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Genetic Inactivation of the α-Galactosidase Locus in Carriers of Fabry's Disease

Abstract. *Skin fibroblasts from a patient with Fabry's disease showed deficient activity of α-galactosidase. Fibroblasts from his mother and sister had two distinct clonal populations, one with enzymatic activity and the other enzyme deficient. This provides evidence of genetic inactivation at the α-galactosidase locus and makes possible the detection of carriers of Fabry's disease even when the enzymatic activity in their leukocytes and uncloned fibroblasts is within the range of controls.*

The Lyon hypothesis (*1*) proposes that in each somatic cell of the female, one of the two X chromosomes is genetically inactive and that the same inactive X is maintained in its progeny. Compelling evidence supporting this hypothesis has come from the demonstration of two populations of clones in heterozygotes for X-linked glucose-6-phosphate dehydrogenase (G6PD) variants (*2*), hypoxanthine-guanine phosphoribosyltransferase (HGPRT) deficiency (*3*), and Hunter's syndrome (*4*). Studies designed to test for inactivation at the Xg^a blood group locus have not produced definitive results (*5*). Since some X-linked genes may escape inactivation, we have tested the Lyon hypothesis at another X-linked locus by determining the phenotype of clones of skin fibroblasts from heterozygotes for Fabry's disease. This locus is of interest since it is located within measurable linkage distance of the Xg^a locus (*6*), whereas the G6PD and HGPRT loci are not (*7*). Our data show that carriers of Fabry's disease have two distinct clonal populations of cells, providing evidence of genetic inactivation of one of the two X chromosomes at this locus in female somatic cells.

Table 1. Mean specific activities of α- and β-galactosidase in clones, uncloned fibroblasts, and amniotic cells. The values are expressed as nanomoles of substrate hydrolyzed per hour per milligram of protein. Each culture was assayed in triplicate. There were six control individuals and a total of ten amniotic fluids.

Source	Cultures		Specific activity	
	Type	No. examined	α-Galactosidase	β-Galactosidase
Controls	Uncloned	7	45.0 ± 13.0	184 ± 69
Proband	Uncloned	9	5.0 ± 4.3	237 ± 99
Father	Uncloned	4	30.0 ± 4.2	
Mother	Uncloned	4	43.1 ± 18.8	
	(−) Clones*	13	4.4 ± 2.3	237 ± 58
	(+) Clones	8	54.0 ± 10.2	249 ± 62
Sister	Uncloned	2	21.7 ± 3.3	
	(−) Clones*	16	5.2 ± 3.0	269 ± 74
	(+) Clones	11	25.9 ± 3.7	276 ± 85
Amniotic fluids	Uncloned	19	36.2 ± 10.8	408 ± 165

* Negative clones are those with α-galactosidase activity in the proband's range.

Fabry's disease is an X-linked inborn error of metabolism characterized by the abnormal accumulation in several tissues of a neutral glycolipid, ceramide-trihexoside (galactosylgalactosylglucosyl ceramide) (*8*). Accumulation of this glycolipid has been observed also in cultures of fibroblasts of patients affected with the disease (*9*). The enzyme ceramide-trihexosidase, which is deficient in these patients (*10*), hydrolyzes the glycosidic bond of the terminal galactose residue in the ceramide-trihexoside molecule. If artificial nonspecific substrates are used instead of the naturally occurring one, patients with Fabry's disease and some carriers show different degrees of α-galactosidase deficiency (*11*). Activity of α-galactosidase has been found in several tissues of rat and man (*12*), and enzymes active on artificial α-galactoside substrates have been purified from fungal and vegetable sources (*13*).

We have studied a family whose proband, M.M. (J.H.H. 1165904) is an 18-year-old boy in whom the diagnosis of Fabry's disease had been made on clinical grounds, including a conjunctival biopsy which showed lipid deposits in the endothelial cells of the vessel walls.

Leukocytes separated according to the method of Fallon *et al.* (*14*) were lysed by ultrasonic vibration and centrifuged. A portion of the clear supernatant (0.1 ml) was incubated with 5 μmole of *p*-nitrophenyl-α-D-galactoside and 25 μmole of acetate buffer, *p*H 4.5, in a final volume of 0.2 ml for 4 hours at 37°C. The reaction was stopped by addition of 0.2 ml of 1*M* tris (*p*H 10.5), and the mixture was centrifuged to obtain a supernatant free of denatured protein. The absorbancy of each sample was determined at 410 nm in a Zeiss spectrophotometer. The assay was linear under the conditions used. No detectable activity was found in the proband's leukocytes, while those of his mother and sister had specific activities of 45 and 13, respectively (as defined in Table 1). Assay in leukocytes from the proband and his mother repeated 3 months after the initial assay gave similar results. The mean specific activity of six controls was 53 (range 43 to 61).

Skin fibroblast cultures were established from all members of the family as described (*3*). Clones were obtained according to the dilution technique of Ham and Puck (*15*), and those that were well isolated from neighboring clones were transferred by trypsiniza-

tion into 60-mm petri dishes. When the cultures had reached confluence, they were washed in saline and the cells were gently scraped from the petri dishes into 0.4 ml of 0.01*M* acetate buffer at *p*H 4.5. The resultant cell suspensions were broken up by ultrasonic vibration. Without prior centrifugation the sonicates were assayed for α-galactosidase, as described for leukocytes. As experimental control, the activity of β-galactosidase was measured (*11*). Protein content was determined in duplicate samples using the Folin-Ciocalteau reagent (*16*).

Fibroblasts from the affected boy showed low α-galactosidase activity in comparison to controls (Table 1). As in leukocytes, the α-galactosidase activity in uncloned fibroblasts of the proband's sister was below the range of the controls, whereas those of the mother had activity within the normal range, so that she could not have been identified as a carrier solely on the basis of these values. This is not surprising since carriers of X-linked diseases often show a wide range of phenotypic variation, which has also been observed in regard to the clinical manifestations in heterozygotes for Fabry's disease (*17*).

A total of 48 clones were isolated from the fibroblast cultures of the proband's mother and sister. Two populations of clones with respect to α-galactosidase activity were found in each woman: those with values similar to the ones found in the proband's fibroblasts (negative clones) as well as those with significantly higher activity (positive clones) (Table 1). On the other hand, both women showed only one cell population with respect to β-galactosidase. These results demonstrate the inactivation of the α-galactosidase locus in female somatic cells and, at the same time, provide evidence that both the proband's mother and sister are carriers of the disease.

Both in leukocytes and uncloned fibroblasts the α-galactosidase activity was higher in the proband's mother than in his sister. Furthermore the enzymatic activities in positive clones from the two women differ significantly ($P < .001$). Although both women have in common the X chromosome carrying the mutant α-galactosidase locus, they do have different paternal X chromosomes, so that the difference between the two positive clonal populations could be due to genetic heterogeneity of the two wild-type alleles of paternal origin. We have looked for evidence of structural variation of the enzyme in the positive clones from these two women by studying some functional properties of α-galactosidase. As judged from the fluorometric assay (*11*), the rate of inactivation of the enzyme at 50°C for periods ranging from 1 to 20 minutes was similar for the two positive clonal populations, and the apparent K_m's were not significantly different (3.0 and $2.6 \times 10^{-3} M$). The twofold difference in specific activity, therefore, cannot be ascribed for the moment to genetic heterogeneity.

Using the colorimetric assay described above, we have also determined the activity of α-galactosidase in mixed populations of fibroblasts and epithelioid cells derived from amniotic fluid (*18*) (Table 1). Since α-galactosidase activity is deficient in our proband's fibroblasts, one can reasonably apply this assay to the prenatal diagnosis of Fabry's disease.

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Antitumor Activity in Mice of Tentacles of Two Tropical Sea Annelids

Abstract. *Crude extracts of tentacles of two polychaetous annelids completely inhibit growth of Erlich ascites tumor in 60 to 100 percent of treated mice. Dialyzed extracts of one of these annelids,* Lanice conchilega, *show activity in the retentate after pronase digestion, suggesting that antitumor activity is associated with a nonprotein component of the crude tentacle extract.*

During a screening program (*1*) designed to test the pharmacologic activity of ancient Polynesian medicines, tentacles of the tropical sea worm, kaunaoa (*Lanice conchilega*) (*2*), were gathered for antitumor assay at the suggestion of an elderly woman of the Hawaiian race who had experienced many of the Hawaiian medical practices on the island of Molokai (*3*). According to her, cancer patients had shown clinical improvement after drinking an infusion of cooked kaunaoa tentacles daily for several weeks. Which kinds of cancer were treated and the exact results obtained were unknown to the informant. Similar accounts of the anticancer use of kaunaoa body fluid, extracted from the live worm by sucking through a fine bamboo tube, were also found among the Hawaiians in the Kona district of the island of Hawaii. *Lanice conchilega* is shown in Fig. 1, at top.

Worms and tentacles for assay were gathered in 0.5 to 4 m of water in coral reef areas in three sites, two on Oahu, and one at Puako, Hawaii. Whole worms were secured by digging them