. A thicker membrane with well developed minute tubular structures coexisted in the same lesions with thinner membranes without tubular structures. This thicker membrane is ordinarily described as the gathering of numerous microtubular structures perpendicularly arranged to the inner surface. These findings are consistent with those of previous reports (Yagishita et al. 1976, Akai et al. 1977). In addition, electron dense substances were observed at the lipid-cytoplasmic interface of the degenerated adipose cells of which basement lamina usually disappeared. These findings were thought to be characteristic of the initial or early changes of the membranocystic lesions. The experimental study of the saponin-induced membranocystic lesions in rabbit bone marrow adipose tissues has revealed that membranocystic lesions were initially formed at the lipid-cytoplasmic interface of degenerated adipose cells (Suganuma 1978).

Our histochemical studies showed that membranocystic lesions reacted with carbohydrates, lipids and phospholipids. The same results have been reported by conventional histochemical studies (Nasu 1978, Fujiwara 1979). Our present investigation employing lectin conjugates provides more precise information about the components of the carbohydrates in the membranocystic lesions. Among the lectins used in this study, MPA which specifically binds α-D-galactose residues, strongly stained the membrane of typical membranocystic lesions, while the other lectins reacted only slightly or failed to stain the same lesions. These results suggest that most terminal carbohydrate residues of the membrane of typical membranocystic lesions are composed of α-D-galactose residues. However, the membranes of degenerated adipose cells, which were thought to be the initial or early stage of membranocystic lesions in our electron microscopic examinations, stained with HPA-HRP but they did not react with MPA-HRP. These results suggest that the lack of affinity of the membranocystic lesions towards HPA implies that GalNAc residues are fundamental structures of saccharide in the lesions, and that a certain change of carbohydrate residues occurs during the formation of the membranocystic lesions. These findings are in agreement with those in another case of membranous lipodystrophy (Suganuma et al. 1987).

The ultrastructural cytochemical study using MPA-HRP-CG conjugate and Lowicryl K4M resin succeeded in showing that the outerface of the membrane consisting of the minute tubular structures in the membranocystic lesions was intensely labelled with this lectin CG conjugate. This result confirmed the presence of α-D-galactose residues at the the membrane of the minute tubular structures. There were few a irregularly spaced CG particles on the thinner membranes and also on the membranes of the degenerated adipose cells. No CG particles labelled the cell membranes of normal adipose cells. These findings suggest the affinity of membranocystic lesions towards MPA, and imply that α-D-galactose residues increase with the formation of the membranocystic lesions of this disease. This study is the first to succeed in demonstrating the precise localization of α-D-galactose residues in the membranocystic lesions at the electron microscopic level.

Similar membranocystic lesions unrelated to membranous lipodystrophy, have been found in adipose tissues of patients suffering from various diseases including dermatomyositis, malignant tumours and limb necrosis (Nasu et al. 1977; Fujiwara 1979; Machinami 1983, 1984). These changes were thought to result from environmental disturbances of the adipose tissue due to chronic circulatory disturbance. Machinami (1983, 1984) concluded that membranocystic lesions characteristic of membranous lipodystrophy were non-specific changes in adipose tissues. However, there are some differences between the membranocystic lesions of membranous lipodystrophy and those of patients unrelated to this disease. The "non-specific" membranocystic lesions were usually localized in areas associated with malignant neoplasmas, granulomatous and/or arteriosclerotic changes causing circulatory disturbance. In contrast to this, the lesions of membranous lipodystrophy are found in the various adipose tissues throughout the body, where no remarkable lesions indicating circulatory disturbance are observed (Nasu et al. 1973, Harada 1975). Although the Finnish group have reported vascular degeneration in various tissues of membranous lipodystrophy, no histopathological evidence for vascular lesions was demonstrated in their reports (Sourander 1970, Hakola 1972, Hakola and Partanen 1983). In addition, bone histomorphometry of biopsied iliac bones containing membranocystic lesions taken from case 1 and case 2 revealed mild osteoporosis with normal bone formation, exhibiting normal tetracycline labeling (Kitajima et al. 1987). Therefore it was postulated that cystic bone lesions of membranous lipodystrophy might result from physical compression by the membranocystic lesions. In addition, it is also difficult to explain the brain lesions (leukodystrophy) of membranous lipodystrophy

by the hypothesis of vascular degeneration (Harada 1975). Consequently, the membranocystic lesions of membranous lipodystrophy are still one of the characteristic profiles of this disease. Further clinical and histopathological investigation is essential to elucidate this peculiar disease.

Although the aetiology of these lesions and the pathogenesis of membranous lipodystrophy are still in debate, we believe our lectin histochemical study to be useful for the characterization of membranocystic lesions in this disease.

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