LYSOSOMAL GLYCOGEN STORAGE DISEASE WITHOUT ACID MALTASE DEFICIENCY—A LECTIN-HISTOCHEMICAL STUDY OF AN UNUSUAL TYPE OF LYSOSOMAL GLYCOGEN STORAGE DISEASE

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Lectin histochemical studies were performed on frozen sections of biopsied skeletal muscle from two unrelated patients with lysosomal glycogen storage disease without acid maltase deficiency. The clinical manifestations of these patients, myopathological features and biochemical characteristics are similar to those first reported by Danon et al. in 1981. Routine myopathological findings showed vacuolar myopathy and excess of glycogen with or without glycogenosomes resembling acid maltase deficiency (AMD), but the biochemical activity of acid α-glucosidase was normal.

Ten lectins with varying sugar specificities were used as probes. The results were compared with normal muscle and with one patient who had the typical manifestations of adult onset AMD. Though myofibers themselves were never stained in normal muscle, positively stained myofibers with or without vacuolations were found in diseased muscle. Vacuolar membranes and their contents were strongly stained by almost all the lectins applied. Ulex europaeus agglutinin-I (UEA-I) specific to L-fucose stained the perimeter of the disturbed muscle in addition to the vacuoles.

The staining pattern of the vacuolations and perimeter of disturbed myofibers was different from that found in AMD. In AMD, Triticum vulgaris agglutinin (WGA) and Limax flavus agglutinin (LFA) faintly stained the vacuolar membranes and contents. These preliminary investigations of abnormal lectin staining patterns may provide a key to understanding the pathomechanism of this unique lysosomal glycogen storage disease.

Lysosomal glycogen storage (glycogenosome) is a characteristic pathological feature recognized in acid maltase deficiency (AMD), which is caused by a defect of acid α-glucosidase, a lysosomal enzyme completely hydrolyzing 1,4- and 1,6-linked α-D-glucose polymers (20). In 1981, however, Danon et al. reported two unrelated patients whose biopsied skeletal muscle showed lysosomal glycogen storage resembling AMD, but whose acid α-glucosidase activity was normal (14). The clinical manifestations were childhood-onset mental retardation, hypertrophic cardiomyopathy, and proximal myopathy. Similar syndromes have been reported later by several investigators (9, 19, 28, 30). Histochemical study of biopsied skeletal muscle showed many vacuolations in myofibers, and electron microscopic study revealed accumulations of free and membrane-bound glycogens. The specific biochemical defect of this syndrome is not yet known.

As lectins are useful cytochemical markers for structures containing carbohydrate residues, they would become very powerful cytochemical probes in the search for membrane organizations and stored materials which carry carbohydrate residues. Accumulation of glycoconjugates in lysosomes in various storage diseases could thus be visualized (1, 13).

We thus applied lectin histochemistry to frozen...
sections of biopsied skeletal muscle from this lysosomal glycogen storage disease, and we report on the abnormal lectin staining patterns in membranes and stored materials.

**MATERIALS AND METHODS**

**Source of tissues:**
Biopsy specimens were obtained from two unrelated patients, a 21-year-old man (case 1) and a 23-year-old man (case 2), who had lysosomal glycogen storage disease without acid maltase deficiency. In both cases, the diagnosis was established through clinical manifestations (mental retardation, cardiomyopathy and muscular weakness), biochemical studies (normal acid α-glucosidase activity) and morphological findings (excess of free and membrane-bound glycogen) (24).

An AMD muscle was obtained from a 34-year-old women, whose diagnosis was established on the basis of laboratory and morphological investigations. Control muscle specimens were obtained through diagnostic biopsy from three patients who were deemed to be free of muscle disorders.

**Tissue preparation:**
A biopsy was obtained from the right quadriceps under local anesthesia. Portions of the tissues were quick-frozen in liquid nitrogen-cooled isopentane. Unfixed transverse cryostat sections (8 μm) were prepared using Reichert Frigocut N. After rehydrating in Dulbecco's phosphate-buffered saline (PBS) (15), the tissues were dipped in 0.3% hydrogen peroxide in pure methanol for 20 min to inhibit the activity of endogenous peroxidase, washed briefly in PBS, and then rinsed in 1% bovine serum albumin (BSA) in PBS for 10 min. Subsequently, the sections were incubated with the appropriate horse-radish peroxidase (HRP) directly labeled lectin solutions diluted at a concentration of 25 μg/ml with PBS for 60 min. The lectins used in this study were *Maclura pomifera* agglutinin (MPA), *Arachis hypogaea* agglutinin (PNA), *Ricinus communis* agglutinin-I (RCA-I), *Glycine max* agglutinin (SBA), *Helix pomatia* agglutinin (HPA), *Dolichos biflorus* agglutinin (DBA), *Triticum vulgaris* agglutinin (WGA), *Limax flavus* agglutinin (LFA) and *Ulex europaeus* agglutinin I (UEA-I). All these lectins were purchased from E-Y laboratories (San Mateo, CA). With *Canavalia ensiformis* agglutinin (Con A), we used the indirect method. After rehydration, rinsing with 1% BSA in PBS, and washing with PBS, the sections were incubated in biotinylated Con A (also from E-Y) solution for 60 min. After washing briefly in PBS, the sections were dipped in 1% BSA in PBS for 10 min, and incubated with streptavidin-HRP (purchased from Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD) solution for 30 min. After further rinsing in PBS, the sections were incubated in the diaminobenzidine (DAB) hydrogen-peroxide medium, pH 7.6, as described by Graham and Karnovsky (18). Finally, with or without hematoxylin-counter staining, the sections were dehydrated, cleared, and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ). Sections were examined with an Olympus Photomicrography BH2-RFCA equipped with Nomarski interference optics. All these procedures were performed at room temperature.

As a control, the HRP-labeled lectins and the

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Source</th>
<th>Specificity</th>
<th>Haptenic sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td><em>Canavalia ensiformis</em></td>
<td>α-D-Man, α-D-Glc</td>
<td>α-D-Man</td>
</tr>
<tr>
<td>MPA</td>
<td><em>Maclura pomifera</em></td>
<td>α-D-Gal</td>
<td>α-D-Gal</td>
</tr>
<tr>
<td>PNA</td>
<td><em>Arachis hypogaea</em></td>
<td>β-D-Gal(1 → 3)-D-GalNAc</td>
<td>β-D-Gal</td>
</tr>
<tr>
<td>RCA-I</td>
<td><em>Ricinus communis</em> I</td>
<td>β-D-Gal</td>
<td>β-D-Gal</td>
</tr>
<tr>
<td>SBA</td>
<td><em>Glycine max</em></td>
<td>α-GalNAc, α-α-D-Gal</td>
<td>GalNAc</td>
</tr>
<tr>
<td>HPA</td>
<td><em>Helix pomatia</em></td>
<td>α-GalNAc</td>
<td>GalNAc</td>
</tr>
<tr>
<td>DBA</td>
<td><em>Dolichos biflorus</em></td>
<td>α-GalNAc</td>
<td>GalNAc</td>
</tr>
<tr>
<td>WGA</td>
<td><em>Triticum vulgaris</em></td>
<td>β-D-GlcNAc, α-NeuNAc</td>
<td>GlcNAc</td>
</tr>
<tr>
<td>LFA</td>
<td><em>Limax flavus</em></td>
<td>α-NeuNAc</td>
<td>NeuNAc</td>
</tr>
<tr>
<td>UEA-I</td>
<td><em>Ulex europaeus</em> I</td>
<td>α-L-Fuc</td>
<td>L-fucose</td>
</tr>
</tbody>
</table>

**Table 1. Lectins Used in This Study and Their Specificities**

Abbreviation used:
Man; mannose, Gal; galactose, Glc; glucose, Fuc; fucose, GlcNAc; N-acetyl-glucosamine, GalNAc; N-acetyl-galactosamine, NeuNAc; N-acetyl-neuraminic acid (sialic acid)
unusual lysosomal glycogen storage disease

biotinylated lectin were preincubated with their haptenic sugars (0.2M D-galactose [Gal] for MPA, SBA and RCA-I, 0.2M N-acetyl-galactosamine [GalNAc] for HPA, and 0.2M N-acetyl-glucosamine [GlcNAc] for WGA, 0.3M α-methylmannoside for Con A, for 30 min, and then applied onto the sections. A matching set of tissue sections incubated with PBS alone served as nonspecific negative controls. The lectins, major carbohydrate specificities of each lectin, and the binding inhibitors used in this study are listed in Table 1. The sections were evaluated in a blind manner for staining intensity with each lectin. Staining intensity was graded as very strong (+++), strong (++), moderate (+), slight (±), or negative (−) by three persons.

RESULTS

The lectin binding patterns and intensities in normal muscle, AMD and glycogen storage disease without acid maltase deficiency are summarized in Table 2. In sections from all three types of normal muscle, Con A, MPA, PNA, RCA-I, SBA, WGA and LFA consistently produced similar patterns of staining although intensities varied. These staining patterns corresponded with previous findings (11, 26). With all lectins, myofiber peripheries were clearly delineated and endo- and perimysial connective tissues

Table 2. Summary of Lectin Histochemical Study

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Myofibers</th>
<th>Connective Tissues</th>
<th>Perimeter of Myofibers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>AMD</td>
</tr>
<tr>
<td>ConA</td>
<td>±</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>MPA</td>
<td>±</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>PNA</td>
<td>±</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>RCA-I</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>SBA</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>HPA</td>
<td>±</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>DBA</td>
<td>±</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>LFA</td>
<td>±</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>UEA-I</td>
<td>±</td>
<td>±</td>
<td>−</td>
</tr>
</tbody>
</table>

Abbreviation used: 1; Case 1, 2; Case 2, AMD; Acid Maltase Deficiency, N; Normal

Extent of staining: ++; very strong reaction, +++; strong reaction, ++; moderate reaction, ±; slight reaction, −; negative, nd; not detected

Table 3. Summary of Lectin Histochemical Study—continued—

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Vacular Membrane</th>
<th>Vacular Content</th>
<th>Blood Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>AMD</td>
</tr>
<tr>
<td>ConA</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>MPA</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>PNA</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>RCA-I</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>SBA</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>HPA</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>DBA</td>
<td>±</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>WGA</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>LFA</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>UEA-I</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>
Figs. 1A–C. Light microphotographs.

1A. Case 1, 1B. Case 2, 1C. Acid Maltase Deficiency (AMD)

Cryostat sections stained with PNA-HRP. Each section shows cytoplasmic vacuolations. Note that the vacuolar membrane and vacuolar contents (arrows) are stained in case 1 (1A) and case 2 (1B). In AMD, vacuolations (arrow heads) are not stained with this lectin at all. (1C). ×410  Bar=25 μm.
FIGS. 2A–C. Light microphotographs.

2A. Case 1, 2B. Case 2, 2C. AMD

Cryostat sections stained with WGA-HRP. Note that the cytoplasmic vacuolations (arrows) are heavily stained in case 1 (2A) and case 2 (2B). Weakly positive structures (arrow heads) can be seen in AMD (2C). ×410  Bar=25 μm.
FIGS. 3A-C. Light microphotographs.

3A. Case 1, 3B. Case 2, 3C. AMD
Cryostat sections stained with UEA-I. Each section shows UEA-I-HRP stained blood capillaries and blood vessels (small arrows). Note that the perimeters of myofibers with vacuolations (arrows) are stained with this lectin in case 1 (3A) and case 2 (3B). In AMD, vacuolations (arrow heads) and periphery of myofibers are not stained at all (3C). ×410  Bar=25 μm.
were always positively stained. Blood vessels and capillaries were also labeled. No myofiber staining was observed in normal muscle. DBA and UEA-I gave different staining patterns in normal muscle. In DBA reacted sections, the myofibers themselves were not stained by the lectin, but there was a slight staining associated with the peri- and endomysial areas of connective tissue, blood vessels and capillaries. The staining of myonuclei by DBA observed by Pena et al. (26) was not observed. In UEA-I reacted sections, though perimysial and endomysial areas of connective tissues were almost all negative, blood vessels and capillaries were strongly stained.

Abnormal lectin binding patterns from patients with lysosomal glycogen storage disease without acid maltase deficiency are also summarized in Table 2. In both specimens from this disease almost all the lectins stained the vacuolar membranes and their contents (Figs. 1A-3A, 1B-3B). UEA-I, which binds specifically to α-L-fucose, stained the perimeter of the disturbed muscle in addition to the vacuoles (Figs. 3A, 3B). These staining patterns were quite different from those of AMD. Most of the lectins did not stain in AMD muscle (Figs. 1C, 3C). LFA and WGA (Fig. 2C) stained small vacuolations weakly; larger vacuoles were not stained at all.

Granular staining of the interior of some diseased myofibers was revealed by UEA-I, WGA (Fig. 2A) and LFA, making a clear contrast with normal myofibers, where no staining of the interior was observed. Extensive staining in both the perimysial and endomysial areas was demonstrated in all diseased specimens studied by Con A, PNA, HPA, RCA-I and WGA but the excess of staining in connective tissues was more prominent in sections from AMD. Muscle from AMD showed variation in fiber size.

Incubation of each lectin with its corresponding binding inhibitor completely blocked the binding of the lectin to tissue sections. Incubation with PBS alone resulted in no nonspecific staining. Dunn et al. (16) have reported positively stained structures in individual isolated cells within the perimysial area with a positive reaction to DAB alone. In the present study, we did not encounter such staining.

DISCUSSION

In the present study, we investigated lectin binding patterns in frozen sections of biopsied skeletal muscle from a rare vacuolar myopathy, lysosomal glycogen storage disease without acid maltase deficiency, which was first reported by Danon et al. (14). The histopathology of muscle in this disease resembles AMD, but acid α-glucosidase is normal. No specific biochemical defect has yet been identified.

Electron microscopic studies of biopsied skeletal muscle revealed the presence of membrane-bound glycogen deposits (i.e. glycogenosomes) in muscle cells (9, 14, 19, 24, 28, 30). Intralysosomal glycogen storage has been regarded as a valuable pathological finding in the diagnosis of AMD (6). Most cases of glycogen storage disease which show intralysosomal glycogen storage are AMD, although a few glycogenosomes have been reported in debrancher deficiency, Anderson’s disease and muscle phosphorylase b kinase deficiency (22, 23). The acid α-glucosidase activity in this disease was normal with both an artificial and a natural substrate. Debrancher activity and phosphorylase activity were also normal (24). Other glycolytic enzyme deficiencies were excluded in other reports (9, 14, 30). In order to explain the presence of glycogenosomes in this disease, we must search for an unknown specific biochemical defect.

Some reports have described the application of lectin histochemistry to normal and pathological skeletal muscles at both the light microscopic (11, 16, 25, 26) and electron microscopic levels (7, 8, 10, 11). These previous studies and our present studies demonstrate that in normal muscle, PNA and RCA-I specific to β-D-galactosyl residues, and Con A specific to α-D-mannosyl residues stained the surface of the muscle fibers, perimysial and endomysial connective tissue, and capillaries. WGA specific to β-D-N-acetyl-glucosamine, MPA specific to α-D-galactosyl residues and LFA specific to sialic acid exhibited similar patterns of staining for myofibers, while the others showed slightly different staining patterns in surrounding connective tissues. In contrast, UEA-I specific to α-L-fucose, and DBA specific to α-N-acetyl-galactosamine gave different staining patterns from those shown above. They did not stain the perimeter of the muscle fibers. UEA-I stained vascular endothelium strongly. These observations reconfirm previous reports.

Our study demonstrated the specific lectin binding pattern of lysosomal glycogen storage disease without acid maltase deficiency. The vacuolar membranes and contents were stained by almost all the lectins we applied, whereas vacuoles in AMD were stained only by LFA, WGA, MPA and SBA, and the staining was very weak. Interestingly, UEA-I, which had been thought not to bind to muscle fibers, stained the perimeter of disturbed muscles. These characteristic lectin binding patterns indicate the specific composition of the cytoplasmic membrane, vacuolar membrane and its contents.
Recently, lectin histochemistry has been used successfully for diagnosis of three glycoprotein storage diseases: α-mannosidosis, α-fucosidosis and sialidosis (1, 21). It has also been used for further characterization of various human and animal glycolipid storage diseases (2-5, 12, 17, 31, 32). In all cases, different patterns of lectin binding were seen for different diseases and for the same disease in different species. The unique lectin-binding patterns seen with specific lysosomal storage diseases make it possible to use this technique in the diagnosis of these diseases (1, 13).

The results of this investigation suggest that the stored material in biopsied skeletal muscle with glycogen storage disease without acid maltase deficiency has terminal moieties consisting of β-galactose, N-acetyl-neuraminic acid, and N-acetyl-galactosamine, α-fucose and neuraminic acid in the cytoplasmic vacuoles in addition to the glycogen (13). UEA-I stained the perimeter of disturbed muscle in addition to the vacuoles. These staining patterns have not been reported previously.

Duchenne muscular dystrophy (DMD) rarely exhibits positively stained vacuoles with WGA (16). In DMD, the perimysial and endomysial connective tissues were heavily stained with various lectins reflecting the increase of connective tissue. These findings are different from those of our patients with lysosomal glycogen storage disease without acid maltase deficiency. In the present study, vacuolar contents were also stained with WGA in AMD. Recently, Sakai et al. reported that ferritin-conjugated WGA labeled the autophagic vacuoles in mouse hepatocytes (29). It is conceivable that lysosomal glycoproteins are one source of the WGA-HRP positive materials. According to UEA-I lectin staining, the cytoplasmic membrane is also assumed to be disturbed in this disease. The segregating membranes of autophagic vacuoles have most often been assumed to arise from pre-existing membranous structures of the cell. The cytoplasmic membrane could be a source of the autophagic vacuole membrane like the cell organelle membranes (27). In this disease, a wide variety of lectin affinity in vacuolations was observed. The disturbed cytoplasmic membrane may provide an explanation for this phenomenon.

Further biochemical and electron microscopic lectin cytochemical investigations are needed to find out the composition of carbohydrates and proteins in the cytoplasmic membrane, vacuolar membrane and its contents.

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