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"Reducing body"-like inclusions in skeletal muscle in childhood-onset acid maltase deficiency

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Summary. Unusual inclusions with some of the features of "reducing bodies" were encountered in the skeletal muscle biopsy of a 2.5-year-old boy with childhood-onset acid maltase deficiency. The biopsy revealed a vacuolar myopathy with lysosomal storage of glycogen and eosinophilic refractile inclusions in myofibers, which appeared dark blue with the menadione-nitroblue tetrazolium reaction. The significance of the association of inclusions with reducing properties in the setting of acid maltase deficiency is discussed.

Key words: Muscle – Ultrastructure – Lysosome – Metabolic disorder – Glycogenosis

In 1972, Brooke and Neville [1] described two patients with a congenital myopathy characterized by unusual structures within skeletal muscle fibers, which they termed "reducing bodies". Since that time, there have been cases reported of this entity in the literature [3–6, 8–11, 14]. In the original descriptions of this entity by Brooke and Neville, the authors allude to and illustrate membrane-bound glycogen as part of the pathology [1]. In the present report we describe unusual structures with many of the features of reducing bodies in skeletal muscle in a patient with childhood-onset acid maltase deficiency (AMD) and address the significance of this association.

Case report

This 2.5-year-old boy is the fifth child of healthy nonconsanguinous French-Canadian parents. The pregnancy was uncomplicated and the delivery was by elective Cesarean section (performed because of prior Cesarean) at 38 weeks gestation. The birth weight was 2640 g. Apart from mild hyperbilirubinemia there were no postnatal problems. The infant's general health was good except for an episode of severe bronchiolitis at 6 months of age which required hospital admission for 1 month with steroid therapy. There had been no physical findings suggestive of heart failure.

His parents had been concerned about gross motor development from early infancy. The patient was felt to have been weaker than his siblings. He sat by 13 months and began walking unaided at 17 months. His gait, however, was unsteady and awkward. He had difficulty walking upstairs and fatigued easily. He developed a pincer grasp by 10 months and by 14 months was able to use a spoon expertly to feed himself. His first words were spoken at 10 months and he was speaking in phrases before the age of 2 years. Fine motor and speech skills were, thus, felt to be appropriate for age. He has continued to acquire new skills. He was noted also to have decreased appetite by the age of 2 years. In summary, developmental progress except for gross motor milestones was within normal limits for age.

The patient has two living sisters aged 6 and 12 years who are well and in particular have no neuromuscular problems. Of the two other siblings, there was one female stillbirth and one female infant who died in the newborn period of presumed pneumonia. There is no other known history of neuromuscular disease.

Examination at 2 years and 10 months revealed a small but generally well-appearing boy. The weight was at the 3rd percentile, standing height below the 3rd percentile, and the occipitofrontal circumference at the 2nd percentile. There was no facial dysmorphism nor any evidence of facial myopathy. The muscle bulk and tone were diminished. The patient had a waddling gait and a positive Gower's sign. There was generalized moderate muscle weakness, the proximal groups being more severely affected, with power being 3–4/5. Deep tendon reflexes were all hypoactive and the plantar responses were downgoing. Cranial nerve examination, indirect ophthalmoscopy, and the rest of the neurological examination were within normal limits.

General examination revealed normal cardiovascular and respiratory function with a regular pulse rate of 72/min. There were no abnormal heart sounds or murmurs. The only notable abnormality on general systemic examination was that the liver was palpable 2 cm below the right costal margin and abnormally firm.

Normal investigations included a hemogram, blood film, venous acid-base, serum electrolytes, urea and creatinine, blood glucose, thyroid function tests, serum bilirubin and γGT, serum ammonia, serum lactate, plasma total and free carnitine, urine amino and organic acid screens, and urine acylcarnitines both before and after an oral carnitine load (100 mg/kg). Urine mucopolysaccharide and oligosaccharide screens were normal. The serum AST was 193 U/l (normal < 45), the serum ALT was 184 U/l, (normal < 40), and the serum creatine kinase was 653 U/l (normal < 255). Leukocyte acid α-glucosidase was 19.7 nmol/mg per h (normal range 11.3–55.5).

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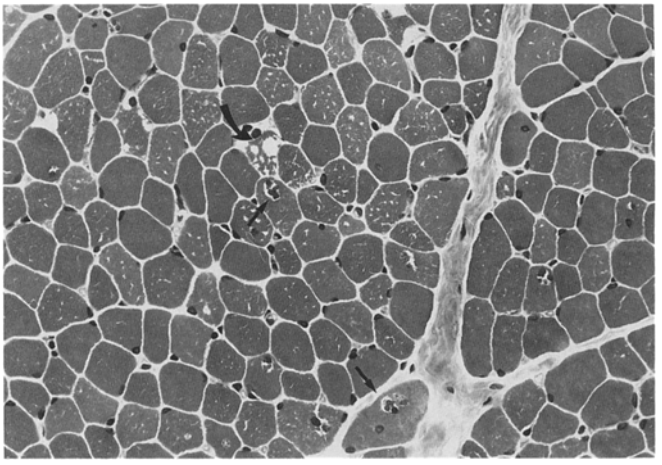

Fig. 1. Light micrograph of skeletal muscle revealing eosinophilic inclusions within empty spaces in several myofibers (*straight arrows*) and a rare vacuolated myofiber (*curved arrow*), H&E, × 225

A chest X-ray revealed normal heart contour and pulmonary vascularity. A computerized tomographic scan of the head was normal. Abdominal ultrasound revealed no abnormalities of the liver, spleen, pancreas and kidneys. Nerve conduction studies of the left posterior tibial, left peroneal and left median motor nerves and electromyography of the left tibialis anterior, gastrocnemius, quadriceps femoris, triceps, and biceps muscles were within normal limits.

A biopsy of skeletal muscle was obtained from the right quadriceps muscle at 26 months of age. A closed liver biopsy was performed at 31 months of age.

Pathology

Muscle biopsy

Methods. The muscle biopsy specimen was collected and processed for enzyme histochemistry, conventional histology, and electron microscopy as described elsewhere [15]. For enzyme histochemistry, the specimen was snap frozen in isopentane cooled to 160 °C in liquid nitrogen. The frozen block was oriented in cross section and mounted on a cryostat chuck and sections were cut at 7-μm intervals. The following stains were performed [15]: adenosine triphosphatase (ATPase at pH 9.4, 4.6, 4.3), succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), phosphorylase, acid phosphatase, hematoxylin-eosin (H&E), periodic acid Schiff (PAS), Oil red O, menadione-nitroblue tetrazolium (M-NBT) and modified Gomori trichrome. For paraffin sections, muscle was fixed in Bouin's fixative and after processing, 3-μm sections were cut and stained with H&E, phosphotungstic acid/hematoxylin (PTAH), Masson trichrome, and PAS stains. The following immunostains were performed by the avidin-biotin-complex or peroxidase-antiperoxidase techniques [2]: desmin (1:20, monoclonal, Dako), myosin (1:300, monoclonal, Sigma), actin (1:1000, monoclonal, Enzo), α-actinin (1:200, monoclonal, Sigma), ubiquitin (1:100, polyclonal, Dako). A histogram was generated for fiber sizes and ratios for type 1 and type 2 fibers utilizing the Nuvision image analyzer system. For electron microscopy, tissue was fixed in

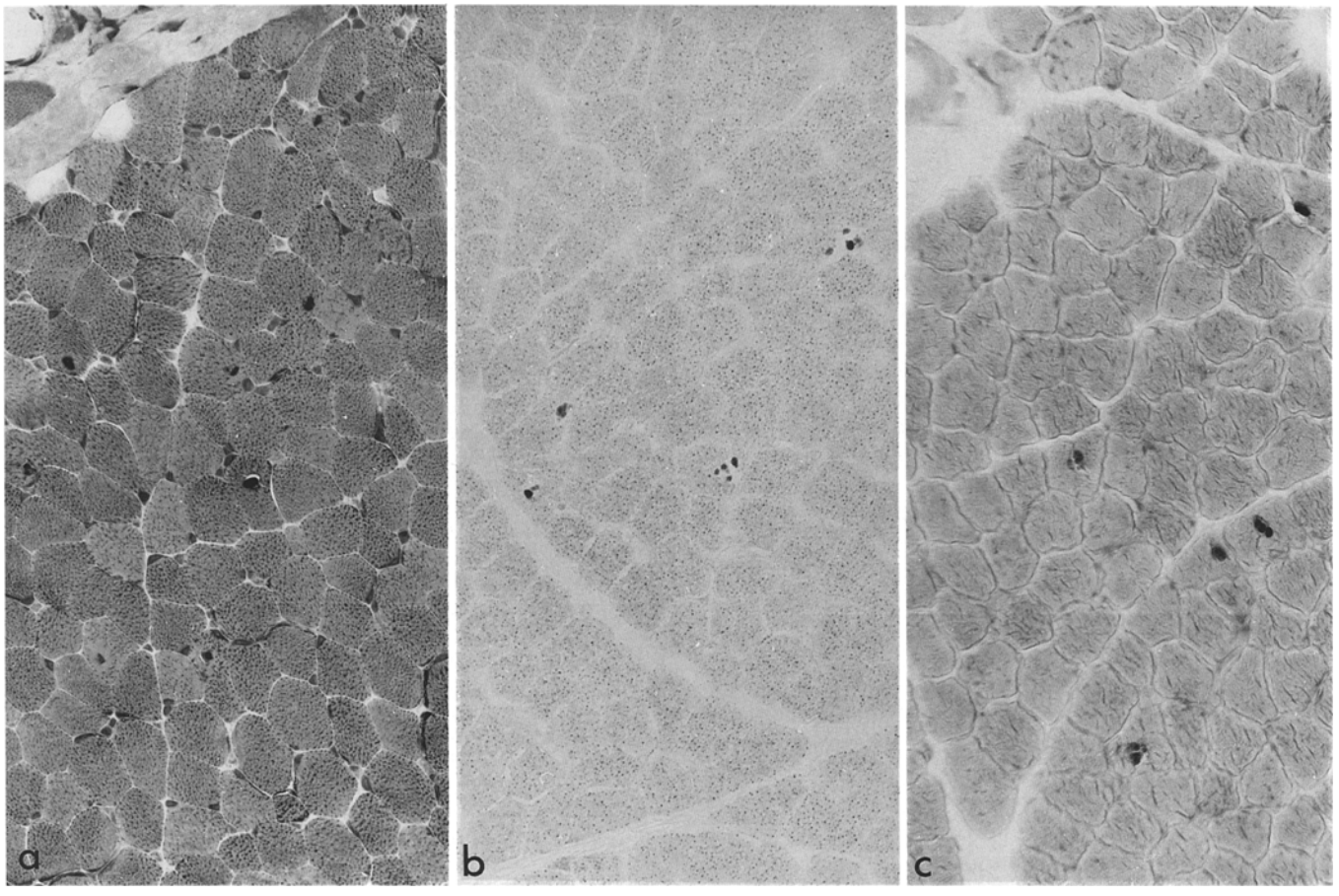

Fig. 2. Inclusions within myofibers are highlighted by the modified Gomori trichrome (**a**), menadione-nitroblue tetrazolium (**b**), and acid phosphatase (**c**) reactions. **a–c** × 280

the Universal fixative (mixture of 4 % formaldehyde – 1 % glutaraldehyde prepared in 200 mOsm phosphate buffer), postfixed in osmium tetroxide, and stained with uranyl acetate, and the thin sections were examined in a Phillips 400 microscope.

Pathological findings. Transverse and longitudinal sections of frozen and paraffin-embedded muscle revealed randomly scattered atrophic fibers with no tendency to grouping. Nuclei were peripherally placed. There was no increase in endomysial or perimysial connective tissue and no evidence of inflammation or vasculitis.

There were three notable alterations on the H&E-stained frozen and paraffin sections. First, the most striking finding was that of eosinophilic refractile oval, circular, or rod-like inclusions with several myofibers often surrounded by an empty space (Fig. 1). The inclusions were single or multiple, usually within the myofibers with no significant subsarcolemmal or perinuclear predilection, although some inclusions were encountered in the vicinity of the nucleus. The inclusions appeared to lie within apparently normal fibers or were associated in some fibers with vacuoles containing bluish granules (see next paragraph). Often, they were surrounded by a clear space. The inclusions appeared pink on H&E, red on modified Gomori trichrome (Fig. 2), red with Masson trichrome, blue with PTAH, dark blue with the M-NBT (Fig. 2) and green with Verhoeff-vanGieson. Acid phosphatase activity was demonstrable within the inclusions (Fig. 2). There was a rim of PAS positivity surrounding the inclusions, which themselves were unstained. The inclusions remained unstained in the following reactions: SDH, LDH, ATPase, phosphorylase, Oil red O, methyl green pyronin, and Congo red. There was type 1 fiber predominance (63 % type 1 fibers) with inclusions demonstrable in both fiber types. The atrophic fibers belonged to both fiber types. The inclusions exhibited faint green autofluorescence and showed prominent apple green fluorescence with acridine orange. Immunostaining for myosin, actin, α-actinin, and desmin revealed no immunoreactivity within the inclusions. On the resin sections, the inclusions appeared bluish and were surrounded by a clear space.

Second, also present in myofibers with or without inclusions were vacuoles with basophilic granules having the appearance of "rimmed vacuoles". Many of these vacuolar spaces contained one or more of the eosinophilic inclusions described above. The granules were associated with acid phosphatase activity. Rare granules were also positive for α-actinin by immunostaining. Ubiquitin immunoreactivity was seen within the granules and surrounding the inclusions which in themselves remained unstained. Other reactions were noncontributory.

The third notable feature was the scattering throughout the muscle of vacuolated (Fig. 1) and occasional degenerating myofibers. The former were randomly dispersed with no preferential location in any portion of the fascicle and represented less than 10 % of myofibers. The vacuolation was apparent on both frozen, paraffin, and resin sections and replaced normal myofibrillar elements. There was no significant glycogen accumulation in most fibers by the PAS reaction.

Electron microscopy

Inclusions. Electron microscopy revealed most of the large inclusions to be composed of intensely dense granular or clumped osmiophilic material, with or without a limiting membrane (Fig. 3). The inclusions were surrounded by particulate glycogen which was often membrane bound. Some inclusions contained lamellar leaflets and rarely, fingerprint-like profiles. In some inclusions, glycogen occurred within the osmiophilic material. No filamentous structures were seen within the inclusions. Examination of a large number of inclusions revealed no association with the nucleus and the morphology was distinct from that of the nuclear chromatin or nucleolus.

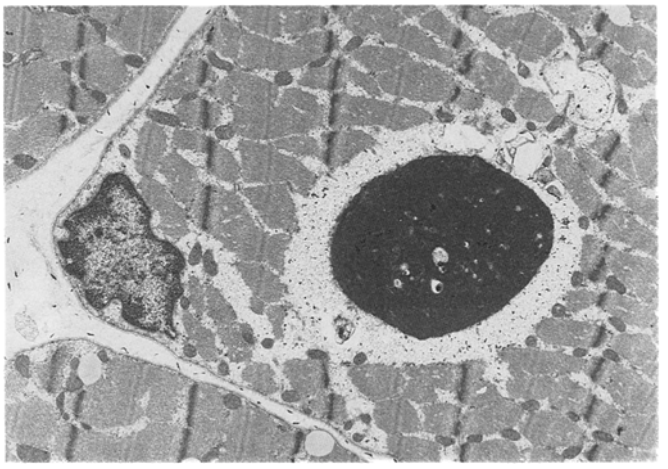

Fig. 3. Electron micrograph of skeletal muscle reveals an intracytoplasmic inclusion containing dense clumped osmiophilic material. × 6840

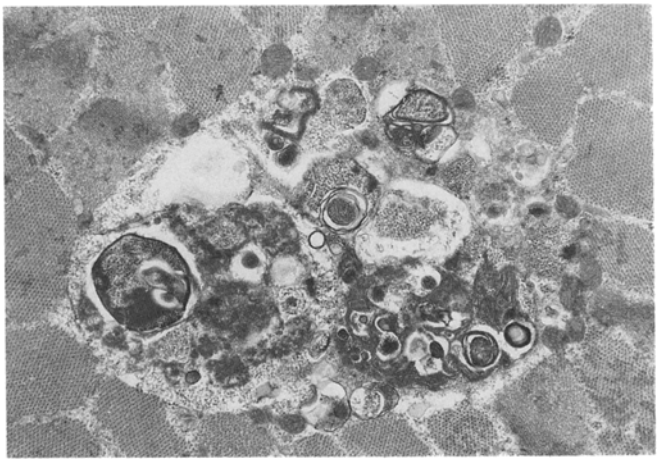

Fig. 4. Electron micrograph demonstrating membrane-bound glycogen within the myofiber. × 15200

Basophilic granules. The basophilic granules appeared as membrane-bound dense clumped or granular osmiophilic material or myelinoid bodies associated with glycogen accumulation. Autophagic vacuoles and secondary lysosomes were common.

Glycogen accumulation. Both nonvacuolated and vacuolated fibers revealed predominantly membrane-bound glycogen as well as some intermyofibrillar free glycogen (Fig. 4). Many myofibers were spared and revealed no excess glycogen.

Vacuolated fibers. This change affected scattered fibers to various degrees. Some fibers revealed vacuoles which contained membrane-bound and free glycogen with myofibrillar elements still identifiable. Other fibers showed almost complete replacement by empty spaces, some of which contained glycogen (Fig. 5).

Other nonspecific findings included a cluster of concentric laminated bodies which appeared as cylindric structures some of which enclosed glycogen [6].

Liver biopsy

The liver biopsy was normal by light microscopy. Electron microscopy revealed increased numbers of glycogen-containing lysosomes, consistent with a glycogen storage disorder, but the appearance was not typical of infantile Pompe's disease. Many of the glycogen-containing lysosomes had lamellar myelin-like profiles.

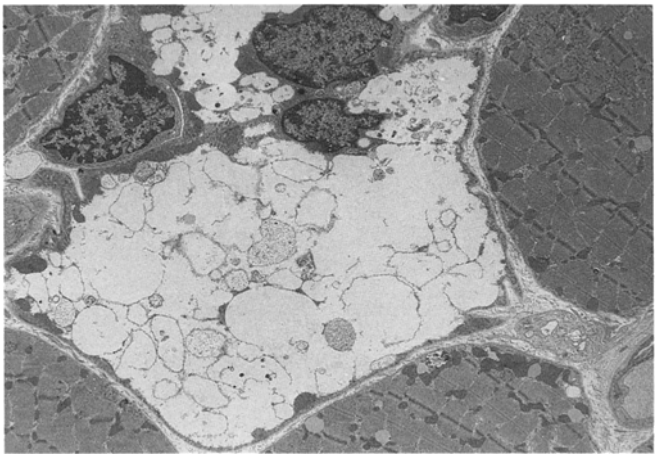

Fig. 5. Electron micrograph demonstrating the vacuolated myofiber containing membrane-bound glycogen as well as virtual replacement of myofibrillary components. × 3230

There was an increase in smooth endoplasmic reticulum. Peroxisomes were normal in size and frequency. No other abnormal inclusions were observed, in particular, the unusual inclusions seen in the muscle biopsy.

Because of the skeletal myopathy, hepatomegaly, and abnormal ultrastructural findings, a skin biopsy was done to assay acid α-glucosidase in cultured fibroblasts. The activity of acid α-glucosidase measured in cultured skin fibroblasts was 16% of controls at pH 4.0 (3.4 nmol/min per mg, normal range 20.4 ± 2.3; $n = 50$) and 67% of controls at pH 6.7 (4.4 nmol/min per mg, normal range 6.56 ± 0.56; $n = 50$). Cultured fibroblasts were also assayed for activity of the phosphorylase kinase system which was normal (tests kindly performed by Dr. Y. T. Chen of Duke University, Durham, North Carolina).

Discussion

With a clinical presentation of progressive myopathy and hepatomegaly, biochemical documentation of low acid α-glucosidase activity in cultured skin fibroblasts, and a vacuolar myopathy with lysosomal glycogen storage in the skeletal muscle and liver, this 2.5-year-old child best fits the childhood-onset type of acid maltase deficiency (AMD) or glycogenosis II [7]. The infantile form (generalized AMD) is associated with lysosomal glycogen accumulation in various tissues, especially the heart, skeletal muscle and the central nervous system and has a poor prognosis with death usually by 2 years. The clinical course for the childhood- and adult-onset forms (the muscular forms) is quite heterogeneous. Childhood-onset AMD usually presents as a slowly progressive proximal limb weakness as in this patient. The α-glucosidase activity is virtually absent in patients with the generalized form, whereas residual activity of 1.5% to 26% is found in other forms of AMD in the liver, muscle, or cultured skin fibroblasts, while in mixed leukocyte assays, enzyme activity may be normal [7, 12] as was seen in the case reported here.

In contrast to infantile Pompe's disease, which is characterized by extensive vacuolation of muscle, the late-onset AMD cases reveal a spectrum of pathological severity and the extent and degree of involvement may show variance between different muscle groups. Childhood-onset AMD cases in general, have less severe changes compared to infantile AMD, and in the adult-onset types, the changes may be subtle. From a pathological perspective, the extent of vacuolation and degree of glycogen accumulation in our patient is more in keeping with the milder adult-onset type, with less than 10% fibers affected.

In the present case, the most intriguing finding on muscle biopsy was the presence of unusual eosinophilic refractile cytoplasmic inclusions in a number of myofibers, sometimes in association with rimmed vacuoles. These inclusions had unique staining characteristics, many of which are those of the so-called "reducing bodies" [1, 3–6, 8–11, 14]. In particular, they appeared dark blue with the M-NBT reaction, which has been the diagnostic basis for recognizing "reducing bodies". They were faintly autofluorescent and exhibited bright apple green fluorescence with acridine orange. Both the inclusions and the basophilic granules were acid phosphatase positive. A rim of ubiquitin immunoreactivity was encountered around the inclusions with the cores remaining unstained. Ubiquitin immunoreactivity was also seen within the granules, the latter being clearly lysosomal in nature with an investing unit membrane.

The inclusions in the present case were not immunoreactive for desmin, actin, α-actinin, or myosin. They had no predilection for a perinuclear or subsarcolemmal location, a feature often associated with reducing bodies [1, 3,–6, 8–11, 14]. By electron microscopy, the inclusions were seen to have densely osmiophilic granular or clumped material, with no discernible filamentous components. Rarely, trapped T-triads, sarcoplamic components, and glycogen was identified within inclusions. There was an intimate association of the inclusions with glycogen, which was either membrane bound or occurring in pools surrounding the inclusions, contributing to the clear space observed on the H&E stain. No membrane was seen around some inclusions, while others demonstrated a partial or complete single membrane.

The diagnosis of "reducing bodies" in the reported cases in the literature is based on the M-NBT reaction, as no other structure in normal or pathological muscle is said to be reactive. These structures were termed "reducing bodies" in the original descriptions, as they have a sulfhydryl-containing compound capable of effecting the reduction of NBT when mediated by menadione. On the basis of this staining property, the inclusions in the present case could be regarded as "reducing bodies". It is apparent that , although there are some similarities, the absence of pyroninophilia by the methyl green pyronin stain and the demonstration of an investing membrane by electron microscopy indicates that these are not identical to structures described by Brooke and Neville [1]. It is also apparent from a review of the literature that structures described as "reducing bodies" have varied histochemical and ultrastructural features and have been encountered in patients with a different clinical course, varying from a mild to a fatal myopathy. The myopathy described by Sahgal and

Sahgal [13], is quite distinct morphologically from other descriptions of "reducing bodies".

The pathogenesis of reducing bodies is unclear. Tubular filamentous morphology has been a prominent feature of cases in the report of Carpenter et al. [4], who also described an association with rimmed vacuoles. Based on the pyroninophilia, the reducing bodies were felt to contain RNA. This and the filamentous composition of some reducing bodies has led to speculation of a possible viral etiology. Dubowitz [5] also described a case in which the inclusions revealed tightly packed rounded particles, which to the author suggested a resemblance to the Coxsackie virus [5]. No viral or filamentous particles were observed in the present case. The presence of a limiting membrane in at least some inclusions and acid phosphatase reactivity indicates a lysosomal origin in our case.

In conclusion, we describe a patient with childhood-onset AMD with unique inclusions in skeletal muscle. In a exhaustive literature review of the pathological spectrum of AMD of the infantile, childhood, or adult-onset types and other glycogenoses, we have not encountered descriptions of inclusions with reducing properties. Basophilic granules which are lysosomal in origin and autophagic vacuoles have been described, as encountered also in our case. The heterogeneity in the clinical course and in the pathological descriptions of myopathies with reducing bodies suggests that inclusions with reducing properties may be encountered in a variety of unrelated myopathies. It is of interest that an association with increased glycogen has been observed in some cases of reducing body myopathy [1, 8, 9]. In the report of Brooke and Neville [1] increased glycogen, sometimes membrane bound, is described with some muscle fibers having massive glycogen accumulation with normal elements of muscle replaced by vacuoles, glycogen, and cytoplasmic bodies. Hübner and Pongratz also described vacuolar degeneration in muscle fibers with empty spaces containing glycogen in addition to inclusions with reducing properties [8, 9]. While this glycogen excess may be an incidental finding, the present case indicates that inclusions with reducing properties may be also encountered in the setting of a glycogen-storage disorder.

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