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Activity of the Fragile X in Heterozygous Carriers

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SUMMARY

Chromosome analyses with conventional stain, Q- and G-banding, and R-banding with 5-bromodeoxyuridine (BrdU) incorporation were performed on the lymphocytes of two sisters who are heterozygous for the fragile X chromosome and clinically diagnosed as slow learners. Two heterozygous relatives with normal intelligence were used as controls. The frequencies of the active fragile X for the "slow" females were 100/129 (77.5%) and 85/120 (70.8%) compared with 40/78 (51.3%) and 10/32 (31.3%) for controls, the differences being highly significant. These observations are consistent with the Lyon hypothesis: activity of the abnormal X could account for the reduction in mental ability of some heterozygous females. Similar to retarded males with the fragile X chromosome, our slow learners had verbal scores that were lower than performance scores.

INTRODUCTION

The history of the fragile X mental retardation syndrome has been succinctly traced by Hecht and Kaiser-McCaw [1] and Gerald [2] from the first significant but neglected observation of a marker X chromosome by Lubs [3] to the important experimental contributions by Sutherland [4–6] that stimulated sudden and widespread interest in this relatively common X-linked disorder. The details need not be repeated here. Suffice it to say that a fragile site at the distal end of the long arm of X is revealed when lymphocytes are cultured in medium deficient in folic

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acid, affected males have a variable phenotype, and some carrier females have been shown to be slow learners.

One kindred with two retarded males, both carriers of the fragile X, was first investigated in Calgary, Alberta, Canada, where one retarded boy was identified [7]. Special interest in this family was aroused when it was learned that two sisters, cousins of this boy, had been diagnosed as "semiretarded." An ideal opportunity presented itself to test the Lyon hypothesis with a morphologically identifiable X chromosome.

FAMILY HISTORY

The members of this kindred have been able to trace their origin to 15th-century England before the family immigrated to "New England," and then to Ontario, Canada, after the Revolutionary War. One member of the founding family in Ontario married a woman who is thought to have been a North American Indian and who is the maternal grandmother of an obligate carrier of the fragile X (fig. 1, subject I-2). The latter now resides in British Columbia. Two of her progeny moved to Calgary (subjects II-1 and II-5). Only two retarded males have been identified, but more remote branches of this kindred have not yet been investigated.

The studies reported here are focused on the family with the two slow learners (subjects III-3 and III-5). They were ages 26 and 21 when they were karyotyped, and their mother (subject II-4), age 50. All three are tall and slim. No physical abnormalities were noted. The two girls are shy and reticent and prefer to stay at home together in the evenings. Their occupations are simple: the older works in a welfare institution and the younger in a workshop for retardates. Their brother (subject III-4), age 23, is normal in appearance and intelligence. Their mother had no other pregnancies.

MATERIALS AND METHODS

Eleven members of this kindred were evaluated in our laboratory. Eight subjects were karyotyped in Calgary, four of whom were among the 11 seen here. Ten-ml blood samples were drawn from all subjects and handled in routine fashion as follows: two macrocultures with 2 ml of lymphocyte-rich plasma from each sample were set up in 10 ml of TC medium

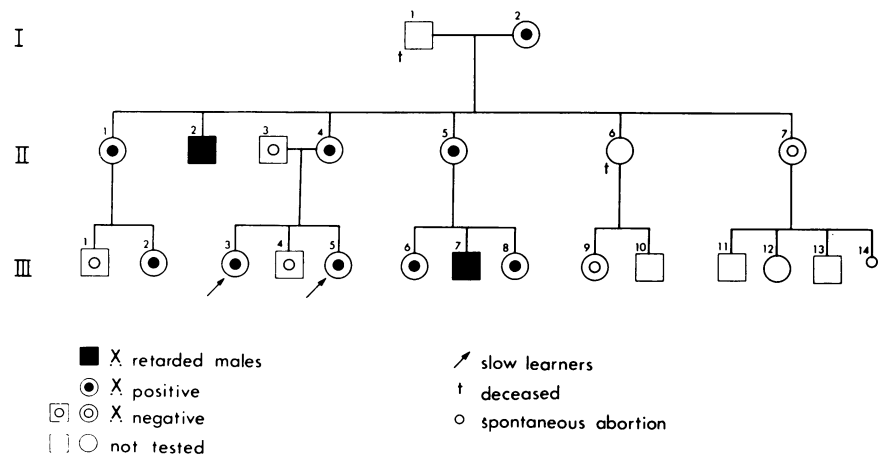

FIG. 1.—Pedigree of fragile X family with two semiretarded females

199 that had been adjusted to pH 7.8. Two ml of human AB serum was added to one culture and none to the other. This procedure is routine in our laboratory because some lymphocytes appear to grow better without adding serum of any kind. Fetal calf serum is never used. Conventional Giemsa staining was done first to screen for C-group chromosomes with fragile sites in the distal region of the long arm. Q- or G-banding followed to identify the marker chromosome. Because it is difficult to see a fragile site in overly contracted chromosomes, only cells with fairly long metaphase chromosomes were analyzed.

The lymphocytes from additional blood samples obtained from the two mentally handicapped girls were tested for inactivation of the X chromosome by BrdU incorporation [8]. Lymphocytes of their mother and an aunt (subject II-5), who is an obligate carrier, were similarly treated and used as controls. Six hrs before harvest, BrdU was added to each cell culture to a final concentration of 23 mg/l. Colcemid was added 4 hrs later. All slides were first stained with Giemsa to identify cells with fragile chromosomes. They were then counterstained with acridine orange to verify the marker chromosome to be an X and to determine which was the early replicating or active X chromosome. All slides were coded before analysis.

Both slow-learner girls had been tested with the Wechsler Adult Intelligence Scale (WAIS) by their psychiatrist before the cytogenetic diagnosis was made.

RESULTS

Frequencies of Fragile X

Examples of X chromosomes with fragile sites are shown in figure 2. The defect can be seen to occur at or distal to the interface between bands q27 and q28 near the tip of the long arm. It is therefore designated Xq28 [9]. No differences were observed in the quality of the preparations or in the frequencies of the fragile X between cultures with and without AB serum. The results for the 11 individuals tested are shown in table 1. Also listed are the frequencies reported by Martin et al. [7]. There is good correlation between the two laboratories for the affected males but our frequencies for the carrier females are somewhat higher. Relatively high frequencies for the marker chromosome in the two handicapped girls were observed.

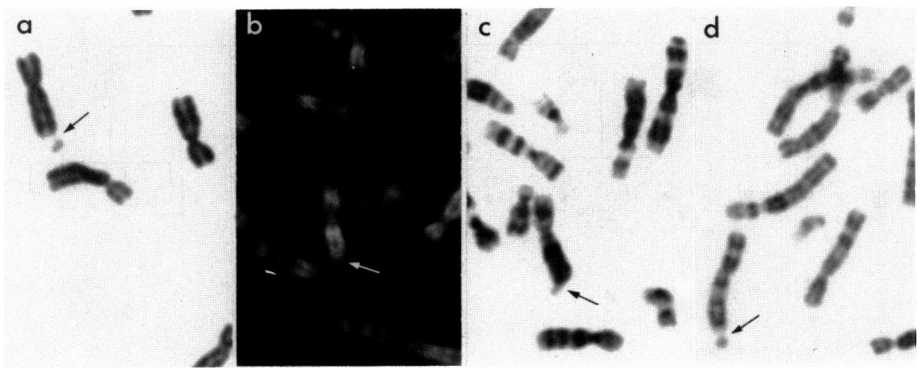

FIG. 2.—Marker X chromosomes of an affected male: *a*, conventional Giemsa stain; *b*, QFQ banding; *c*, GTG banding; *d*, RBG banding.

TABLE 1

FREQUENCIES OF FRAGILE X IN LYMPHOCYTES OF FAMILY REPORTED HERE. BRDU NOT ADDED TO CULTURES

PEDIGREE NO.	SEX	AGE	NORMAL X	FRAGILE X		
				No.	%	% (Calgary)*
I-2	F	79	...	...	...	0.5
II-1	F	53	110	4	3.6	1.5
II-2	M	52	97	3	3.0	3.0
II-3	M	52	100	0	0	...
II-4 (J. R.)	F	50	177	7	3.8	...
II-5 (S. H.)	F	48	107	3	2.7	1.0
II-7	F	39	100	0	0	...
III-1	M	27	...	...	...	0
III-2	F	25	...	...	...	6.5
III-3 (L. R.)	F	26	167	14	7.7	...
III-4	M	23	100	0	0	...
III-5 (S. R.)	F	21	167	17	9.2	...
III-6	F	28	...	...	...	0.5
III-7	M	26	92	16	14.8	15.5
III-8	F	19	...	...	...	0.5
III-9	F	24	120	0	0	...

NOTE: Initials refer to subjects in tables 2 and 3.
* Frequencies obtained by Martin et al. [7].

Active and Inactive X Chromosomes

Examples of active and inactive X chromosomes are shown in figure 3. In table 2 are presented the frequencies of fragile X chromosomes found after BrdU incorporation. BrdU has been shown to inhibit almost completely the expression of fragile sites [5]. Evidence of this phenomenon can be seen by comparing the frequencies for the same subjects listed in tables 1 and 2, but the reductions in frequencies were not such as to interfere with our investigation. However, thousands of cells had to be screened to obtain samples large enough for statistical treatment.

The fragile X is active more frequently than the normal X in the two daughters (table 3), the increases over the expected 1:1 ratio being highly significant (subject S. R.: $\chi^2 = 10.4, P < .002$). The frequency of the active marker X in one control (subject S. H.) is significantly lower than expected: $P < .04$. It was not possible to obtain more cells to increase the sample size of the latter. The difference between subject S. R. and her mother, subject J. R., is significant at the .006 confidence level ($\chi^2 = 7.8$).

Wechsler Adult Intelligence Scale

WAIS scores for the two girls were provided by their psychiatrist. The results for subject L. R. were: verbal 62, performance 81, and full score 68; for subject S. R.: verbal 66, performance 73, and full score 67. For both girls, the verbal scores were lower than the performance scores.

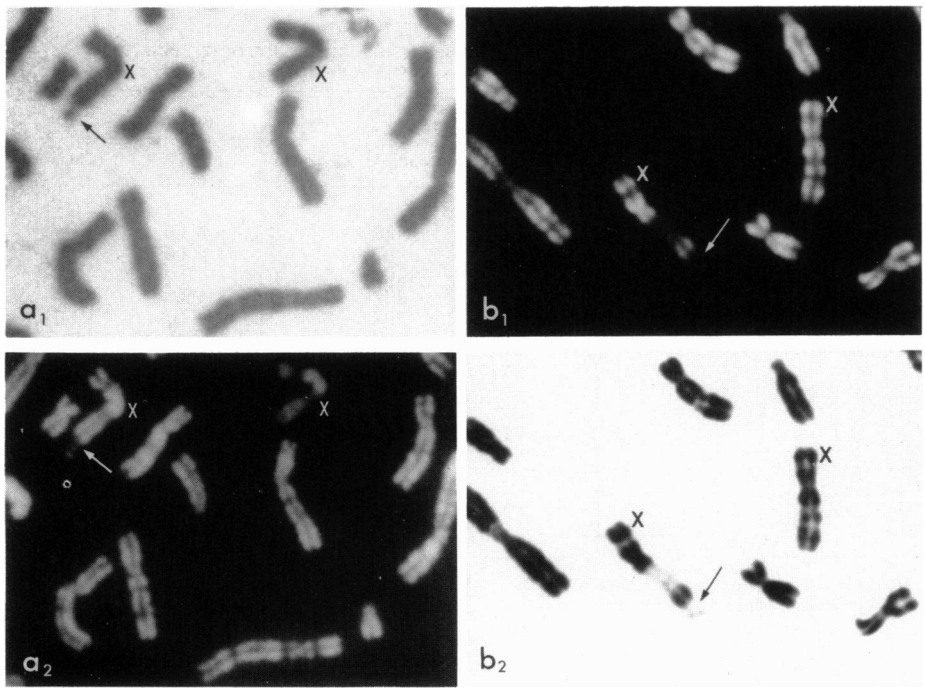

FIG. 3.—Examples of active and inactive fragile and normal X chromosomes. The same partial spreads are shown in a_1 and a_2 *on the left* and in b_1 and b_2 *on the right* following BrdU incorporation: a_1, conventional Giemsa stain without BrdU; a_2, RBA banding showing active fragile X and inactivated normal X; b_1, RBA banding; and b_2, RBG banding showing active normal X and inactivated fragile X.

DISCUSSION

With the identification of a fragile site in the X chromosome of mentally retarded males, a significant step has been taken toward the understanding of a serious but relatively common problem. A more tantalizing observation is the presence of slow learners among some female carriers of the marker X in kindreds of these retarded males [10]. The question posed is why some heterozygous carriers are mentally handicapped while others have normal intelligence. The obvious explanation is that the difference may be due to the activation or inactivation of the fragile X chromosome. The marker X and its variable expression in female carriers presented the first opportunity to test the Lyon hypothesis with a morphologically distinctive variant of the X chromosome.

The two affected sisters tested here were found to have higher frequencies of identifiable fragile chromosomes than do their normal relatives. Similar increased frequencies in semiretarded females were noted by Jacobs et al. [11] who postulated that mental status may be correlated with the frequency of marker X chromosomes, but no such correlation was found by Turner et al. [10]. Our study provides good evidence that the abnormal X is more frequently the functional chromosome

TABLE 2

FREQUENCIES OF FRAGILE X FOUND AFTER BRDU TREATMENT

SUBJECT	AGE	TOTAL CELLS SCREENED	NORMAL X	Fragile X	
				No.	%
L. R. III-3	26	2331	2173	142	6.1
S. R. III-5	21	3360	3204	126	3.8
J. R. II-4	50	2915	2811	82	2.8
S. H. II-5	48	1240	1202	33	2.7

in slow learners but not in normal heterozygotes. The same trend can be seen in the very limited data provided by Jacobs et al. [11]. These results are more compatible with the probability that the expression of chromosome fragility and mental ability are both secondary to X-chromosome inactivation.

The interpretation of the fragile X phenomenon must take into consideration other complicating factors. The detection of fragile X chromosomes is said to be more difficult with increasing age of heterozygous females [12]. This inverse correlation between fragility and age is reported to be supported by regression analysis [11]. The combined Hamilton-Calgary data, however, are not wholly consistent with an age-related increased resistance to fragility. Studies of the kindreds of mildly retarded female probands also do not support an age factor [10]. In our study, age-matched controls were not available for the repeat testing required. Moreover, it was not feasible to include cousins with a fragility frequency of 0.5%. Until less speculative evidence of the importance of age is forthcoming, age-matching of subjects and controls does not appear imperative.

An ascertainment bias in the selection of relatives of retarded male probands may account for the conflicting age data. Among older women, obligate carriers are more likely to be examined than are women who have no retarded sons, while among younger females, it is too early to identify obligate carriers. When a different method of ascertainment is used [10], the age factor is no longer evident. This problem cannot be resolved without conducting long-term longitudinal studies or unless more objective comparisons are made among subjects of the same sibship or even of the same generation rather than between generations.

TABLE 3

FREQUENCIES OF ACTIVE AND INACTIVE FRAGILE X CHROMOSOMES IDENTIFIED WITH RBA BANDING TREATMENT

Subject	Active	Inactive	% active	Noninformative
L. R.	100	29	77.5	13
S. R.	85	35	70.8	6
J. R.	40	38	51.3	4
S. H.	10	22	31.3	1

NOTE: Subject S. R. vs. expected: $\chi^2 = 10.4$, $P < .002$. Subject S. H. vs. expected: $\chi^2 = 4.5$, $P < .04$. Subject S. R. vs. J. R.: $\chi^2 = 7.8$, $P < .006$.

Whether fragile sites that are seen in lymphocyte chromosomes can affect intelligence is a moot question. A more reliable indicator would be frequencies derived from fibroblasts. Jacky and Dill [13] and Tommerup et al. [14] have reported success in inducing fragile sites in fibroblasts but the yield is low.

Of additional interest are the scores obtained from IQ testing of the two "slow" girls described here. In males with X-linked mental retardation, the WAIS test revealed lower verbal scores than performance scores [15–17]. Our subjects provided similar results for females. The verbal deficiencies probably account for their reticence and inability to make friends among their peers.

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