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Fabry disease: ultrastructural lectin histochemical analyses of lysosomal deposits

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Abstract Fabry disease is an X-linked inborn error of glycosphingolipid catabolism resulting from a deficiency of lysosomal α-galactosidase activity. Globotriaosylceramide accumulates predominantly in lysosomes of various tissues. Former studies have clarified the nature of this disease, and the accumulated materials in the lysosomes have been analyzed using biochemical techniques. In the present study, transmission electron microscopy was used to reveal the fine structure of these lysosomal deposits, and sugar residues in the lysosomal deposits in Fabry disease were examined by lectin histochemistry combined with enzyme digestion. This is the first report to describe the lysosomal sugar residues in Fabry disease analyzed using lectin histochemistry at the ultrastructural level. With these techniques, we were able to detect α-galactosyl, β-galactosyl and glucosyl sugar residues in the lysosomal deposits. The experimental procedures used in this study have considerable potential for use in investigations of glycolipid and glycoprotein storage diseases without the need for complex methodology and expensive materials.

Key words Fabry disease · Lysosomal deposit · Glycosphingolipid · Lectin histochemistry · Enzyme digestion

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Introduction

Fabry disease is an X-linked glycosphingolipid storage disease [2, 10, 27] caused by a deficiency of α-galactosidase [6, 20]. The gene encoding α-galactosidase has been localized to the X-chromosomal region, Xq22. The full-length cDNA and entire genomic sequences have been isolated [4, 5, 21] and used to analyze the mutations causing Fabry disease. A variety of lesions and mutations underlying the molecular genetic heterogeneity of this disease have been identified [8]. α-Galactosidase is one of the exoglycosidases that hydrolyze a terminal α-linked galactosyl moiety, and the enzyme defect leads to systemic accumulation of glycosphingolipids with a terminal α-D-galactosyl moiety, predominantly globotriaosylceramide [28]. These materials accumulate gradually, particularly in the lysosomes of vascular endothelial cells of most organs, and lead to various clinical manifestations, which include the onset of pain and paresthesias in the extremities, angiokeratoma in skin and mucous membranes, hypohidrosis, and cardiac and renal dysfunction, especially in hemizygous male patients with classic Fabry disease [8]. Heterozygous female subjects show various levels of enzymatic activity. They are usually asymptomatic, but some of them also show the corneal opacities and other clinical symptoms. These clinical manifestations result from the progressive accumulation of glycosphingolipid in the vascular endothelium. Many studies have revealed the characteristics of these accumulated materials using various histochemical and immunohistochemical approaches [7, 9, 11–13, 19, 22, 25, 30]. Generally, identification of the stored material using histochemical approaches involves considerable difficulty, because a number of solvents readily extract these lipid-laden materials [1, 23]. Morphological studies using electron microscopy have also demonstrated the fine structure of these deposits [16, 29]. At the ultrastructural level, the need for post-fixation with osmium tetroxide, which is a good fixative for lipid, greatly hampers the usefulness of many electron microscopic histochemical techniques owing to its nature as a strong oxi-

Table 1 Brief summary of the cases with Fabry disease

Age/sex	α-Galactosidase activity[a]	Clinical manifestations	Biopsy site
27 M	0.15	Acroparesthesia, hypohidrosis, cardiomyopathy	Anterior chest
18 M	0.30	Acroparesthesia, hypohidrosis, cardiomyopathy	Back of thigh
34F	1.36	Acroparesthesia, hypohidrosis	Lower abdomen
36F	0.76	Hypohidrosis, cardial hypertrophy, corneal opacity, angiokeratoma	External genitalia
53F	2.12	Hypohidrosis, cardial hypertrophy, corneal opacity, angiokeratoma	Lower abdomen
59F	1.82	Acroparesthesia, hypohidrosis, cardiomyopathy	Lower abdomen

[a]Normal enzyme activity 2.88–16.15 nmol/h per ml in plasma

dizing agent. Some studies have revealed the localization of globotriaosylceramide using immunohistochemical or lectin histochemical methods at the light microscopic level, but for the above reasons, definite staining of glycosphingolipid materials in Fabry disease employing ultrastructural lectin histochemistry has not yet been reported.

In the present investigation, we examined the skin tissues of six patients in whom Fabry disease had been diagnosed by enzyme assay, pedigree analysis and several clinical features. We also analyzed lysosomal deposits of eccrine sweat gland cells using transmission electron microscopy and lectin histochemistry. We used three kinds of lectins: GS-I B_4 (*Griffonia simplicifolia* I B_4), which has specific affinity for the terminal a-D-galactosyl residue, RCA-I (*Ricinus communis* I) for the terminal α/β-D-galactosyl residue, and ConA (*Canavalia ensiformis*) for the terminal glucosyl/mannosyl residue. First, we ascertained the expression of the terminal α-galactosyl moiety in cytoplasmic lysosomal deposits using GS-I B_4 and RCA-I lectins. These lectin histochemical methods were found to be very useful for the study of Fabry disease.

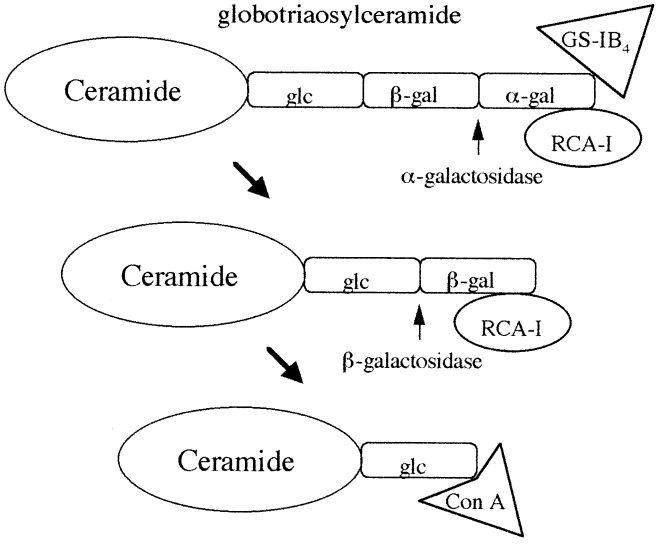


Fig. 1 Schema representing the relationship between enzyme digestion and lectin staining. Without enzyme digestion, α-galactosyl residues of globotriaosylceramide are recognized by GS-I B_4 and RCA-I lectins. After initial α-galactosidase digestion, only β-galactosyl residues are stained by RCA-I. After sequential β-galactosidase digestion, glucosyl residues become identifiable with ConA

Materials and methods

Six patients with Fabry disease were studied. Their clinical manifestations are summarized in Table 1. All specimens were taken by skin biopsy. Each biopsy block was divided into two portions. One portion was fixed with 10% formalin, dehydrated in alcohol, embedded in paraffin and used for hematoxylin-eosin staining. The other portion was cut into small pieces, and fixed in half-Karnovsky's fixative for 12 h at 4°C. They were embedded in Epon 812 resin (Taab, Berks., UK) after post-fixation in osmium tetroxide, and some of them were embedded in Epon 812 resin without osmium tetroxide post-fixation.

Pure and biotinylated GS-I B_4, biotinylated RCA-I, and pure ConA were purchased from EY Laboratories (San Mateo, Calif.), and pure RCA-I from HONEN (Tokyo, Japan). For light microscopic analyses, we cut sections from the samples embedded in Epon 812 at 2 µm thickness and mounted them on silanized slides (Dako Japan, Kyoto, Japan). After removal of the epoxy resin with Maxwell's solution [24], semi-thin sections were bleached with 10% hydrogen peroxide for 10 min at room temperature according to the procedure of Aparicio and Marsden [3] with minor modification. Endogenous peroxidase activity was inhibited by dipping the sections in 0.3% hydrogen peroxide in methanol for 20 min. The sections were washed briefly twice with PBS, then immersed in 1% bovine serum albumin (BSA) in PBS for 10 min and incubated in biotinylated GS-I B_4 and biotinylated RCA-I, both at a concentration of 25 µg/ml, overnight at 4°C. After washing with PBS, they were dipped in streptavidin-horseradish peroxidase conjugate (GIBCO, Gaithersburg, Md.) diluted 1:100 in 1% BSA. After a further rinse with PBS, the sections were developed with 3, 3'-diaminobenzidine tetrahydrochloride [15].

Ultrathin sections of the skin tissues were cut with a 2088 LKB Ultratome V (LKB-Produkter AB, Bromma, Sweden) and a diamond knife (DiATOME, Biel, Switzerland), and mounted on 150 gold mesh grids (Stork Veco B.V., Eerbeek, The Netherlands). Grids were floated on top of one or two drops (40~80 µl) of the reagents consecutively in a moist chamber. Before lectin staining and enzyme digestion, the sections were pretreated with 10% hydrogen peroxide for 10 min at room temperature to remove any residual osmium [3]. These sections were washed with PBS for 10 min, incubated with 1% BSA in PBS for 10 min, rinsed twice with PBS at room temperature for 5 min each time, and incubated with pure GS-I B_4 (50 µg/ml in 1%BSA in PBS), pure RCA-I (50 µg/ml) and pure ConA (100 µg/ml) overnight at 4°C. These sections were cleansed with PBS and incubated in biotinylated rabbit anti-GS-I antibody (10 µg/ml), anti-RCA-I antibody (20 µg/ml) and anti-ConA antibody (25 µg/ml) for 2 h at room temperature. All antibodies were obtained from EY Laboratories. After washing with PBS, the sections were labeled with 10 nm streptavidin–colloidal gold complex (British BioCell, Cardiff, UK) diluted ten times with 1%BSA in PBS for 60 min at room temperature, and then counterstained with uranyl acetate and lead citrate before observation in a Hitachi H-7000 transmission electron microscope.

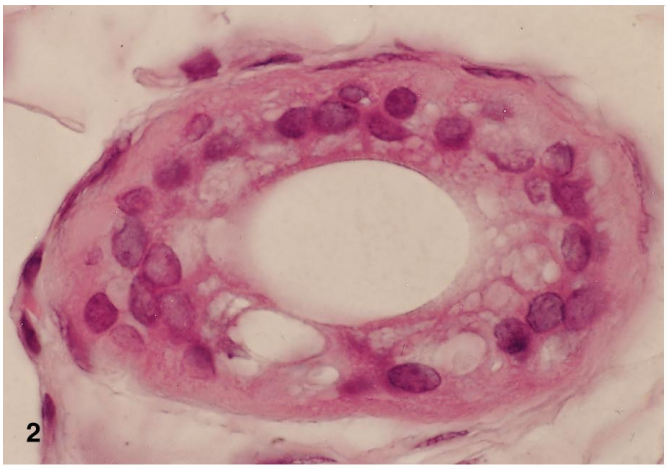
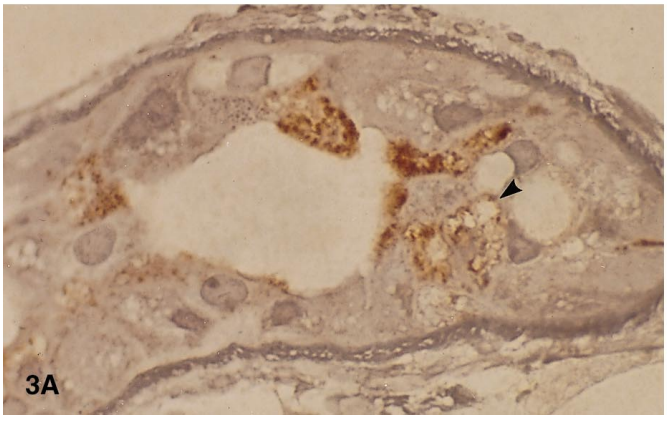


Fig. 2 Eccrine gland cells from the anterior chest. There are many cytoplasmic vacuoles without glycolipid contents. H&E, ×250



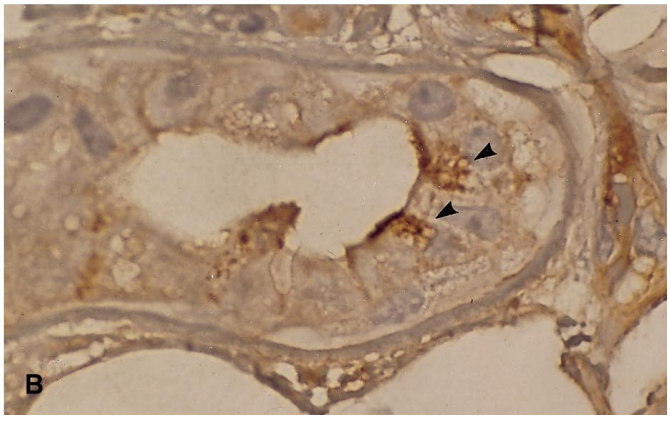


Fig. 3A, B Lectin histochemical stainings of eccrine gland cell vacuoles. Epon-embedded semithin sections are stained with biotinylated GS-I B_4 (**A**) and biotinylated RCA-I (**B**) after removal of resin and osmium tetroxide. The peripheral vacuolar regions are positively stained with both lectins (*arrowheads*). **A, B** ×250

To analyze the structure of sugar residues existing in the lysosomal deposits, sequential digestion with α-galactosidase from green coffee beans (Sigma, St. Louis, Mo.) and β-galactosidase from *Charonia lampas* (Seikagaku Kogyo, Tokyo, Japan) was also performed according to the previous reports [17, 18]. Digestion with α-galactosidase was performed by incubating tissue sections in 3.2 M $(NH_4)_2SO_4$ solution, pH 6.0, containing α-galactosidase (2 U/ml) for 20 h at 37°C. Digestion with β-galactosidase was carried out by incubating the sections at a concentration of 1 U/ml enzyme in 0.2 M citrate phosphate buffer containing 0.5 M NaCl, pH 4.0 for 20 h at 37°C. After washing with PBS, each section was stained with three kinds of lectins in the same way as described above. A relationship between enzyme digestion and lectin histochemistry is shown in Fig. 1 as a brief schema.

As a control, to verify the specificity of the sugar binding, tissue slices were treated in the same manner, except that they were incubated in the presence of 0.2 M D-(+)-galactose. In addition, biopsied normal skin tissues from the left sole of a 15-year-old girl and the right shoulder of a 61-year-old man were observed after processing in the same way. Epon sections of a 27-year-old male patient and a 53-year-old female patient without osmium post-fixation were stained with GS-I B_4 and RCA-I.

Results

Paraffin sections stained with hematoxylin-eosin showed many cytoplasmic vacuoles without any glycolipid contents in eccrine sweat gland cells (Fig. 2). Peripheral vacuolar regions in the eccrine gland cells were positively stained with biotinylated GS-I B_4 and biotinylated RCA-I in Epon-embedded semithin sections (Fig. 3A, B). All specimens were observed by transmission electron microscopy. They displayed lysosomal inclusion bodies of various sizes, with a concentric lamellar configuration in the endothelial cells, fibroblasts and eccrine sweat gland cells (Fig. 4A, B).

Lectin histochemistry with pure GS-I B_4 and pure RCA-I, biotinylated antibodies against each lectin, and a combination of streptavidin–colloidal gold in ultrathin sections of Epon-embedded specimens revealed the specific staining pattern of sugar residues in lysosomal deposits (Figs. 5A, 6A). As these lectin stainings were performed after bleaching treatment with a 10% solution of hydrogen peroxide to remove any interference from the electron-dense osmium, some of the lysosomal deposits lost their clear lamellar structures.

The results of enzyme digestion and lectin staining are shown in Table 2. We performed lectin stainings with GS-I B_4, RCA-I and ConA each time before enzyme digestion, after initial α-galactosidase digestion, and after sequential β-galactosidase digestion (Figs. 5–7). Before enzyme digestion, lysosomal deposits were stained positively with GS-I B_4 and RCA-I (Figs. 5A, 6A). After initial enzyme digestion with α-galactosidase, GS-I B_4 lectin did not identify any cytoplasmic material (Fig. 5B), but lysosomal deposits were still stained by RCA-I lectin (Fig. 6B). After sequential β-galactosidase digestion, colloidal gold labeling of RCA-I was markedly reduced (Fig. 6C), whereas labeling of ConA lectin was remark-

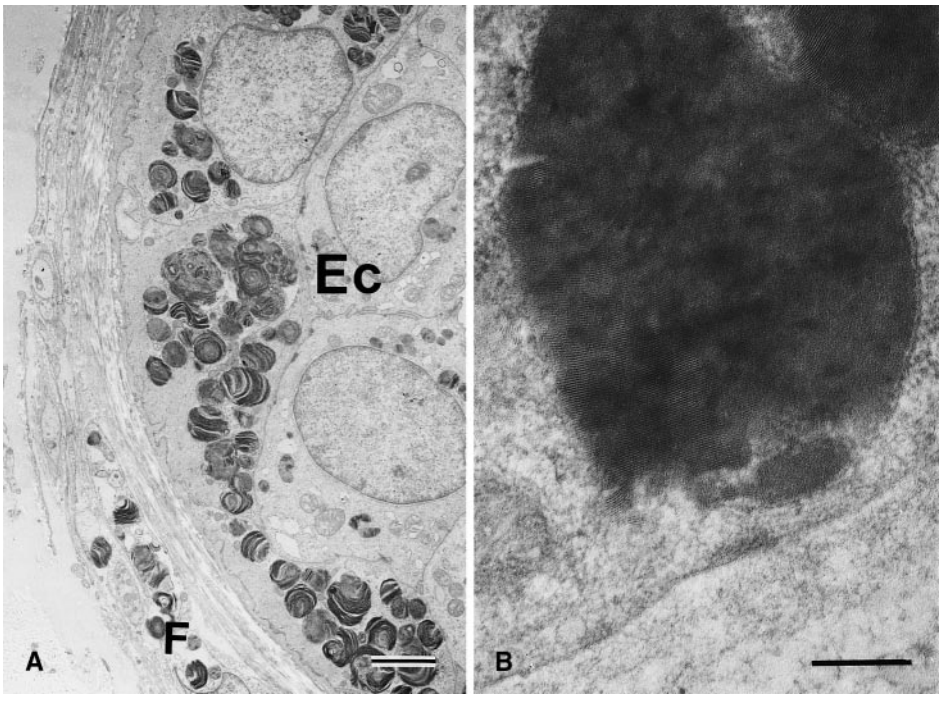


Fig. 4A, B Transmission electron micrographs of the lysosomal deposits in various cell types. **A** Osmophilic polymorphous lysosomal deposits in an eccrine gland cells (*Ec*) and in a fibroblast (*F*). ×4,120, *bar* 2 μm **B** Lysosomal deposit of glycosphingolipid in the endothelial cell showing a typical lamellar structure with regular periodicity of about 5.5 nm. ×63,000, *bar* 0.2 μm

ably increased (Fig. 7A–C). These results were similar in vascular endothelial cells and eccrine sweat gland cells in all six cases. Two specimens of normal skin tissue did not display any lysosomal deposit with a lamellar structure anywhere, and no lectin staining pattern was revealed either before or after enzyme digestion. Upon incubation of sections with 0.2 M D-(+)-galactose no lysosomal deposit was stained by GS-I B_4 or RCA-I. Ultrathin sections of Epon-embedded specimens without osmium post-fixation exhibited many amorphous vacuoles, but no staining pattern with GS-I B_4 and RCA-I was detected in these regions.

Discussion

The purpose of this study was to characterize the sugar residues of lysosomal storage materials in Fabry disease using ultrastructural lectin histochemistry and enzyme digestion. The main material that accumulates in lysosomes is a glycosphingolipid named globotriaosylceramide, which has a sugar chain of Gal(α1→4)Gal(β1→4)Glc(β1→) attached to ceramide. This material would be expected to display the characteristics of both lipids and carbohydrates. In general, histochemical analysis of these materials is difficult because they are extracted during specimen dehydration procedures [1, 23]. At the ultrastructural level, fixation with osmium tetroxide greatly hampers the usefulness and specificity of many electron microscopic histochemical techniques [11]. A comparison of various fixation and embedding techniques that preserve the storage material has been reported [11, 12]. Also, there have been several reports on the analysis of lysosomal deposits in Fabry disease using immunohistochemistry [7, 13, 19, 25] and lectin histochemistry [9, 11, 22, 26, 30]. So far, owing to technical difficulties, no published study has attempted to elucidate the characteristics of lysosomal deposits in Fabry disease using ultrastructural lectin histochemistry. We investigated six biopsied skin materials from patients in whom Fabry disease had been diagnosed (Table 1), performing lectin staining in combination with enzyme digestion using some additional techniques reported previously [3, 24], and obtained excellent results.

In paraffin-embedded sections many vacuoles were observed in the peripheral cytoplasm of eccrine gland cells stained with hematoxylin-eosin (Fig. 2). These vacuoles were thought to be the sites of lysosomal deposit of glycosphingolipid extracted by certain reagents during the embedding and deparaffinization.

For light microscopic analysis, we stained 2-μm-thick sections of Epon-embedded specimens with bio-

Fig. 5. A Eccrine gland cells stained with GS-I B_4, biotinylated rabbit antibody against GS-I and 10 nm streptavidin-colloidal gold combination. Cytoplasmic lysosomal deposits (*LD*) are strongly labeled with this lectin. *M* mitochondria, ×23,700 *bar* 0.5 μm). **B, C** After initial α-galactosidase digestion (**B**) and after sequential β-galactosidase digestion (**C**), positive labeling has diminished in both sections. **B** ×25,200, **C** ×23,200, *bars* 0.5 μm

Fig. 6. A, B Lectin stainings of eccrine gland cells with RCA-I. **A** Positive labeling is discernible in the lysosomal deposits (*LD*) before digestion, ×23,700. **B** Labeling of significant intensity is still evident after α-galactosidase digestion. ×39,800. **C** After sequential β-galactosidase digestion, no labeling is apparent, ×25,800. *Bars* 0.5 μm

Fig. 7A–C ConA stainings of lysosomal deposits (*LD*). **A** Before digestion and **B** after α-galactosidase digestion, very little colloidal gold is evident, whereas **C** positive labeling is observed after sequential digestion. **A** ×29,000, **B** ×22,800, **C** ×46,300. *Bars* 0.5 μm

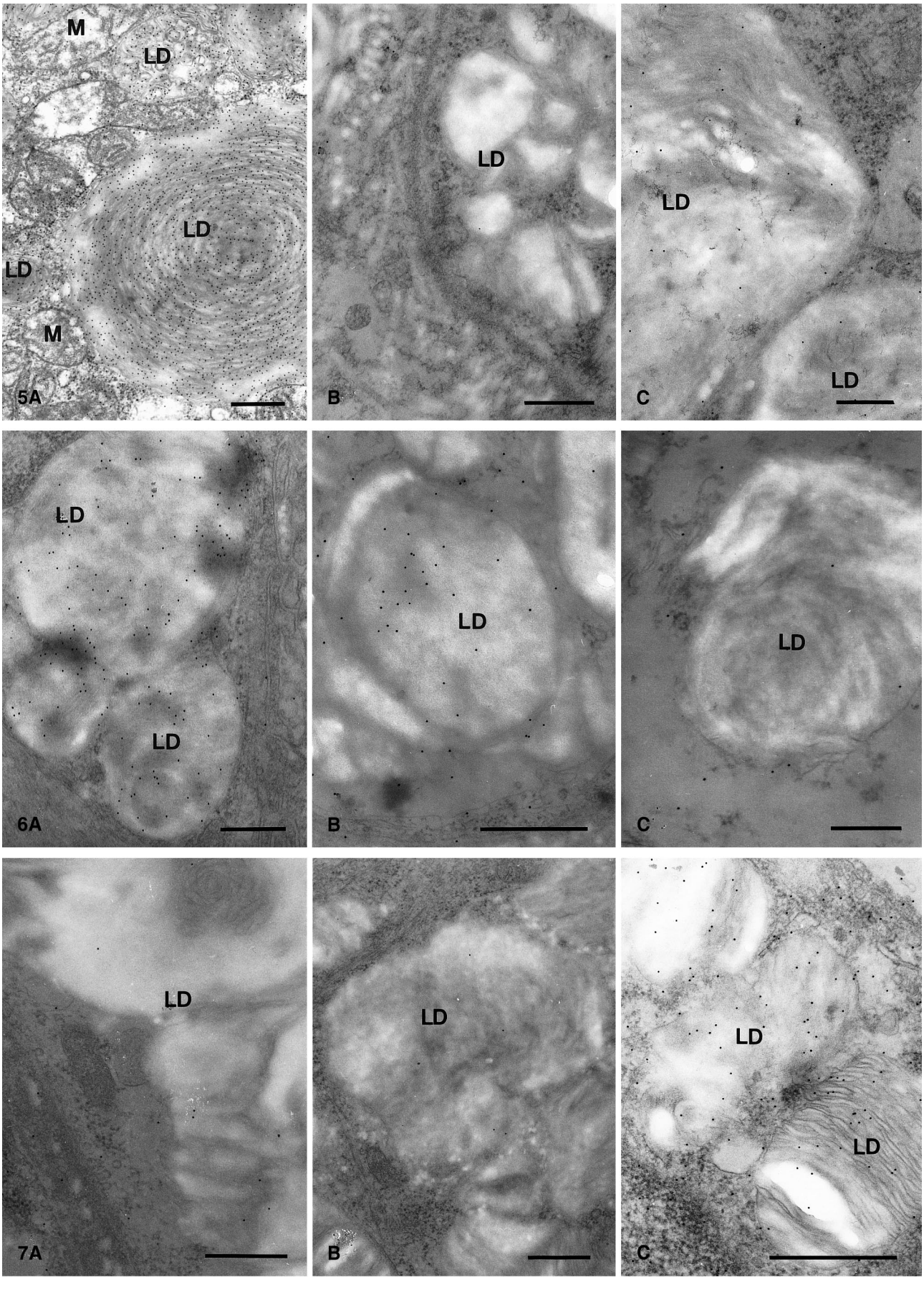

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Table 2 Results of enzyme digestion and lectin staining

Enzyme digestion	GS-I B_4	RCA-I	Con A
None	Positive	Positive	Negative
α-Galactosidase	Negative	Positive	Negative
α-Galactosidase→β-galactosidase	Negative	Negative	Positive

tinylated GS-I B_4 and biotinylated RCA-I lectins after removal of the epoxy resin and osmium tetroxide. Both lectins, which have specific affinity for terminal α-D-galactosyl residues, were detected around the peripheral vacuolar membrane in eccrine gland cells (Fig. 3A, B). This indicated that most of the globotriaosylceramide had been extracted, but that glycosphingolipid retained at peripheral sites expressed α-D-galactosyl moieties.

Electron microscopy showed that all the specimens revealed numerous pleomorphic lysosomal deposits with a concentric lamellar structure in capillary endothelial cells and eccrine gland cells (Fig. 4A, B). To analyze the characteristics of these lysosomal deposits ultrastructurally, we performed enzyme digestion experiments with α-galactosidase and β-galactosidase combined with histochemistry using three lectins; GS-I B_4, RCA-I and ConA. Specific affinities of lectins have been described by Goldstein and Hayes [14]. The results of this series of experiments are summarized in Table 2, and Fig. 1 shows a brief schema representing a relationship between enzyme digestion and lectin histochemistry. Before enzyme digestion, lysosomal deposits were stained with GS-I B_4 and RCA-I (Figs. 5A, 6A), which have affinity for terminal α-D-galactosyl moieties and α/β-D-galactosyl moieties, respectively. After initial enzyme digestion with α-galactosidase, which hydrolyzes the terminal α-binding galactosyl moiety, GS-I B_4 lectin did not stain any cytoplasmic material (Fig. 5B), but RCA-I lectin still recognized the lysosomal deposits (Fig. 6B). After sequential β-galactosidase digestion, which hydrolyzes the terminal β-binding galactosyl residue, colloidal gold labeling of RCA-I was markedly reduced (Fig. 6C) and GS-I B_4 staining pattern was the same as that after initial digestion (Fig. 5B, C). This indicated that there were no galactosyl residues in the deposits owing to hydrolysis by the two enzymes (Fig. 1). Staining with ConA lectin, which can recognize the terminal glucosyl moiety, was markedly increased after sequential digestion (Fig. 7A–C). Based on current knowledge, these results suggest that the sugar chain structure in the lysosome deposits in Fabry disease might be "Gal α→Gal β→Glc" and are in agreement with earlier results obtained from various biochemical studies. The results of several control experiments also supported this proposed sugar sequence. The present experiment revealed that osmium post-fixation preserves lysosomal globotriaosylceramide well. In addition, since ultrathin sections of Epon-embedded specimens without osmium post-fixation showed many amorphous lysosomal deposits, but GS-I B_4 and RCA-I lectins did not identify specific sugar residues in the deposits, it was also ascertained that osmium tetroxide is essential for the preservation of glycolipid in lysosomes.

In conclusion, we have demonstrated the expression of sugar residues in the lysosomal deposits of Fabry disease, and clarified the chemical nature of the sugar residues. We believe that the present procedures using Epon-embedded specimen would be useful for the further histochemical analyses of glycolipid and glycoprotein storage diseases, in addition to providing practical support in diagnosis.

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