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A Comparison of Fragile X Expression in Lymphocyte and Lymphoblastoid Cultures

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SUMMARY

This study compares fragile X expression in peripheral blood lymphocyte cultures with expression in lymphoblastoid cell lines established from 23 individuals from families in which the fragile X is segregating. Most patients expressed the fragile X in lymphoblastoid cell lines treated with FUdR under optimal conditions at approximately the same frequency as in peripheral blood cultures from the same individual. No fragile X cells were seen in the lymphoblastoid cell lines from three phenotypically normal males who had transmitted the fragile X gene to offspring or in the lines from three phenotypically normal obligate-carrier females, all of whom were also negative in peripheral blood cultures. Two individuals, however, who expressed at high levels in peripheral blood lymphocytes expressed in lymphoblastoid cells only at low levels or not at all. We describe the considerations needed for the consistent demonstration of the fragile X in lymphoblastoid cell lines.

INTRODUCTION

The fragile X [fra(X)] syndrome is a relatively common type of X-linked mental retardation that is distinguished by the expression of a fragile site at Xq27 when

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cells of affected individuals are cultured under conditions of thymidylate stress [1, 2]. Although intracellular thymidylate availability appears to be critical to the expression of the fragile site, the mechanism by which shortage of thymidylate produces a gap or uncoiled region at the distal end of Xq is unknown. The fragile site is expressible in essentially all males having other features of the syndrome and also in most mentally retarded heterozygotes. It is expressed, however, in only some of the heterozygotes who are not mentally retarded, its expression being inversely related to the age of the individual [3]. Extensive recent evidence indicates that the fra(X) syndrome can be transmitted through nonaffected males who are neither retarded nor manifest characteristic facies or large testes. Moreover, such males do not express the fra(X), at least in cultures of their peripheral blood lymphocytes [4].

The fragile site at Xq27 has been detected in a variety of different tissues from affected individuals, although the specific conditions necessary to elicit expression vary among tissues and cell types. The easiest cells in which to demonstrate the fra(X) are peripheral blood lymphocytes in short-term culture. In such cells, the fra(X) is demonstrable by reducing the concentration of serum [5] and using a culture medium low in folic acid and thymidine, such as medium 199 or modified F-10. Initial attempts at demonstrating the fragile site in cells other than blood cells were largely unsuccessful [6, 7, 9]. Subsequently, however, more stringent conditions such as the addition of methotrexate (MTX) or fluorodeoxyuridine (FUdR) to the culture resulted in fra(X) expression in fibroblasts, amniocytes, and lymphoblastoid cells from affected individuals [9–11]. The fragile site is seen in only a proportion of cells of affected individuals regardless of the cell type used, and this proportion seldom, if ever, exceeds 50% [9, 12].

Since initially demonstrating the fra(X) in lymphoblastoid cell lines (LCL) [9], we have established many LCL from individuals in families with the syndrome. These lines provide an excellent immortalized source of cells for the study of fra(X) expression as well as a source of genomic DNA for linkage studies and to investigate the molecular nature of the fragile site. However, no systematic study of a comparison of fra(X) expression in peripheral blood lymphocyte cultures and LCL has been reported. Here, we report our data on fra(X) expression in short-term cultures of peripheral blood lymphocytes and in LCL established from 23 individuals from families in which the fra(X) is segregating.

MATERIALS AND METHODS

The 23 individuals whose blood and LCL we studied consisted of 11 mentally retarded males, all of whom expressed the fra(X) in their peripheral blood lymphocytes, six heterozygous females whose peripheral blood lymphocytes were fra(X) positive (two independent LCL being established from two of them), and six individuals, three males and three females, known from pedigree information to carry the fra(X) gene but in whom the fra(X) had not been demonstrated in peripheral blood lymphocytes.

Lymphoblastoid Cell Lines

All LCL except the following were established from peripheral blood lymphocytes at the Massachusetts General Hospital as previously described [9]: PO-1 provided by Dr.

S. Purvis-Smith, New South Wales, Australia; TL 11075 provided by Dr. T. W. Glover, Tempe, Ariz.; GM 6892 and GM 6906 obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, N.J. The lines were maintained in RPMI 1640 medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 20 mM Hepes buffer, pH 7.2, and penicillin/streptomycin. Experiments to score the fra(X) were initiated by suspending 3×10^5 cells/ml in either modified F-10 medium containing no thymidine, folic acid, or hypoxanthine (Gibco formula no. 78-5227, Grand Island, N.Y.) or in medium 199. Both media were supplemented with 5% fetal bovine serum and penicillin/streptomycin. Originally, we treated all cultures with 10^{-7}, 10^{-8}, and 10^{-9} M FUdR for 24 and 48 hrs. We found, however, that the fra(X) was seldom seen in cultures treated with 10^{-9} M FUdR and that its expression was consistently lower after exposure for 48 hrs than after 24 hrs. We therefore discontinued the use of 10^{-9} M FUdR and the longer FUdR exposure. Most cultures were brought to 10^{-7} M and 10^{-8} M FUdR at the time the cells were resuspended in modified F-10 or medium 199. The cultures were harvested 24 hrs later. For lines with a slow growth rate, FUdR was added 24–48 hrs after the cells had been transferred to modified F-10 or medium 199, since this seemed to reduce the cytotoxic effect of FUdR on these slow-growing cell lines. Colcemid was added to the cultures for the last 30 min of the incubation period and the cells harvested by standard cytogenetic techniques.

Lymphocyte Cultures

Lymphocyte cultures were established by injecting 0.2 ml of whole blood into 5 ml of modified F-10 medium supplemented with 5% fetal bovine serum, phytohemagglutinin, and penicillin/streptomycin. The cultures were incubated for 3–4 days, and, in a few instances, FUdR at a final concentration of 10^{-7} M was added for the last 24 hrs of culture. Colcemid was added for the last 45 min and the cells harvested by standard cytogenetic techniques.

In our experience, the proportion of lymphocytes showing the fra(X) is relatively low in cultures harvested after 2 or 2½ days at 37°C, increases to a plateau after 3–5 days incubation, and tends to fall somewhat in cultures incubated for longer than 5 days. The addition of FUdR to lymphocyte cultures did not increase the proportion of fra(X) positive cells although it did result in the maximum being reached earlier in the culture period. Since none of the variables used in our culture of peripheral blood lymphocytes made any systematic difference to the maximum proportion of fra(X) positive cells, we have pooled our results from a number of different cultures on each patient.

Fra(X) Scoring

Fra(X) scoring on both LCL and lymphocyte cultures was done blindly by two independent observers on nonbanded preparations stained either with orcein or 2% Giemsa, or on Q-banded slides.

RESULTS

Peripheral blood lymphocytes from 11 retarded males expressed the fra(X) in a high proportion of cells (table 1). In nearly every patient, however, the proportion of fra(X) positive cells varied considerably from culture to culture, the most extreme being MGL 50 in whom the proportion of fra(X) positive cells ranged from 16% to 51% on different occasions. We could find no systematic basis for this variability.

LCL from all 11 patients were fra(X) positive. In nine patients, the average proportion of fra(X) positive cells at the optimum FUdR concentration was very similar to that in peripheral blood lymphocytes. Patient MGL 44, however, expressed the fra(X) at high frequency in peripheral blood lymphocytes

TABLE 1

FRAGILE X IN PERIPHERAL BLOOD CULTURES (PBC) AND LCL FROM EXPRESSING MALES

PATIENT NO.	AGE*	MENTAL STATUS†	PBC			LCL			
			No. cells‡	% fra (X)	Range	FUdR	No. cells‡	% fra (X)	Range
MGL 29§	22	Mod	340 (3)	28	22–43	10^{-7} M	723 (10)	47	29–63
MGL 44	17	Mod	228 (4)	44	38–55	10^{-7} M	151 (3)	2	0–5
						10^{-8} M	150 (1)	0	...
MGL 48	19	Mild	296 (4)	41	31–54	10^{-7} M	58 (1)	38	...
MGL 50	39	Mild	865 (4)	33	16–51	10^{-7} M	63 (2)	29	23–32
MGL 51	36	Mod	306 (1)	29	...	10^{-7} M	20 (1)	25	...
						10^{-8} M	50 (1)	24	...
MGL 64	16	Mild	270 (2)	26	21–36	10^{-7} M	39 (1)	33	...
						10^{-8} M	91 (1)	32	...
MGL 75	51	Sev	637 (3)	25	18–36	10^{-8} M	172 (3)	46	34–60
MGL 83	11	Mild	495 (3)	24	18–30	10^{-8} M	50 (1)	24	...
MGL 84	14	Mod	135 (2)	43	30–52	10^{-8} M	62 (2)	36	32–41
MGL 89	37	Mod	135 (2)	29	25–34	10^{-7} M	50 (1)	24	...
						10^{-8} M	156 (2)	0	...
PO-1	12	Mod	272 (4)	45	27–51	10^{-7} M	50 (1)	34	...
						10^{-8} M	75 (1)	32	...

* When LCL established.
† Sev = severely retarded, IQ 20–35; Mod = moderately retarded, IQ 36–50; Mild = mildly retarded, IQ 51–70.
‡ No. in parentheses equals the no. experiments.
§ PBC data from [13], family P individual III-1.
PBC data from T. W. Glover, personal communication, 1985.

but in only 2% of his LCL cells examined on four occasions, twice in parallel with a fra(X) positive control. No fra(X) positive cells were seen in three attempts, and only two of 44 cells were positive in another sample. The reason for the low expression in the LCL from this patient is obscure. In MGL 89, fra(X) expression was not elicited by 10^{-8} M FUdR, although positive results were obtained in a control line simultaneously tested. Furthermore, MGL 89 could not be scored after treatment with 10^{-7} M FUdR because metaphases were absent, although the positive control grew well at this concentration. Therefore, we subsequently transferred the cells from RPMI to medium 199 for 48 hrs to allow this slow-growing line to adjust to the new medium before adding FUdR for an additional 24 hrs. Under these conditions, the mitotic index in MGL 89 was adequate at both 10^{-8} and 10^{-7} M FUdR. The fra(X) was expressed in 24% of MGL 89 cells at 10^{-7} M FUdR but was still not expressed at 10^{-8} M.

Table 2 shows data from six expressing females, from two of whom we established two independent LCL. While all were fra(X) positive in peripheral blood cultures, the proportion of fra(X) positive cells in different cultures from the same patient varied over a considerable range. Five patients showed a reasonable correspondence between the levels of fra(X) expression in peripheral blood lymphocytes and in LCL. However, in MGL 58, who showed the highest proportion of expression (26.2%) in peripheral blood cells, there was no expression after exposure at 10^{-8} M FUdR for 24 hrs and only 5.8% expression

TABLE 2

FRAGILE X IN PERIPHERAL BLOOD CULTURES (PBC) AND LCL FROM EXPRESSING FEMALES

PATIENT NO.	AGE*	MENTAL STATUS†	PBC			LCL			
			No. cells‡	% fra (X)	Range	FUdR	No. cells‡	% fra (X)	Range
MGL 43	41	Mild	139 (2)	6	0–8	10^{-7} M	295 (4)	14	13–14
MGL 45	47	N, slow	296 (2)	25	20–31	10^{-7} M	60 (1)	18	...
MGL 46	39	N, slow	393 (3)	7	6–13	10^{-7} M	60 (1)	2	...
MGL 88	42	Duplicate of MGL 46		...	...	10^{-8} M	50 (1)	4	...
MGL 47	71	N, slow	470 (4)	9	1–21	10^{-7} M	65 (1)	9	...
MGL 73	73	Duplicate of MGL 47		...	...	10^{-7} M	100 (2)	27	24–30
						10^{-8} M	291 (3)	24	6–38
MGL 58	50	Mod	401 (3)	26	22–30	10^{-7} M	103 (2)	6	4–8
						10^{-8} M	50 (1)	0	...
MGL 76	57	N	165 (2)	13	4–17	10^{-7} M	100 (1)	10	...
						10^{-8} M	50 (1)	16	...

* When LCL established.
† N = normal intelligence, IQ > 70; Mild = mildly retarded, IQ 51–70; Mod = moderately retarded, IQ 36–50.
‡ No. in parentheses equals no. experiments.

at 10^{-7} M. LCL were established on two separate occasions using blood from MGL 46, a patient with a relatively low level of expression. The proportion of fra(X) positive cells in both lines was similarly low. LCL were also established on two different blood samples taken 2 years apart from MGL 47, another patient with a relatively low expression. The proportion of fra(X) positive cells in the first was very similar to that in peripheral blood cultures, while the second showed a considerably higher proportion of expressing cells.

Table 3 gives data from the three nonexpressing males who had transmitted the gene to progency and from the three nonexpressing obligate-carrier females. No fra(X) positive cells were observed in LCL from any of these six patients after exposure to either 10^{-7} or 10^{-8} M FUdR. The very low levels of fra(X) expression found in blood cultures from two obligate-carrier females and one transmitting male are from nonbanded orcein-stained slides and, hence, could not be confirmed by destaining. We have not seen a fra(X) on a banded preparation from any of these individuals.

DISCUSSION

The great majority of patients tested expressed the fra(X) in approximately the same proportion of cultured peripheral blood lymphocytes as in LCL treated with FUdR under optimal conditions. This suggests that the proportion of cells in which the fra(X) can be demonstrated may be an innate feature of the patient and not of the cell type. It is particularly noteworthy that no fra(X) cells were seen in the three LCL from the phenotypically normal transmitting males or from the three obligate-carrier females, all of whom were also fra(X) negative in peripheral blood lymphocytes. As in peripheral blood cultures, we found no case in which the proportion of fra(X) positive lymphoblastoid cells was significantly greater than 50%.

TABLE 3

FRAGILE X IN PERIPHERAL BLOOD CULTURES (PBC) AND LCL FROM NONEXPRESSING OBLIGATE-CARRIER FEMALES AND TRANSMITTING MALES

PATIENT NO.	AGE*	MENTAL STATUS†	PBC			LCL		
			No. cells‡	% fra (X)	Range	FUdR	No. cells‡	% fra (X)
Females:								
MGL 65	37	N	518 (4)	0.4	0–1	10^{-7} M	115 (2)	0
						10^{-8} M	66 (1)	0
MGL 92	44	N	394 (3)	1.5	0–3	10^{-7} M	100 (2)	0
						10^{-8} M	75 (1)	0
TL 11075§ ...	54	N	714 (4)	0	...	10^{-7} M	100 (2)	0
						10^{-8} M	100 (2)	0
Males:								
MGL 66	69	N	365 (3)	0.5	0–1	10^{-7} M	200 (4)	0
						10^{-8} M	266 (4)	0
GM 6892‖	84	N	275 (2)	0	...	10^{-7} M	100 (2)	0
						10^{-8} M	100 (2)	0
GM 6906‖	52	N	275 (2)	0	...	10^{-7} M	100 (2)	0
						10^{-8} M	100 (2)	0

* When LCL established.
† N = normal intelligence, IQ >> 70.
‡ No. in parentheses equals no. experiments.
§ PBC data from T. W. Glover, personal communication, 1985.
‖ PBC data from [14], family D1, individuals II-5 and IV-22.

There were, however, two exceptions to the general level of concordance of the proportion of fra(X) positive cells between peripheral blood lymphocytes and LCL. One male and one female who expressed at consistently high levels in peripheral blood lymphocytes consistently expressed at low levels or not at all in their LCL. A second female, with low-level expression in peripheral blood cultures and her first LCL, expressed at a moderately high level in a second independently derived lymphoblastoid line. The reasons are not known for the discrepancy between the peripheral blood lymphocytes and LCL on these three occasions.

While the technical requirements for the consistent demonstration of the fra(X) in peripheral blood cultures are reasonably well documented, the requirements for a reproducible fra(X) demonstration in LCL are not well established. FUdR or MTX must be added to demonstrate the fra(X) in LCL, and LCL are clearly very individualistic with respect both to their sensitivity to FUdR and to their rate of growth. To compensate for the cytotoxic effect of FUdR, it is advisable to treat LCL with FUdR only when they are in a vigorous growth phase as determined for each individual LCL [15]. Moreover, FUdR exposure should be limited to the 24 hrs prior to harvest, since fra(X) expression declined on the few occasions when we exposed cells for longer periods. This decline has been demonstrated by others and shown to correspond to an increase in thymidylate synthase activity [16]. In addition, we found that the concentration of FUdR necessary to optimize fra(X) expression varied between lines. Variability in response to FUdR also occurs in fibroblasts and amniocytes [17]. Thus, in all these tissues, it is desirable to test a range of

FUdR concentrations to determine the minimum concentration of FUdR needed for fra(X) expression. This variability in response to FUdR may reflect differences in intracellular nucleotide pools.

In summary, lymphoblastoid cultures with few exceptions manifest the same level of expression of the fragile site at Xq27 as do lymphocyte cultures from the same individuals and therefore provide very useful experimental material for the study both of the mechanism of expression of the fra(X) and of its molecular structure.

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