Fucosidosis: ultrastructural study of conjunctiva and skin and enzyme analysis of tears

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Conjunctival and skin biopsies from two new patients with fucosidosis were studied by electron microscopy. In both tissues, the connective tissue cells and the capillary endothelial cells were filled with single membrane limited inclusions of two types: (1) Clear inclusions containing a fibrillogranular reticulum. (2) Dark inclusions with a dense granular material. Specific stainings in ultrastructure suggest that these inclusions contain oligosaccharide chains. The ultrastructural aspect is characteristic for fucosidosis. Enzyme studies on tears realize an easy and secure technique for the diagnosis of the disease.

The interest of conjunctival biopsy in the study of storage diseases is now abundantly documented. Pathologic changes have been demonstrated in the systemic mucopolysaccharidoses, 1 several mucolipidoses, 2-5 Fabry's disease, 6, 7 Niemann-Pick's disease, 8 and cystinosis. 9

The usefulness of tears for the enzymatic screening of inborn lysosomal diseases was recognized about two years ago and applied to Tay-Sachs and Fabry's diseases. 7

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Supported by the Belgian Fonds de la Recherche Scientifique N° 20,126 and N° 20,183, and by the United States Public Health Service (Research Grant N° AM 9235).

Submitted for publication Jan. 6, 1976.

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Most of the lysosomal acid hydrolases can be assayed in tears, and their deficiency allows the diagnosis of several other inborn lysosomal diseases. 10-18

Fucosidosis, an autosomal lysosomal disease, is characterized by the absence or the profound deficiency of α-L-fucosidase, 19-21 which results in the widespread accumulation of fucose-containing glycosphingolipids, oligosaccharides, and polysaccharides, together with an increased excretion of fucosides in urine. 22 Clinically, a severe and a mild phenotype have been distinguished. 23, 24 The patients suffering from the severe form present a progressive psychomotor retardation and a moderate chondrodystrophy somewhat reminiscent of the mucopolysaccharidoses. Death occurs around the age of three to five years in the severe phenotype. Survival to adolescence and beyond has been reported in the mild phenotype, which is characterized by the presence of angiookeratoma corporis diffusa. 25-26 Ocular signs, as thin and
tortuous vessels in the conjunctiva and the fundus have been described in this group.\textsuperscript{26, 27}

No histopathological studies of the eyes have been made, to our knowledge, in fucosidosis. The purpose of the present paper is to describe the ultrastructural abnormalities of the conjunctival and skin biopsies, and the enzymatic findings in tears from two patients suffering from the severe form of fucosidosis, and to stress the importance of these investigations in the diagnosis of the disease.

**Brief case reports**

Case 1 is the younger sister of the patient described by Loeb and co-workers in 1969,\textsuperscript{25} the first patient in whom the deficiency of \(\alpha\)-L-fucosidase was evidenced. Their Italian parents are first cousins; one sibling is unaffected. The diagnosis was made at the age of five months by clinical examination. Their Italian parents are non-consanguineous. At the age of 2.5 years old, somatic and psychomotor development are delayed, but there is no other sign of neurological deterioration. The face has a mild gargoyl aspect. She has a lumbar hyperlordosis and a protruding abdomen with umbilical hernia. The spleen and the liver are slightly enlarged. Corneae and fundi are normal.

Case 2, a girl, is the first child of healthy non-consanguineous Belgian parents. At the age of 2.3 years, the face has a mild gargoyl aspect; the thorax is broad; the spleen and the liver are slightly enlarged. Hypotrophy and psychomotor regression are evident. A spastic paraplegia of the lower legs is noted. X-ray examination shows a delayed skeletal maturation and abnormalities of the lumbar vertebral bodies. In the peripheral blood, 31 per cent of the lymphocytes are vacuolized and foam cells are present in the bone marrow. The diagnosis was first made on typical alterations in conjunctival biopsies and on the absence of \(\alpha\)-L-fucosidase activity in tears. It was later confirmed by liver biopsy and enzymatic studies in leukocytes and plasma. A skin biopsy was also performed for fibroblast culture and ultrastructural study.

**Material and methods**

The conjunctival biopsies were obtained under topical anesthesia with oxybuprocaine hydrochloride (Novesine). They were taken with a smooth jaw forceps and scissors from the upper bulbar conjunctiva. No suture was necessary, phenylephrine and an antibiotic ophthalmic ointment were placed in loco as postoperative care.

The skin biopsies were taken aseptically from the forearm after local anesthesia with an ethyl chloride spray. The specimens were cut with a curved forceps and a scalpel, divided into two fragments for fibroblast culture and ultrastructural study.

The tissue fragments were immediately placed in 4 per cent glutaraldehyde (Millonig buffer, pH 7.4) and divided into smaller fragments. After two hours in the fixative, the tissue was washed in Millonig buffer containing 5.4 per cent glucose. Postfixation was conducted for 30 minutes with 2 per cent osmium tetroxide (Millonig buffer, pH 7.4). After washing, the tissue was dehydrated in ethanol and propylene oxide, and embedded in an epoxy resin (Epon).

Semithin sections were performed in order to orient the ultrathin sections perpendicularly to the epithelial surface. Ultrathin sections were cut with diamond knives on a Reichert Om U 2 microtome and stained as follows: (1) uranyl acetate and lead citrate according to Reynolds;\textsuperscript{29} (2) silver proteinate after periodic acid oxidation according to Thiéry;\textsuperscript{30} this latter reaction reveals vic-glycol groups, thus mainly polysaccharides. Some conjunctival fragments were treated separately, dehydrated in acetone and embedded in Vestopal W. The ultrathin sections were stained with phosphotungstic acid in aqueous solution (10 per cent weight per volume) at pH 1.5.\textsuperscript{31} PTA stains glycoproteins and mucopolysaccharides.

For the colloidal iron staining, the specimen was fixed in 6.25 per cent glutaraldehyde (cacodylate buffer, pH 7.4). After one hour in the fixative, the tissue was washed in cacodylate buffer containing 5.4 per cent glucose. It was then prepared and stained according to Hardin and Spicer.\textsuperscript{32} This technique is specific for acid mucopolysaccharides and sialic acid-rich glycoproteins.

The sections were thereafter coated with a carbon film and examined with a Philips EM 300 electron microscope.

For the enzymatic study, biopsies were immediately frozen. Homogenates were performed in conical sintered glass tissue grinders, in the presence of 99 volumes of bidistilled water, and without addition of detergent. Acid hydrolases were assayed as described earlier,\textsuperscript{20} or with similar techniques using substrates derived of 4-methylumbelliferone. The same techniques were applied to plasma and leukocytes. The latter were isolated either by collection of the buffy coat, after centrifugation of heparinized blood, or by the dextran sedimentation technique.

Tears were collected on strips of Whatman's no. 44 filter paper, which were allowed to dry and kept as such for one to five days in the
Fig. 1. Patient 2. Conjunctival biopsy. Semi-thin section stained with toluidine blue. The epithelium displays an increasing number of cytoplasmic vacuoles from the basal to the apical layer. In the stroma, the capillary endothelial cells and the connective tissue cells also contain many clear vacuoles. *1,000.

Results

Morphology. Biopsies of both patients displayed no evident differences in the extension of the storage process.

Light microscopy. In the conjunctiva, the epithelial cells in the apical layers were distended by numerous clear vacuoles of various sizes, while the basal layer seemed unaffected (Fig. 1). The skin epithelium had a normal aspect. In both conjunctiva and skin biopsies, a severe vacuolation was evident in the endothelial cells of the capillaries. The sweat glands of the skin were also heavily involved (Fig. 2).

Electron microscopy. CONJUNCTIVAL BIOPSY. In the epithelium, small vacuoles were observed in the basal layer. In the adjacent layers, all cells were filled with abnormal inclusions of greater dimension (diameters between 0.3 and 4.0 microns). A marked tendency to confluence was noted in the apical layer (Fig. 3). Goblet cells showed numerous clear inclusions aside from their normal mucous secretions (Fig. 4). The inclusions were limited by a unit membrane, and contained a fine granular material and a few small concentric lamellar structures, often organized in half rings in contact with the limiting membrane (Fig. 5).

In the stroma, numerous cytoplasmic inclusions distended the fibroblasts (Fig. 6), the endothelial cells of the blood (Fig. 7), and the lymphatic capillaries (Fig. 8), the
Fig. 2. Patient 2. Skin biopsy. Semi-thin section stained with toluidine blue. The secretory coils of the sweat glands are filled with clear intracytoplasmic vacuoles. The blood capillary endothelial cells are also severely involved. ×1,000.

Fig. 3. Patient 1. Conjunctival epithelium. Uranyl acetate, lead citrate. The epithelial cells of the superficial layer are filled with clear inclusions, which contain a fine protein-like reticulum and some lamellar profiles that appear at this magnification as dense half rings (arrows). ×8,000.

perivascular cells and the perineural cells (Fig. 9). The Schwann cells and the axonal processes of the nerve fibers were unaffected. The inclusions, limited by a unit membrane, had two distinct aspects: clear inclusions were prominent and contained a weakly osmiophilic reticulum. Dense inclusions were less numerous and
Fig. 4. Patient 1. Conjunctival epithelium. Uranyl acetate, lead citrate. The goblet cells show numerous clear inclusions aside from their normal mucous secretions; the latter appear as denser oval or ribbon-shaped striated bodies (arrows). ×10,000.

Fig. 5. Patient 1. Conjunctival epithelium. Uranyl acetate, lead citrate. This higher magnification shows details of the lamellar structures and of the finely reticular content of the clear vacuoles, which are limited by a unit membrane. ×60,000.

exhibited a moderately contrasted homogenous material at low magnification, which appeared to be finely granular at high magnification. Some lamellar profiles (Fig. 7) and some dense aggregates (Fig. 6) were present in both types. Occasionally, the inclusions contained rather dense spherules whose ultrastructure resembles that of the neutral lipids (Fig. 8). Other cellular organelles were normal.

Skin biopsy. Epidermal cells seemed unaffected. Histiocytes and fibroblasts of the superficial dermis displayed clear and dense inclusions, identical to those observed in the conjunctival stroma. The endothelial cells of the blood and lymphatic capillaries, and the perineural cells were also involved, while the Schwann cells and the axonal processes appeared to be normal.

In the sweat glands (Fig. 10), the myoepithelial cells contained few small clear inclusions. The clear and dark cells of the secretory coils were filled with numerous clear vacuoles containing a fine osmiophilic reticulum and small lamellar lipid struc-
Fig. 6. Patient 2. Conjunctival stroma. Uranyl acetate, lead citrate. The connective tissue cells have a characteristic aspect in fucosidosis: They contain both clear and dense inclusions with sometimes dense aggregates. ×9,600.

tures. Some inclusions displayed a dense homogenous material and resembled the dense inclusions of the connective tissue cells. In the sweat ducts, the cells of the superficial and of the deep layers were distended by numerous clear and dense inclusions.

SPECIFIC STAININGS. Silver proteinate. In the conjunctival epithelial cells, the inclusions contained a fine granular material of variable density, which was markedly stained with silver (Fig. 11). The lamellar structures, observed with usual staining techniques, were poorly contrasted. The mucus and the inclusions of the goblet cells showed a similar contrast and were only distinguishable by their shapes.

In both conjunctiva and skin, the connective tissue cells and the endothelial cells of the capillaries contained some inclusions that were strongly contrasted with silver as a dense granular material, but many of the vacuoles remained clear with a faintly stained reticulum (Fig. 12).

Similar clear and dense inclusions were evident in the secretory coils and ducts of the sweat glands (Fig. 13).

Colloidal iron. Iron staining showed a positive reaction in the periphery of the vacuoles of the conjunctival epithelial and stromal cells. In the goblet cells, the mucus was strongly contrasted with this technique while the inclusions had an aspect rather similar to that of the adjacent epithelial cells (Fig. 14).

Phosphotungstic acid. PTA contrasted the collagen fibers and the tear film of the epithelium surface. The stored material and the inclusion's membrane were markedly stained. A variable density of the inclusions was here also recognizable (Fig. 15).

Biochemistry.

PROPERTIES OF α-L-FUCOSIDASE IN NORMAL TEARS. α-L-fucosidase is relatively abundant in tears, where the enzyme displays a broad pH activity curve, usually with 3 pH optima around 5.2, 6.3, and 7.4 (Fig. 16). The mean normal activity is 1.1 U per gram of protein at the first two pH and 0.73 at pH
7.4, as well in children as in adults. The liver enzyme, for comparison, reaches its highest activity (1.6 U per gram of protein) at pH 4.4, with a second peak around 6.4. The affinity of the tear enzyme for 4-methylumbelliferyl-\(\alpha\)-L-fucopyranoside is high, but depends on the pH. A \(\text{Km}\) value of 0.04 mM has been measured at pH 5.1; 0.13 mM at pH 6.5, and 0.24 mM at pH 7.6. In the liver, the \(\text{Km}\) is around 0.06 mM at pH 4.4. Thermostability (50°C) of tear \(\alpha\)-fucosidase is similar at the three peaks of the pH activity curve.

ACID HYDROLASES IN TEARS FROM PATIENTS AND PARENTS. Fig. 16 illustrates the activity of \(\alpha\)-fucosidase at various pH, in tears from a normal subject and from both patients and their parents. In the tears of the pa-
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Fig. 8. Patient 1. Conjunctival stroma. Uranyl acetate, lead citrate. The lymphatic vessels are recognizable by their clear, acellular lumen and by the absence of basement membrane under their endothelium. The two characteristic inclusion types are evident in this lymphatic endothelial cell. Some dense aggregates and lipid spherules are also disclosed. (L: lumen) x13,000.

In patients, there was no detectable activity at any pH between 3.0 and 9.7. Seven other lysosomal enzymes (see methods) were assayed in the lacrimal fluid of the patients and their parents and found to be normally active.

OTHER ENZYMATIC STUDIES. A liver needle biopsy was obtained from patient 2. There was no detectable activity of α-fucosidase, while the other lysosomal enzymes assayed were hyperactive: α-glucosidase displayed a 9-fold increase over the mean normal activity, while α- and β-galactosidase and N-acetyl-β-glucosaminidase were six times more active than normal. No liver biopsy was performed in patient 1 because of the results already obtained in her brother.

Leukocytes were analyzed in both patients. There was no detectable α-fucosidase activity at any pH between 3.1 and 8.6.

Activity of this enzyme at pH 5.1 (peak of the pH curve), in leukocytes from the parents of case 1 was, respectively, 60 per cent of the control for the father and 54 per cent for the mother. Two other lysosomal hydrolases (α-galactosidase and β-hexosaminidase) were measured in these leukocytes and found to be normally active in patients and parents.

In blood plasma from the patients, α-fucosidase was not completely absent but reduced to less than 3 per cent of the mean normal value of controls in patient 1 and to less than 1.2 per cent in patient 2. This deficiency extended all over the pH curve. Plasma α-fucosidase activity represented, respectively, 120 and 79 per cent of the normal value for the father and the mother of case 1. Other lysosomal enzymes assayed in patients and parents were normally active.

α-Fucosidase was assayed in urine of patient 1. The enzyme was barely detectable between pH 5 and 6. Other lysosomal hydrolases (α- and β-galactosidase, β-glucuronidase, β-hexosaminidase, and acid phosphatase) were normal.

Discussion

The two new cases of fucosidosis, described here, obviously belong to the severe phenotype of the disease. Mild Hurler-like dysmorphia without mucopolysacchariduria, psychomotor retardation, and neurologic disturbances were their symptoms. As the other reported cases of severe phenotype fucosidosis, they did not display angiokeratoma corporis diffusum nor vascular tortuosities in the conjunctiva and in the fundus. In Fabry's disease, where the vascular symptoms are barely similar, vascular abnormalities also are late symptoms, appearing only during adolescence or adulthood.7 Patients with the severe phenotype of fucosidosis possibly do not survive long enough to develop them.

The ultrastructural changes in our biopsies resemble those reported in Hurler disease. They consist mainly of vacuoles or
Fig. 9. Patient 2. Conjunctival stroma. Uranyl acetate, lead citrate. The perineural cells (arrows) show many inclusions similar to those of the fibroblasts and endothelial cells in the vicinity. The myelin sheets, the axons and Schwann cells do not present any significant alteration. ×6,000.

Fig. 10. Patient 2. Skin biopsy. Uranyl acetate, lead citrate. The myoepithelial cells of the sweat glands contain small clear inclusions (arrow). The cells in the secretory coil are distended by numerous clear and dense inclusions. Little normal cytoplasm remains between the inclusions. Fibroblasts and endothelial cells in the vicinity show the characteristic lesions already described in the conjunctiva. ×4,000.
inclusions resulting from the overloading of the lysosomal system. The connective tissue cells and the conjunctival epithelium are heavily involved, while the pathological process seems to spare the axones and the Schwann cells of the peripheral sensitive nerves. Contrary to the mucopolysaccharidoses with mucopolysacchariduria, the endothelial cells of the blood capillaries are filled with abnormal inclusions in fucosido-
sis. This storage in the vessels’ walls is probably responsible for the vascular tortuositites and the angioretinal in the mild phenotype of the disease.

The fine structure of the stored material in fibroblasts and endothelial cells is quite pathognomonic. It differs from what is observed in the mucopolysaccharidoses, other forms of mucolipidoses, and sphingolipidoses. In the present study, two distinct inclusion types are evident: clear vacuoles which contain a finely reticular structure, similar to those seen in the mucopolysaccharidoses, and less numerous dark inclusions exhibiting a dense almost homogenous content. The existence of these two types of inclusions had already been reported by Loeb and co-workers in astrocytes, macrophages, and endothelial cells in a brain biopsy taken from our first patient’s brother, while the neurones contained only clear inclusions and a few zebra bodies. This heterogeneity is also evident in the vessels of the liver, the rectal, and the bronchial biopsies in our second case. To our knowledge, the presence of these two inclusion types has not yet been described in other lysosomal storage diseases. We assume that this electron microscopic appearance is characteristic of fucosidosis.

The staining properties with silver proteinate and phosphotungstic acid suggest that the major components of the inclusions are polysaccharides. The weakly positive results obtained with colloidal iron could be due to the fact that these molecules are poorly acidic. Alternatively, a loss of water-soluble substances during the incubation in the staining solutions could also explain this result. Biochemically, the storage of fucose containing sphingolipids, oligosaccharides, and polysaccharides has been reported in the liver, the brain, and other tissues. We believe that the
silver positive materials of the inclusions could represent fucose-rich oligosaccharide chains, either free or bound to polypeptides. Variations in the proportions or in the structure of these components might explain the occurrence of different inclusion types. Some lamellar lipid structures were also observed in the inclusions and probably correspond to the multilayered bodies described in the hepatocytes. This latter lipid material has been identified as fucosyl-glycosphingolipids.

The ultrastructural changes of the sweat coils and ducts resemble those reported in Hurler's disease, except for the presence in our cases of some dark inclusions, similar to those of the connective tissue cells. The extensive vacuolation of these glands is not surprising since sweat of secretor subjects is known to contain fucosides as A, B, H, and Le active glycoproteins. This involvement of the sweat glands did not express itself, as in Durand's first patients, by an increase in concentration of sweat electrolytes. Moreover, in our second case, sweat did not contain abnormal amounts of fucosides by comparison with normal secretor controls.

It must be pointed out that the histologic findings in skin biopsies are similar in the second case aged 2.3 years and in the first patient when she was five months old and did not, at this time, clearly manifest the disease clinically. It is likely that ultrastructural alterations are also present in the conjunctiva before the appearance of the first clinical signs.
The enzymatic study, besides of bringing the proof that the two children suffer from fucosidosis, demonstrates the usefulness of tear analysis in this disease. α-Fucosidase activity is of the same magnitude in tears as in liver, on a protein basis. To collect tears is easy and harmless; it can be repeated many times, even at the patient's home, which could be of potential use in therapeutic attempts. The enzyme is relatively stable: 60 to 85 per cent of it remains active when tears are allowed to dry on the paper strips at room temperature (10 to 20 minutes). Once dried, the enzyme is remarkably resistant. If the filter papers are mailed in an envelope in temperate countries, over 50 per cent of the activity are still present after one week. If the paper strips are kept below -18° C, no detectable loss of activity occurs within one year. The method can, however, apparently not be used for the detection of heterozygotes for fucosidosis (Fig. 16) and in this respect, is thus not superior to the analysis of leukocytes.

It is suggested that the complex pattern of the pH activity curve in tears reflects the existence of multiple molecular association forms of a single α-fucosidase, which has been demonstrated in other tissues. The total absence of activity in patients indicates that all these enzyme forms are under the control of a single gene, which is the same that controls the lysosomal α-fucosidase in all the tissues that were investigated in fucosidosis patients.

Conjunctival biopsy and tear analysis are easy to obtain and to send to the appropriate laboratory. These two methods allow the early and secure diagnosis of several lysosomal storage diseases and, among them, of fucosidosis which is probably frequently undetected.

REFERENCES

Fucosidosis

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