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Evidence for Preferential X-Chromosome Inactivation in a Family with Fabry Disease

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INTRODUCTION

During early embryogenesis of females in placental mammals, one of the two X chromosomes in each somatic cell is irreversibly inactivated. This inactivation is maintained in all daughter cells, resulting in cellular mosaicism. Since X inactivation appears to be a random process, each X chromosome is active in about half of all somatic cells. However, the composition of the mosaic may vary depending on the time of X inactivation, restriction of randomness, the size of primordial cell pools at tissue differentiation, and somatic selection. Assuming that inactivation occurs before tissue differentiation [1, 2], the variation caused by early or preferential X inactivation should be identical in all organs and tissues. In contrast, the variation contributed by random sampling of primordial cells is not necessarily equal in different organs because tissues may originate from different numbers of precusor cells. Somatic selection is unlikely to proceed at the same rate in different tissues (due to differences in metabolic activity and cellular environment); selection against X-linked genes may virtually eliminate one of the two cell types in one tissue while leaving a mosaic population in another [3].

Recent observations [4] argue for familial occurrence of symptomatic Duchenne heterozygotes. This finding cannot be explained by early X inactivation alone. However, on the basis of their data, the authors were unable to decide among other models. An explanation might have been possible if there was a biochemical technique that could be applied to multiple tissues of several Duchenne heterozygotes in the same family.

In the present study, we examined several tissues of eight heterozygous daughters whose father died of Fabry disease (angiokeratoma corporis diffusium), an X-linked disorder caused by a deficiency of the lysosomal hydrolase α-galactosidase A. This enzyme can be measured in most tissues and body fluids. Our investigation was triggered by the observation that enzyme activities were not randomly distributed in carriers in this family.

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MATERIALS AND METHODS

Professor Lenz made a tentative diagnosis of Fabry disease in a family from northwestern Germany, which was confirmed in our laboratory by enzyme analyses of fibroblasts of an affected male. In this family, there were nine male Fabry patients and 13 heterozygotes, including eight daughters of one of our patients (fig. 1).

The α-galactosidase activity in the males was determined primarily in white blood cells, but in some and in their maternal grandmother (I-2), fibroblasts, serum, and hair roots were also tested. Serum, leukocytes, hair roots and fibroblasts were analyzed in the eight heterozygous sisters. Slit lamp examination was performed with portable equipment; Xg typing was done in triplicate using two different commercial antisera (Biotest, Frankfurt; Schwab, Villmar-Aumenau). Color vision was tested with Ishihara tables.

Fibroblasts were obtained by skin biopsy and cultivated in MEM (Gibco, Grand Island, New York) supplemented with 10% fetal calf serum. They were subcultivated by trypsinization and analyzed 1 day after reaching confluency. At least two independent biopsies were performed; in a severely affected woman (II-8), six biopsies from different areas of the body were performed to determine heterozygosity. For analysis, cells were harvested with a rubber policeman in Dulbecco's phosphate buffered saline (D-PBS) and homogenized by sonication. After centrifugation for 30 min at 10,000 g, the clear supernatant was used for determination of enzyme activities and protein content (as determined by the Lowry method).

Serum was obtained by defibrinization of venous blood with glass beads and subsequent centrifugation. For preparation of leukocytes, 0.2 ml of 0.3 M Na_2 EDTA and 1 ml of a dextran solution (5 g/dl) was added to 5 ml of venous blood. After sedimentation of the erythrocytes, leukocytes were obtained by centrifugation of the supernatant (600 g for 10 min). The cells were washed with D-PBS and taken up in 1 ml of D-PBS; an aliquot was removed for cell counting. The suspension was sonicated and centrifuged as described for fibroblasts. Slight contamination with erythrocytes was disregarded.

Single hair roots of the scalp were stored on dry ice and analyzed within 24 hr after pulling. Normal hair roots did not lose any activity with this procedure.

Enzyme activities in serum, leukocytes, and fibroblasts were determined fluorometrically using artificial methylumbelliferyl substrates (Koch-Light, Colnbrook) for measurement of α- and β-galactosidase, α-mannosidase, and hexosaminidase, as described earlier [5]. All assays were performed in duplicate; in each series, eight to 10 normal samples were assayed in parallel. Alpha-galactosidase activities were referred to protein content, cell number, and a number of lysosomal enzymes (fibroblasts: protein, hexosaminidase, and β-galactosidase; leukocytes: cell number, hexosaminidase, α-mannosidase; serum: hexosaminidase and α-mannosidase). The method for determining α-galactosidase activity in hair roots [6] allows the determination of enzyme activity and protein content in single hair roots. Five to 22 hair roots from each individual were examined.

For comparison of enzyme activities in different tissues, all values were expressed in percent of the mean activity of α-galactosidase A in healthy individuals. Corrections for the B enzyme were made on the basis of α-galactosidase levels in tissues of affected males ([7] and table 1).

The extent of vascular skin lesions was determined by direct comparison of the heterozygotes. In females of generation III, heterozygosity was ascertained by statistical analysis similar to that described recently [8] and by hair root analysis (table 2). Karyotype analyses were performed in fibroblasts of probands I-2, II-6 and II-8, employing both conventional and Giemsa staining.

RESULTS

Linkage Analyses

Color vision and Fabry disease. The only son (II-9) of subject I-1, was found to be protanopic, indicating heterozygosity of his mother, I-2 (fig. 1). Of three protanopic boys in generation III, two had Fabry disease. As the Fabry trait was of grandpaternal

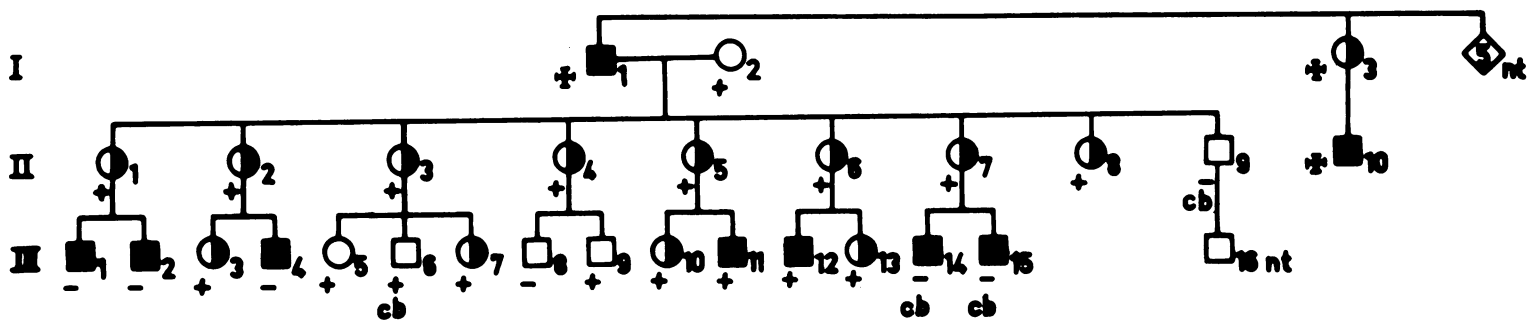

FIG. 1.—Pedigree of family with Fabry disease. *Open squares (circles)* = healthy males (females); *closed squares* = affected males; *halfclosed circles* = heterozygotes; +(−) = Xg(a+) or (a−); cb = color blind; cross = deceased; nt = not tested.

TABLE 1

ALPHA-GALACTOSIDASE ACTIVITY (% OF NORMAL) AND VASCULAR SKIN LESIONS IN HETEROZYGOTES AND AFFECTED MALES

Individuals Examined	White Blood Cells	Serum	Fibroblasts	Hair Roots	Vascular Skin Lesions
II-1	45	49	· · ·	73	(+)
II-2	20	26	· · ·	· · ·	+
II-3	14	40	· · ·	31	+++
II-4	17	28	· · ·	78	++
II-5	47	60	· · ·	31	+
II-6	51	74	69	78	ϕ
II-7	48	58	· · ·	80	ϕ
II-8	5	16	14	18	+++
Affected males ...	7	10.4	14	13.3	Mostly +++
Mean of *n* patients ..	(*n* = 7)	(*n* = 2)	(*n* = 2)	(mean of 23 hair roots of five patients	· · ·

NOTE. — Normal values ± 1 SD: WBC: 5.25 ± 0.75 nmol/10^6 cells/hr; fibroblasts: 2.13 ± 0.35 nmol mg protein. Variation coefficient of serum α-galactosidase in controls: .19. For α-galactosidase activity in normal hair roots, see reference [6].

origin, recombination occurred in two out of three possible cases which agrees with previous data [9], indicating nonlinkage of these loci.

Xg blood group and Fabry disease. All eight heterozygous daughters of subject I-1 were found to be Xg(a+). His only son is Xg(a−), proving heterozygosity of his Xg(a+) mother, I-2. Since the serological examination of his sibs yielded no evidence of illegitimacy, the deceased father (I-1) was probably Xg(a+), because it is highly unlikely that an Xg(a−) man married to an Xg heterozygote would have eight Xg(a+) daughters ($P < .004$).

Previous pedigree studies have indicated that, with a recombination fraction of about .25, the *Xg* locus may be within a measureable distance of the Fabry locus [10, 11]. In this family, however, analysis of the grandsons born to Xg heterozygous daughters revealed six out of seven possible crossovers between the Fabry and the *Xg* loci; this is incompatible with linkage ($P = .0013$ for a recombination fraction of .25), if the grandfather indeed was Xg(a+). However, if one assumes that I-1 was Xg(a−), there

TABLE 2

ALPHA-GALACTOSIDASE ACTIVITY IN TISSUES OF FABRY HETEROZYGOTES

	α-GALACTOSIDASE ACTIVITY (% OF NORMAL)			
INDIVIDUALS EXAMINED	White Blood Cells	Serum	Fibroblasts	FREQUENCY OF α-GAL A NEGATIVE HAIR ROOTS
III-3*	99	98	74 (2)	4/16
III-5	117	100	89 (2)	0/25
III-7*	61	96	26 (1)	· · ·
III-10*	· · ·	80	81 (2)	2/7
III-13†	90	69	78 (2)	· · ·

NOTE. — Nos. in parentheses = no. biopsies performed.
* Heterozygotes.
† Heterozygosity highly probable.

would be 10 informative males in generation III, seven nonrecombinants and three recombinants; this would be in excellent agreement with previous estimates of the distance between the *Xg* and the Fabry loci. Ascertainment of the grandfather's genotype might be achieved by Xg typing if it were possible to perform Xg-genotyping in his daughters' fibroblasts [12].

Clinical and Biochemical Findings

Owing to poor equipment, we were not able to detect corneal clouding in any of the heterozygotes. According to other criteria however, four to six out of eight heterozygous sisters are affected carriers; six have vascular skin lesions, and four experience pain in the extremities. In general, only about 20% of the heterozygotes show skin lesions, and an even smaller percentage have the characteristic pain in fingers and legs [13]. Two of the heterozygotes, II-3 and II-8, manifest the full-blown syndrome with cardiac and renal involvement, vision disturbances, and severe vascular skin lesions.

The mean levels of α-galactosidase in serum, leukocytes, and hair roots of the sisters were less than 50% of normal (serum: 37.4%; white blood cells: 27.3%; table 1), whereas normal activities were found in their mother I-2 and in all healthy boys of generation III. The data suggest that there are two groups of heterozygotes with significantly different mean activities (serum: $P < .05$; white blood cells: $P < .05$, U-test of Mann and Whitney) of α-galactosidase A in the range of 50% and 20% (table 1). If the eight values obtained for serum α-galactosidase are taken as a single group, they are not randomly distributed around their mean (χ^2 adaptation test). This is also true for white blood cells, suggesting that the eight heterozygotes do not belong to a genetically homogeneous group.

Comparison of enzyme activities of different tissues yields high positive correlations which, despite the low number of individuals examined, reaches significance in several combinations (table 3). The figures referring to hair root activities, however, are biased by sampling errors since many hair roots consist entirely of either normal or defective cells (fig. 2). Depending on the number of hair roots examined, the confidence limits of the calculated hair root activities may be narrow (as in II-1, $n = 22$) or wide (as in II-3, $n = 5$). However, when these differences are taken into account, the pertinent correlation coefficients become even higher, which emphasizes the fact that the positive correlations observed are real rather than random. Moreover, in the preliminary experiments when more hair roots were examined by simply classifying them as positive or negative on the basis of their α-galactosidase activities, similar correlation coefficients were obtained.

Continued analysis of the data provides additional evidence against the sisters comprising a genetically homogeneous group of heterozygotes. If random X inactivation in early female embryonic development leads to a deviation from the expected 50:50 ratio of paternal:maternal X chromosomes in each cell, then the deviation will be demonstrated in all organs [1]. Thus, multiple tissue studies in heterozygotes permits the estimation of the number of cells (N) in the embryo when X differentiation occurs since this is inversely related to the covariance of the distribution of mosaic composition in different organs. Because our data yield such low estimates for N (table 4), it is logical to assume that the sisters are genetically heterogeneous.

In white blood cells and fibroblasts of the most severely affected woman (II-8), the activity of α-galactosidase A is virtually zero. Repeated hair root analyses however, revealed that a small portion of her hair roots have α-galactosidase A activity which proves that she is heterozygous. Her karyotype was normal, ruling out the possibility that some form of X autosome translocation is responsible for the findings in this family.

DISCUSSION

Statistical analyses of the data in our family reveal that early random X inactivation is not sufficient to explain our results because they would argue for X differentiation in man occurring around the six cell stage of embryonic development. This estimate is probably too low since it would predict that complete hemizygous expression of X-linked genes occurs in slightly less than 2% of heterozygous woman, and no example of complete hemizygous gene expression has been found [14].

We have considered four possible models to explain our findings. (1) The mother (I-2) of the eight sisters is heterozygous for partial deficiency of α-galactosidase A. (2) Somatic selection favors the expression of the Fabry gene in some of the heterozygotes. (3) Some of the sisters carry a mutation at a gene locus regulating α-galactosidase activity. (4) Preferential X inactivation accounts for genetic heterogeneity of the heterozygotes.

The first hypothesis is incompatible with the observation that the healthy males born to severely affected sisters (II-3 and II-4) have completely normal enzyme activities in white blood cells. If this hypothesis were correct, one could not expect to find normal enzyme activities since their mothers would be doubly heterozygous for complete and partial α-galactosidase deficiency. Examination of the grandmother (I-2) yielded no evidence to support this hypothesis.

TABLE 3

CORRELATION OF α-GALACTOSIDASE A LEVELS AND SKIN LESIONS

	Product Moment Correlation Coefficient (r)	r Corrected for Small No. of Individuals Tested ($= r'$)	Rank Correlation Coefficient (R)
White Blood Cells: Serum	.92*	.93*	.88*
White Blood Cells: Hair roots	.57	.62	.82†
Serum: Hair roots	.47	.52	.57
Skin Lesions: Hair roots	. . .	. . .	.87†
Skin Lesions: White blood cells	. . .	. . .	.80†
Skin Lesions: Serum	. . .	. . .	.77†

NOTE. — Vascular skin lesions have not been quantitated; therefore, no pertinent r and r' is given.
* Significant at the 1% level.
† Significant at the 5% level.

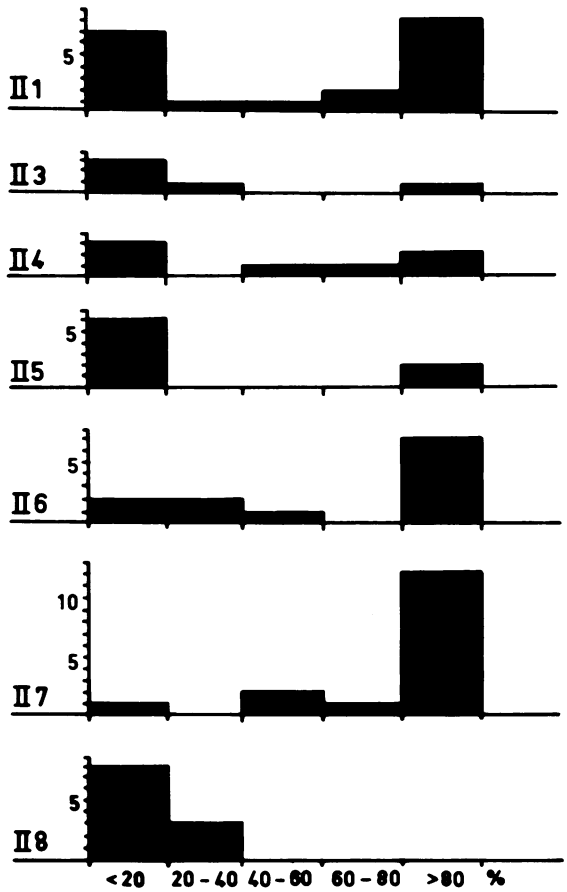

FIG. 2.—Proportion of normal, intermediate, and defective hair roots in heterozygotes (see fig. 1). Abscissa shows relative activities of α-galactosidase A (normal = 100%; values are corrected for α-galactosidase B). Number of hair roots indicated by height of bars.

Several models involving somatic selection were analyzed for their ability to explain the findings in this family. First, there is the possibility of somatic selection favoring α-galactosidase negative cells. This idea would imply that in Fabry heterozygotes α-galactosidase activities would be regularly lower than 50% of normal. Published data [15], as well as our data, do not support this assumption. In heterozygotes of our family, pain in the extremities is associated with mean enzyme activities of less than 30% of normal; however, only a small proportion of heterozygotes exhibit this symptom [13]. It follows that in the vast majority of heterozygotes, mean α-galactosidase activities must be higher than 30% of normal. Thus, somatic selection for α-galactosidase negative cells appears to be no general phenomenon in Fabry heterozygotes. In our family, somatic selection favoring α-galactosidase negative cells would result in a uniform class of heterozygotes since all sisters have obtained the Fabry gene from their father. The apparent nonhomogeneity of the sisters, however, argues against this model.

According to a related model, the heterogeneity might be caused by the presence of a

gene interacting with either α-galactosidase positive or negative cells, so that α-galactosidase positive cells obtain a selective disadvantage or α-galactosidase negative cells are favored. Alternatively, their mother (I-2) may be heterozygous for an X-linked defect causing selective disadvantage of all cells expressing this hypothetical gene. This model implies that in about half of the sisters, most cells would be α-galactosidase negative, and in these, severe clinical signs would be expected. This model is appealing because it is simple, and because it easily explains the unusual sex ratio among the nine children of I-2 (eight females and one male; $P = .017$). Assuming that the postulated maternal gene defect is lethal in affected male embryos, the observed sex ratio has a probability of about 11%. However, for the reasons mentioned above, somatic selection is unlikely to proceed at the same rate in all tissues. In the only example presently known in man of in vivo selection against cells expressing an enzyme defect, selection has been observed in only one tissue: in peripheral blood of females heterozygous for hypoxanthine phosphoribosyltransferase deficiency, more than 99% of the cells exhibit the normal phenotype [16]. In contrast, α-galactosidase activity in all tissues of individual Fabry heterozygotes is lowered by a similar factor. In particular, somatic selection does not satisfactorily explain why the tissues of subject II-8 consist almost exclusively of α-galactosidase negative cells.

It is noteworthy (though not statistically significant) that there are positive intertissue correlations among severely affected females. It is hard to imagine that these correlations reflect the variance generated by early random X inactivation; somatic selection also seems an unlikely explanation in light of these findings, unless one argues that selection is a very early embryonic event.

The observations in this family are easily explained by two other models. In the mouse, the activity of α-galactosidase is controlled by a regulatory gene; recently a mutation at its locus has been observed [17]. If there were a similar gene in man, mutations should be easily detectable since the α-galactosidase activities in half of the healthy children of carrier mothers should be lower than normal. Although this is probably not the case in our family, we cannot rule out this model with the available data.

The other plausible explanation is based on the assumption that one of the maternal X chromosomes (I-2) has a greater tendency to become inactivated during embryogenesis than the paternal (i.e., one of the maternal X chromosomes carries a mutation affecting the genetic factor (receptor?) mediating X inactivation). If this were true, our data would suggest that the probability of the mutant X chromosome escaping inactivation is about .2, which is the mean enzyme activity of severely affected heterozygotes.

Preferential X inactivation enhances the probability of heterozygotes showing hemizygous (or near-hemizygous) gene expression and would explain the findings in proband II-8. According to this model, 50% of the females born to sisters of the lower group should inherit the preferentially inactivated X chromosome. However, preferential X inactivation would only be evident if, in addition, these daughters carried the Fabry gene (by crossing over during maternal meiosis). In this case, most of their cells would be α-galactosidase positive, and the daughters would exhibit no clinical symptoms. It is noteworthy that the heterozygosity of individual III-3, whose mother is

TABLE 4

TIME OF X DIFFERENTIATION

Pairs of Tissues Examined for Covariance of α-Galactosidase A	No. Embryonal Cells at X Differentiation*
WBC/serum	6
Serum/hair roots	7
WBC/hair roots	5

* Estimates are based on data in figure 2.

severely affected, could only be proven by hair root analysis; serum and white blood cell α-galactosidase activity was in the normal range (table 3).

Preferential X inactivation is known in mice [18] but unknown in man [19]. Mutations causing only moderate distortion of the embryonal mosaic need not be associated with a reduced reproductive fitness of heterozygous carriers. Thus, selection might be limited, and therefore the possibility of multiple allelism at this postulated X chromosomal locus cannot be ruled out. In man, different alleles of this kind would be detected only in families with a suitable X-linked marker. Studies of the familial occurrence of symptomatic Duchenne heterozygotes [4], as well as similar analyses dealing with hemophiliacs (A. E. H. Emery, personal communication, 1976), suggest that the expression of X-linked genes in heterozygotes is under some sort of genetic control.

An additional model is concerned with the *time of X-chromosome inactivation;* if it were controlled genetically (as suggested by one of the reviewers of the present paper), mutations leading to early inactivation might also explain the frequent familial occurrence of manifesting carriers. A mutation of this kind might be responsible for the observations in our family, too.

SUMMARY

Severe clinical signs of Fabry disease were observed in four of eight heterozygous daughters of a male patient. Activities of α-galactosidase A in serum, white blood cells, and hair roots of the manifesting carriers were markedly lower than 50% of normal. These findings are not easy to interpret in terms of random X inactivation alone; several alternative models including nonrandom (preferential) X inactivation are discussed.

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