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Genetics

Adrenoleukodystrophy: Evidence for X linkage, inactivation, and selection favoring the mutant allele in heterozygous cells

(glucose-6-phosphate dehydrogenase linkage/long-chain fatty acids/skin fibroblast clones/gene mapping/adrenomyeloneuropathy)

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ABSTRACT **Skin fibroblasts of human males affected with adrenoleukodystrophy (ALD) have previously been shown to be abnormal with respect to C_{26} fatty acid content. Skin fibroblast clones from heterozygotes in three families segregating this mutation have been analyzed and are of two types: clones with normal ratios of C_{26} to C_{22} fatty acids and clones with an excess of C_{26} fatty acids similar to that found in cells of affected males. This indicates not only that the locus is X linked but also that it is subject to inactivation. In most of the heterozygotes there were significantly more clones of abnormal type than those expressing the normal allele, indicating a proliferative advantage *in vitro* for skin fibroblasts of mutant type. The increased levels of fatty acids in plasma in most heterozygotes and the phenotype of blood cells of women heterozygous for both ALD and glucose-6-phosphate dehydrogenase (G6PD) in one family are evidence that selection favoring the mutant allele may occur *in vivo* as well as *in vitro* and may explain why many heterozygotes manifest clinical symptoms of the disease. These studies have also revealed the close linkage between *ALD* and *G6PD* loci, because there are no recombinants among 18 informative offspring of doubly heterozygous mothers. Therefore, the *ALD* locus can be mapped on the human X chromosome near the *G6PD* locus at Xq28.**

Adrenoleukodystrophy (ALD), a lipid storage disease, is characterized by adrenal insufficiency and progressive demyelination of the cerebral white matter (1). The onset of symptoms is usually between 4 and 8 years of age, with death in 1–4 years. ALD is believed to be an X-linked mutation, on the basis of the pattern of inheritance; usually males are affected and no male-to-male transmission has been reported. ALD has many features in common with a more indolent neurological disorder, adrenomyeloneuropathy (AMN), which affects females as well as males. Characteristic cytoplasmic inclusions and the accumulation of very long chain fatty acids are found in both disorders (2). Stronger evidence for a relationship between the two disorders is that they cosegregate in the same family (3), and skin fibroblasts from individuals affected with either ALD or AMN are abnormal with regard to the quantity of C_{26} long-chain fatty acids (4–6).

Because the ALD phenotype is demonstrable in cultured fibroblasts, we initiated studies of heterozygous females to determine if this locus is indeed X linked, if it is subject to X chromosome inactivation (7), and to explore the relationship with AMN. Our strategy was to analyze skin fibroblast clones from heterozygotes to determine if some clones were normal and others expressed the mutant gene, as expected for X-chromosomal loci at which inactivation occurs (8). Studies of families segregating not only the ALD mutation but also electrophoretic variants of glucose-6-phosphate dehydrogenase (G6PD) revealed that the ALD mutation is X linked and that the locus is subject to inactivation and is closely linked to *G6PD*. These studies also suggest that the mutant allele has a selective advantage in some tissues of the ALD heterozygote.

MATERIALS AND METHODS

Subjects. Subjects of these studies were males from three unrelated families (Fig. 1) with clinical and biochemical evidence of ALD as well as obligate heterozygotes and other females at risk in these families. Two of the three families were segregating G6PD electrophoretic variants as well. Skin biopsy samples and blood specimens were obtained from each subject.

Cell Culture. Fibroblast cultures were maintained in minimal essential medium (GIBCO) enriched with 15% fetal calf serum and nonessential amino acids. Fetal skin and amniotic fluid fibroblast cultures were established from tissues of a female abortus at risk for ALD.

Clonal Analysis. Clones were obtained from dilute suspensions of fibroblasts in second subculture. Approximately 10–20 cells were plated into 60-mm (Falcon) tissue culture dishes. At 8–10 days after plating, clones were isolated with cylinders, the cells were removed with trypsin, and clonal cultures were established. Replicate cultures of clones from each female at risk were assayed for G6PD and ALD phenotypes at approximately the second subculture after isolation. The uncloned fibroblasts assayed from each heterozygote were usually the cultures from which clones were derived. Cells of these cultures were harvested and assayed earlier than their clonal derivatives.

Phenotypic Analysis. The G6PD phenotype of fibroblasts, blood specimens, and hair follicles was determined by electrophoresis using cellulose acetate gels in buffer (0.2 M Tris/0.5 mM Na_2EDTA/0.05 M glycine/0.05 M sucrose) at pH 9.2, 350 V for 23–27 min. Leukocytes were separated from heparinized blood specimens drawn less than 2 days previously so that both erythrocyte and leukocyte phenotypes could be determined.

The ALD phenotype was determined (4, 5) on duplicate cultures of fibroblasts (10^6 cells per culture). Total lipid extracts of these clonal fibroblasts were hydrolyzed, their fatty acid composition was determined by gas/liquid chromatography, and the weight ratio of C_{26} to C_{22} fatty acids was calculated. The C_{26}:C_{22} ratio was also determined for plasma from heterozygotes and affected males (9).

RESULTS AND DISCUSSION

Evidence for X Chromosome Inactivation. Table 1 shows C_{26}:C_{22} ratios for skin fibroblast specimens from controls and

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Abbreviations: ALD, adrenoleukodystrophy; AMN, adrenomyeloneuropathy; G6PD, glucose-6-phosphate dehydrogenase.

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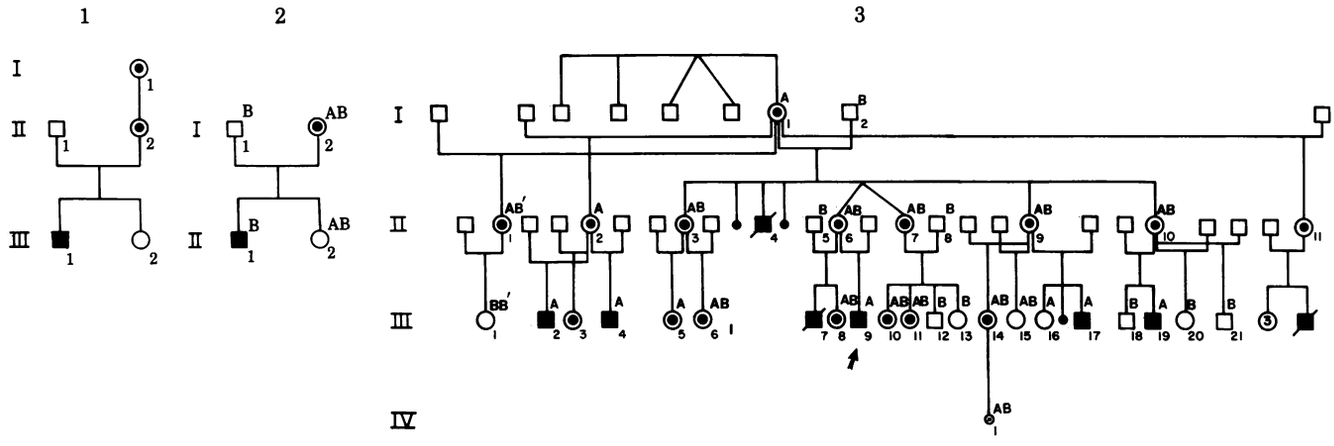

FIG. 1. Pedigrees of families segregating ALD. ■, Affected males; ◉, heterozygotes; A and B, G6PD genotypes.

members of three families segregating the mutation. The C_{26} fatty acid content of uncloned fibroblasts from mothers of affected males in families 1, 2, and 3 was obviously abnormal—i.e., C_{26}:C_{22} ratio significantly greater than 0.077 ± 0.03, the mean value ($\pm$SD) for normal individuals (6). The variability in the increase of C_{26} fatty acids from one assay to another (in affected males as well as heterozygotes) may reflect problems inherent in the assay; nonetheless, assays from replicate cultures were generally consistent. In family 2, heterozygote I-2, mother of II-1, was also heterozygous for G6PD A. Clones obtained from her skin fibroblasts were first assayed for G6PD phenotype and then replicate cultures were analyzed for the ALD phenotype without knowledge of the G6PD type. G6PD A clones from this heterozygote expressed the normal allele at the ALD locus, whereas those with G6PD B were of the mutant ALD type. In contrast, skin fibroblasts from the sister of this affected male, although heterozygous for G6PD, had normal C_{26} fatty acids and clones of both G6PD A and G6PD B type expressed the normal ALD allele (Table 1). Mosaicism with regard to ALD phenotype was also observed in clones from the mother (II-6) of an affected male in family 3. In this case, however, those of the G6PD A type had an abnormal C_{26}:C_{22} ratio, whereas those of the G6PD B type had normal C_{26} fatty acids. Similarly, a female cousin (III-6) of the affected male also had two populations of clones. The mutant ALD phenotype was observed in clones of G6PD A type, although values for her uncloned fibroblasts were not abnormal (Table 1). Because the heterozygote has clones with abnormal amounts of C_{26} fatty acids as well as clones of normal type, we conclude that the ALD locus is X linked and subject to inactivation.

Table 1. Ratio of C_{26} to C_{22} fatty acids in skin fibroblasts from heterozygotes and controls in three unrelated families

Subject	C_{26}:C_{22} ratio*		
	Uncloned fibroblasts	Cloned fibroblasts	
		G6PD A	G6PD B
	Family 1		
III-1, affected male	0.740, 0.594		
II-2, mother of III-1	0.667, 0.650		
	Family 2		
II-1, affected male	0.751, 0.944 0.356, 0.366		
I-2, mother of II-1	0.490, 0.420 0.536, 0.544	0.075, 0.140 0.192, 0.126	0.447, 0.512 0.420, 0.393 0.534, 0.650 0.500, 0.409
II-2, sister of II-1†	0.136, 0.112	0.085, 0.143 0.119, 0.082	0.219, 0.163 0.132, 0.058
	Family 3		
III-9, affected male	0.760, 0.792		
II-6, mother of III-9, obligate heterozygote	0.331, 0.365	0.469, 0.594 0.488, 0.672 0.331, 0.379 0.568, 0.893	0.069, 0.115 0.176, 0.126
III-6, cousin of III-9	0.196, 0.132	0.443, 0.433 0.410, 0.420 0.413, 0.396	0.146, 0.146 0.156, 0.192 0.155, 0.188
III-12, normal male	0.068		
III-18, normal male	0.061		
III-20, normal male	0.069		

* Paired values are for duplicate cultures assayed simultaneously.
† Presumably noncarrier female.

Evidence for *in Vitro* Selection. Further analysis of clones from heterozygotes was carried out to determine the proportion with abnormal C_{26} fatty acids . All clones of the heterozygote from family 1 were of the ALD type and in family 3 most (94%) were of mutant type (Table 2). That the predominance of ALD type clones was attributable to some proliferative advantage of clones expressing the mutant gene and not to an abnormality of clones expressing the normal allele is seen from studies of the heterozygote in family 2. In this case, clones that were picked early, the faster proliferating ones, were predominantly of the ALD type, whereas at 13 days both kinds of clones were present in more equal amounts.

Table 2. Percent of skin fibroblast clones of ALD type from mothers of unrelated affected males

Family	Hetero-zygote	Cloning efficiency*	Number of clones	
			Analyzed	% mutant
1	II-2	0.53	19	100
3	II-6	0.10	32	94
2	I-2	0.44	27†	87†
			18‡	55‡

* Number of clones obtained divided by number of cells plated.
† Clones picked at 8 days.
‡ Clones picked at 13 days.

Table 3. Characteristics of ALD heterozygotes from studies of skin fibroblasts (family 3)

Heterozygote	Age, years	C_{26}:C_{22} ratio in uncloned culture*	Cloning efficiency	No. of clones analyzed	% ALD clones in specimen	G6PD pattern in uncloned culture*†
I-1	59	0.425	0.25	6	100	
II-10	35	0.216	0.49	14	71	A >> B
			0.44	15	80	
II-9	32	0.411	0.16	7	100	A >> B
II-7	31	0.445	0.16	13	69	A >> B
II-6	31	0.485	0.10	32	94	A >>> B
II-3	26	0.323	0.18	12	83	A >> B
II-2	25	0.247	0.14	11	33	
II-1	23	0.348	0.09	11	100	A >> B′
III-14	14	0.664	0.22	11	73	A >>> B
III-11	14	0.098	0.46	19	42	B >> A
III-10	13	0.174	0.42	15	80	A >> B
III-8	8	0.337	0.28	13	54	A ≥ B
III-6	9	0.167	0.65	15	33	A > B
III-5	8	0.171	0.46	10	70	
III-3	6	0.302	0.20	12	42	A = B

* The uncloned cultures were the sources of the clones whose characteristics are given in the other columns.
† In uncloned cultures of double heterozygotes only.

To determine if the mutant gene, in fact, gave a selective advantage to mutant fibroblasts of ALD heterozygotes, we carried out studies of females at risk in the large family segregating both G6PD and ALD variants. The pedigree of family 3 (Fig. 1) shows the typical X-linked transmission of the ALD mutation from grandmother to carrier daughters to affected grandsons and includes an aborted fetus in generation IV. Many females in this family are double heterozygotes, being heterozygous for G6PD as well. This facilitated our ability to look at the proportion of ALD type cells among skin fibroblast cultures. In known heterozygotes in this family, ALD studies were carried out in selected fibroblast clones of G6PD A type, whereas the proportion of ALD type clones was determined on the basis of G6PD type alone.

Table 3 and Fig. 2 compare the C_{26}:C_{22} ratio in skin fibroblast cultures of the heterozygote with the proportion of mutant clones obtained from that specimen. Many of the heterozygous values approached those of an affected male, and these were generally for specimens from which a large percent of mutant clones were obtained. Not all heterozygous values, however, were outside of the normal range, and at least some of the lower values reflect a low percentage of ALD clones in the mixture.

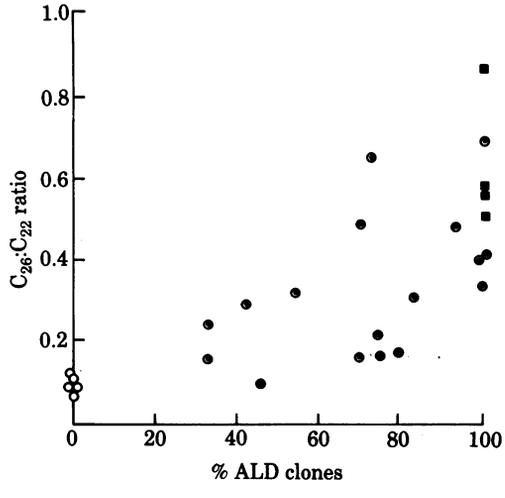

FIG. 2. Relationship between C_{26}:C_{22} ratio in uncloned skin fibroblasts of heterozygotes in family 3 and the proportion of mutant clones derived from that specimen. ○, Normal females; ■, affected males; ●, heterozygotes.

Table 3 also shows the relationship between C_{26}:C_{22} ratio in uncloned specimens and the age of the heterozygotes. It is clear that the abnormal values may occur at all ages. On the other hand, most of the normal values belong to the younger heterozygotes.

Evidence for Linkage with G6PD. Because many of the females we studied are heterozygous for G6PD as well as ALD, they were informative for linkage relationships between the two loci. Table 4 shows the coupling of *G6PD* and *ALD* alleles in progeny of doubly heterozygous mothers. In family 2, only two

Table 4. Coupling of *G6PD* and *ALD* alleles in progeny of doubly heterozygous mothers

Family	Subject	*G6PD* genotype	*ALD* genotype	Coupling	
2*	II-1	*B*	Affected ♂	*G6PD B, ALD*	N.R.
	II-2	*AB* (*A*)†	Normal ♀	*G6PD A*, +	N.R.
3‡	III-9	*A*	Affected ♂	*G6PD A, ALD*	N.R.
	III-17	*A*	Affected ♂	*G6PD A, ALD*	N.R.
	III-19	*A*	Affected ♂	*G6PD A, ALD*	N.R.
	III-12	*B*	Normal ♂	*G6PD B*, +	N.R.
	III-18	*B*	Normal ♂	*G6PD B*, +	N.R.
	III-21	*B*	Normal ♂	*G6PD B*, +	N.R.
	III-5	*A* (*A*)	Carrier ♀	*G6PD A, ALD*	N.R.
	III-6	*AB* (*A*)§	Carrier ♀	*G6PD A, ALD*	N.R.
	III-8	*AB* (*A*)§	Carrier ♀	*G6PD A, ALD*	N.R.
	III-10	*AB* (*A*)§	Carrier ♀	*G6PD A, ALD*	N.R.
	III-11	*AB* (*A*)§	Carrier ♀	*G6PD A, ALD*	N.R.
	III-14	*AB* (*A*)§	Carrier ♀	*G6PD A, ALD*	N.R.
	IV-1	*AB* (*A*)§	Carrier ♀	*G6PD A, ALD*	N.R.
	III-1	*BB′* (*B′*)¶	Normal ♀	*G6PD B′*, +	N.R.
	III-13	*B* (*B*)	Normal ♀	*G6PD B*, +	N.R.
	III-20	*B* (*B*)	Normal ♀	*G6PD B*, +	N.R.

N.R., nonrecombinant.
* Maternal genotype: *G6PD B, ALD/G6PD A*, +.
† Maternal allele based on paternal genotype.
‡ Maternal genotype: *G6PD A, ALD/G6PD B*, +.
§ Maternal allele by clonal analysis.
¶ G6PD variant, migrates slower than B.

Table 5. G6PD phenotypes of heterozygous females in families segregating the ALD mutation

Family	Subject	Age, years	ALD status	C_{26}:C_{22} ratio		Relative skewing of G6PD phenotype* (predominant allele)			
				Plasma†	Skin fibroblasts	Skin fibroblasts	Erythrocytes	Leukocytes	Hair follicles
2	I-2	34	Carrier	0.019	0.492	++ (*B*)	−	−	
	II-2	13	Normal	0.006	0.124	−	−	−	
3	II-1	23	Carrier	0.022	0.348	++ (*A*)	+ (*A*)	± (*A*)	
	II-3	26	Carrier	0.047	0.323	++ (*A*)	+++ (*A*)	+++ (*A*)	++++ (*A*)
	II-6	31	Carrier	0.044	0.348‡	+++ (*A*)			
					0.485‡	++++ (*A*)			
					0.700‡	+++ (*A*)			
	II-7	31	Carrier	0.035	0.445	++ (*A*)	+ (*A*)	+ (*A*)	
	II-9	32	Carrier	0.052	0.411	++ (*A*)	+ (*A*)	+ (*A*)	−
	II-10	35	Carrier	0.036	0.216	++ (*A*)	++ (*A*)	+++ (*A*)	++++ (*A*)
	III-3	6	Carrier	0.050	0.302	−	−	−	
	III-6	9	Carrier	0.032	0.167	± (*A*)	++ (*A*)	++ (*A*)	
	III-8	8	Carrier	0.047	0.337	++ (*A*)	+++ (*A*)	++ (*A*)	
	III-10	13	Carrier	0.015	0.174	± (*A*)	−	−	−
	III-11	14	Carrier	0.016	0.098	++ (*B*)	+++ (*B*)	++ (*B*)	++ (*B*)
	III-14	14	Carrier	0.027	0.664	+++ (*A*)	−	± (*A*)	
	IV-1	Fetus	Carrier		1.090	++++ (*A*)			
	III-1	6	Normal	0.005	0.105	−	−	−	
	III-15	13	Normal	0.006	0.100	+ (*B*)	++ (*B*)	++ (*B*)	
	III-16	11	Normal	0.004	0.071	++ (*A*)	−	± (*A*)	

* Plus signs indicate degree of skewing; plus-or-minus signs indicate slight to negligible skewing; minus signs indicate no skewing.
† Plasma C_{26}:C_{22} ratio (mean ± SD): normal, 0.014 ± 0.0023; affected, 0.064 ± 0.0046.
‡ Three biopsy specimens.

individuals are informative, and both are nonrecombinants. The son received his mother's *G6PD B* allele along with her mutant *ALD* allele, whereas the normal daughter must have received the *G6PD A* allele from her mother because her father is of *G6PD B* type (Fig. 1). Sixteen informative individuals have been ascertained in family 3 (Fig. 1). Affected males in this family have the *G6PD A* allele, whereas normal sons are *G6PD B*. Therefore, all of the sons are nonrecombinants. The coupling of maternal alleles in daughters of doubly heterozygous females has been determined for the most part on the basis of analysis of clones—that is, by determining the G6PD type of the clones with abnormal fatty acid content. As was the case with the males, all daughters are nonrecombinants. Because no recombination has been observed in 18 informative individuals, there seems to be a close physical relationship between the *ALD* and *G6PD* loci—at least within 15.7 centimorgans, at the 95% confidence level (B. Keats and N. E. Morton, personal communication). The close linkage between the two loci permits the regional localization of the *ALD* locus to the distal long arm of the X chromosome near Xq28, where the *G6PD* locus has been mapped (10).

G6PD as Marker for ALD Cells. Because of the close linkage between the two relevant loci, the G6PD A isozyme in family 3 reflects the proportion of cells in the heterozygote that are of the ALD type. The C_{26}:C_{22} ratio in skin fibroblasts from double heterozygotes correlates not only with the percent of ALD clones but with the G6PD pattern as well (Tables 3 and 5). For instance, those individuals with the most abnormal ratio of fatty acids frequently have significant skewing of their G6PD patterns with predominance of G6PD A. These observations confirm the proliferative advantage *in vitro* of cells expressing the ALD mutation and show that in double heterozygotes it is reflected as an advantage of the *G6PD A* allele.

Evidence for *in Vivo* Selection. Because the observed proliferative advantage for the mutant allele might be merely an *in vitro* phenomena, we wished to determine if cells expressing the ALD phenotype were selected *in vivo* as well. Initial studies of erythrocytes and leukocytes from heterozygotes in family 3 showed a consistent predominance of *G6PD A* (Fig. 3). However, as further specimens were analyzed, we observed a discordance between the prominence of one clonal population in blood cells of the heterozygote and the presence of the ALD mutation. There was no imbalance in clonal populations of erythrocytes or leukocytes in several heterozygotes, even when ALD clones prevailed in skin fibroblast cultures (i.e., II-1 in family 2 and III-10 in family 3). More important perhaps is that, in one carrier (III-11), the cell population favored was the one expressing the *G6PD B* allele, associated in this female with the wild-type *ALD* allele. Therefore, although the excess of ALD clones observed in heterozygotes from three unrelated families is most likely attributable to a proliferative advantage of fibro-

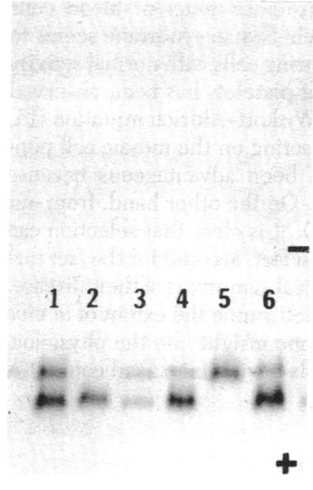

FIG. 3. G6PD phenotype of leukocytes from females in family 3, with *G6PD A* coupled with the mutant *ALD* allele in carriers. The notation is as in Table 5. Lane 1, III-16, noncarrier, ± (*A*); lane 2, G6PD A control; lane 3, G6PD A and G6PD B; lane 4, II-3, carrier, +++ (*A*); lane 5, G6PD B control; lane 6, III-6, carrier, ++ (*A*).

blasts expressing the mutant allele at the *ALD* locus, it is not as certain that the predominance of ALD cells in blood specimens (observed as prominence of the coupled *G6PD* allele) is in fact attributable to the ALD mutation. It may be that the skewing, at least in blood cells, results from the influence of some other gene closely linked to *G6PD* and *ALD* loci that provides a proliferative advantage for blood cells. The existence of such an X-linked gene has been postulated previously on the basis of observations in families segregating an unusual G6PD variant (11). Alternatively, because selective advantage is influenced by all the X-linked genes and modifiers expressed in a cell, it may be that another X gene (or autosomal locus) with major effects on cell proliferation is modifying or masking the proliferative advantage of the ALD mutation in heterozygote III-11. Supporting this genetic hypothesis is that her heterozygous sister, III-10, was also exceptional in that she also did not show the expected predominance of ALD type cells in any of her tissues that we analyzed. Another exceptional heterozygote (II-2) whose ALD mutation was not associated with a proliferative advantage had a different father and hence a different paternal X chromosome than her sisters with predominance of ALD cells.

In any event, even if there is no selective advantage of the ALD mutation in blood cells, it seems likely that there is a predominance of ALD cells in other tissues of the heterozygote. Many heterozygous females have clinical manifestations of the ALD mutation and have been classified as AMN or multiple sclerosis. Heterozygote II-6 in family 3 has evidence of cerebral demyelination as well as peripheral neuropathies and other severe cases have also been reported among females (6). Although the onset of disease occurred relatively late, heterozygotes I-2 (family 3) and I-1 (family 1) also have significant neurological abnormalities and are confined to wheelchairs. Other evidence to support the hypothesis that the mutant allele has a selective advantage *in vivo* is that the plasma C_{26} fatty acids are increased in most of the heterozygotes (Table 5 and ref. 9), perhaps reflecting an excess of liver cells of mutant type. In addition, cultures of liver as well as skin from the fetus (individual IV-1 of family 3) have eliminated cells with the normal fatty acid content. The heterozygous phenotype in this case was demonstrable only in cultures of lung fibroblasts, further evidence that the mutant allele in liver cells of heterozygotes may give a selective advantage.

Selection favoring one cell population in the heterozygote has been observed at other X-linked loci (12). Distribution of G6PD erythrocyte phenotype in an unbiased sample of females heterozygous for the G6PD Mediterranean mutation is skewed in favor of cells expressing the normal allele (13). The paucity of hypoxanthine phosphoribosyltransferase-deficient blood cells in obligate heterozygotes for Lesch–Nyhan syndrome seems to be a consequence of selection favoring cells with normal activity (14). Furthermore, elimination of platelets has been observed in heterozygotes expressing the Wiskott–Aldrich mutation (15, 16). In these instances selection acting on the mosaic cell population in the heterozygote has been advantageous because mutant type cells are eliminated. On the other hand, from our studies of heterozygotes for ALD, it is clear that selection can favor the mutant allele and may, in fact, account for the fact that some heterozygotes manifest clinical symptoms of their disease. Studies of other families should determine the extent of *in vivo* selection and perhaps provide some insight into the physiological basis for the advantage of cells having increased content of long-chain fatty acids.

Because of the well-documented cytoplasmic inclusions in brain and adrenal glands of affected males (1) and observations of morphological abnormalities in skin fibroblasts of heterozygotes (17) we looked for microscopic evidence of cytopathology, but we observed no consistent differences in microscopic appearance of normal and mutant cells. It is, therefore, unlikely that cell morphology will be useful to detect the heterozygote or the affected fetus. However, the close linkage between *ALD* and *G6PD* loci provides the means to detect the presence of the *ALD* allele prenatally in families cosegregating variants at both loci. Furthermore, because skin fibroblasts of the heterozygous fetus that we studied have increased C_{26} fatty acids it should be possible to identify an affected fetus directly by analysis of the fatty acid content of amniocytes (18).

The *in vitro* selective advantage of the mutant allele in females heterozygous for ALD facilitates carrier detection at this locus, in contrast to the difficulty in identifying carriers at other X-linked loci, where the mutant gene is frequently disfavored. As a consequence, studies of families with ALD may be more useful than those of families with other X mutations have been to estimate the incidence of new mutations at X-chromosomal loci.

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