

Replication status of fragile X(q27.3) in 13 female heterozygotes

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SUMMARY Chromosome analysis, after bromodeoxyuridine incorporation and a sequential Leishman acridine orange staining method previously described,¹ was used to assess the percentage of early or active fragile Xs compared with the overall total in informative cells in 13 heterozygous females. The percentage thus obtained was then used to calculate the percentage of early or active fragile X that would be expected in a culture without bromodeoxyuridine, that is: percentage active fragile X \div 100 \times percentage fra(X) in standard 199 or M culture. Five females were normal and eight of below normal intelligence.

In one culture pokeweed mitogen was substituted for phytohaemagglutinin and the percentage of active fragile Xs obtained was compared with that obtained with phytohaemagglutinin. In the same female the effect of addition of fluorodeoxyuridine and methotrexate on the replication ratio of the X was also investigated. R banded and G banded cells from this subject were also scanned for the deletion of Xq27.3 \rightarrow qter.

About one-third of females heterozygous for the fragile X are mentally retarded.²⁻⁴ There is previous evidence of a relationship between mental retardation and the inactivation pattern of fra(X)^{1 5-8} in that mental retardation is due to expression of the fra(X) gene on the active or early replicating X, and that on the late replicating X the normal gene is not active. Mentally retarded females would be expected to show an excess of early replicating fra(X) and normal females the opposite.

This correlation has been disputed by Fryns *et al*⁹ who found an excess of early replicating fra(X) in 10 subjects regardless of IQ. More recently, a highly significant inverse correlation between IQ and early replicating cells with fra(X) in 18 subjects was reported by Paul *et al*.¹⁰

Nielsen *et al*,¹¹ however, suggest that the relationship between IQ and X inactivation is more complicated than this. Although there is a general tendency for an overall excess of early replicating fra(X) in mentally retarded females and vice versa, in females with high fra(X) expression this does not always hold true.

We report here on 13 subjects, 10 with high and three with low fra(X) expression. Of the 10, eight

are mentally retarded and two are of normal IQ. All the low expressing females are of normal IQ.

As a crude estimate of the time of X inactivation in lymphocytes, the inactivation pattern obtained using a B cell mitogen (pokeweed) was compared with that of a T cell mitogen (PHA).

Terminal deletion of the X chromosome in patients with the fra(X) syndrome has been reported by Fitchett and Seabright.¹² As the replication pattern of the terminally deleted X may be important as an indicator of IQ, 50 R banded and 50 G banded cells from a subject with a 'high' percentage of fra(X) were examined for this deletion. Cells containing unattached small fragments were also banded to try to determine the origin of the fragments.

Material and method

Seventy-two hour lymphocyte cultures were established on the day of blood collection. A total of 0.4 ml of peripheral blood was added to (1) TC199 + 2% fetal calf serum (FCS); (2) TC199 + 2% FCS + methotrexate (10^{-7} mol/l) (MTX); (3) TC199 + 2% FCS + FUdR (10^{-5} mol/l); and (4) TC M (modified F10 without folate or thymidine).

In one culture, PHA was omitted and pokeweed mitogen substituted (0.02 ml/ml). This culture was

maintained for 120 hours. BUdR 50 µg/ml was used in earlier cultures and was gradually reduced to 12 µg/ml in later subjects SI, VGr, and JGr. By reducing the level of BUdR it was hoped to increase the fra(X) level in BUdR treated cultures. BUdR was added six hours and colchicine one hour before harvesting. After BUdR addition, all cultures and slides were kept in the dark. On all cultures except MC, slides were made and stained as rapidly as possible. In the case of MC the cells had been stored in fix in the cold and dark for several months. Efforts were made to obtain another blood sample but with no success.

Cells from BUdR cultures were stained using a modification¹ of Howell and McDermott's⁶ sequential Leishman acridine orange Leishman technique for permanent R banding. All slides were first stained with Leishman to identify cells with a fra(X), then stained with acridine orange to determine whether the X was late or early replicating. Permanent R banding was used for checking.

Cells from cultures without BUdR had previously been stained with Leishman and G banded, in order to identify heterozygotes and to determine the level of fra(X). A G banded culture was also used to check for the deletion of Xq27.3→qter.

Results

REPLICATION STATUS OF THE FRAGILE X AND CORRELATION WITH IQ (TABLE 1)

Of the eight mentally retarded females with a high fra(X) expression, four showed a marked excess of active fra(X). Two were around 50% active to inactive X, and two had an excess of inactive fra(X).

Of the two normal females with a high expression of fra(X), one had an excess of active fra(X) (MC) and one an excess of inactive fra(X). MC is 6 years old and is considered to be of normal IQ because she is at a normal school; however, this could change.*

All the three normal females with low fra(X) expression had an excess of inactive fra(X).

Calculation of the percentage of early fra(X) that would be expected in a culture without BUdR should indicate the level of active fra(X) in the culture, and so be comparable with the fra(X) level in a male subject. The low normals all dropped below the 4% cut off level for a positive result used in this laboratory, and all the high subjects remained above this level. The high normal SI now had a similar level to four of the mentally handicapped females. Using percentage active fra(X) only, she had been similar to the low normals.

*MC has since been reported to be 'struggling' at school.

TABLE 1 Replication studies in heterozygous females fra(X)(q27.3).

Subject	Age	Media	IQ	No. of cells + BUdR	% fra(X) + BUdR	% fra(X) - BUdR	+ BUdR		NI	Late		% early - BUdR
							Early	NI		NI	NI	
High, mentally retarded												
FCo	Adult	199	MR	854	1	10	4	4	4	2	7	*
SM	16	M	MR	960	4	31	19	4	4	16	17	7
JW	17	M	MR	606	9	25	39	7	7	11	20	20
TG	11	M	MR	1327	4	20	24	9	9	14	63	13
SG	13	M/199	MR	1363	2	20	17	6	6	7	16	16
GW	19	M/199	MR	798	3	20	4	5	5	10	29	6 ←
VGr	40	M	MR	1434	4	26	15	21	21	26	37	7 ←
JGr	33	M	MR	666	5	13	13	10	10	13	50	7
High, normal												
MC	6	199	N	673	5	32	17	15	15	4	81	26 ←
SI	29	M/199	N	614	5	33	4	9	9	18	18	6
Low, normal												
HF	15	199	N	429	3	4	3	—	—	13	19	1
CB	7	M	N	360	4	6	1	2	2	11	8	0.5
DC	40	M	N	238	4	5	2	4	4	4	33	2 *

NI=non-informative. * =small sample. MR=mental retardation. N='normal' IQ. ← = no inverse correlation between IQ and active fra(X) (all percentages corrected to nearest whole number).

TABLE 2 The effect of addition of FUDR or MTX (72 hours) and use of pokeweed as a mitogen on fra(X) replication in subject JW (figures corrected to nearest whole number).

Subject	Media	No of cells	% fra (X) + BUdR	Early	NI	Late	% early X
JW	M	606	9	39	7	11	78
JW	M + pokeweed	282	10	19	8	2	90
JW	199 + FUDR	480	10	35	6	5	88
JW	199 + MTX	173	14	17	5	2	89

$\chi^2=2.84$ (n=4).

p=0.45, therefore no relation between different media and replication ratio.

EFFECT OF BUdR ON FRAGILE X EXPRESSION
Fra(X) expression was depressed. Reduction of BUdR to 12 $\mu\text{g/ml}$ did not alter the suppression but did lessen autobanding. If autobanding occurs when solid staining with Leishman, it can be difficult to tell whether the early X is autobanded or fragile. Subjects who had the highest levels of fra(X) in the absence of BUdR showed the heaviest reduction (table 1) with its addition.

EFFECT OF POKEWEED ON THE REPLICATION PATTERN IN SUBJECT JW

The percentage of early fra(X) obtained with pokeweed was increased compared to that obtained with PHA, but this result was not significant (table 2). The overall percentage fra(X) with pokeweed (10%) was within 1% of that with PHA (9%). This is in contrast to Marchese *et al*¹³ who found a decrease of fra(X) expression in positive males when pokeweed was used as a mitogen. This shows that Lyonisation took place before stem cell separation into B and T cells.

THE EFFECT OF FUDR AND MTX ON THE REPLICATION PATTERN IN SUBJECT JW (TABLE 2)

Addition of FUDR increased fra(X) expression from 9% to 10%. MTX gave a larger increase (9% to 14%) but the mitotic index was more drastically reduced with MTX. The percentage of early replicating Xs was increased with both MTX and FUDR but not to a significant degree when the size of the sample was taken into account. χ^2 for cultures containing pokeweed, MTX, and FUDR were calculated together (n=4): $\chi^2=2.84$, df 3, p=0.45. Therefore, addition of MTX or FUDR does not alter the replication pattern.

G AND R BANDING AND TERMINAL DELETION OF THE X

G banded cells

In subject JW, 50 cells were analysed. In 22 cells a fra(X) was present and in four cells the terminal band from the X appeared to be absent (fig 1).

R banded cells

In subject JW, 50 cells were analysed for terminal deletion of the X. In 10 cells a fra(X) was present (nine early, one late) and in two cells the terminal band from the early X was absent (fig 2).

Minute fragments

In six cells from an M + BUdR culture (25 $\mu\text{g/ml}$) from subject TG there was a pair of minute fragments. However, when stained with acridine orange the ends of the early X were normal, although the fragments were brightly fluorescent. In a culture from SI there were two such cells; in one the fragments were dull red and could have come from the late X. It seems more likely that the fragments were due to the effect of BUdR.

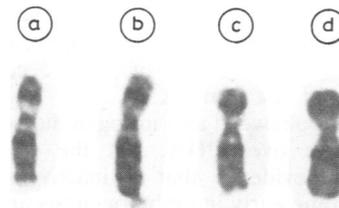


FIG 1 G banded X chromosomes from subject JW. (a) Normal, (b) fragile, (c) and (d) terminal deletion.

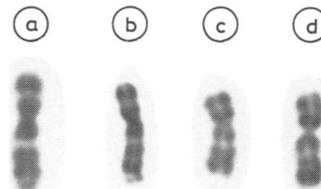


FIG 2 R banded X chromosomes from subject JW. (a) Normal, (b) fragile, (c) and (d) terminal deletion.

Discussion

In the heterozygous fragile X female, high fra(X) expression is usually associated with mental retardation, and low fra(X) expression with a normal IQ. Although there is also a tendency towards an inverse correlation between the replication pattern and the IQ in female heterozygotes, this is not always so. It seems likely that IQ and the manifestation of the fra(X) syndrome is influenced by a number of factors, such as the expression and penetrance of the gene as it is modified by the genetic background and the environment. Also, although there is a good chance of similarity between Lyonisation pattern in lymphocytes and the CNS, the two tissues being derived from adjacent areas of the early embryo, 100% correlation seems unlikely.

In common with Uchida and Joyce⁷ and Nielsen *et al.*,¹¹ we also found that BUdR repressed fra(X) expression in the high percentage groups more than in the low. It may be that this extra sensitivity to BUdR reflects an additional biochemical lesion not present in the low percentage group that is being repaired by the BUdR.

The addition of MTX to induce fra(X) expression does not appear to bias the results. Not only were the results from JW with and without MTX similar, but our results and those of Nielsen *et al.*,¹¹ who employed an MTX block, are alike. This suggests that use of an MTX block is a more economical method to use. However, the amount of BUdR used may possibly influence the number of active fra(X) ascertained as high levels of BUdR can cause autobanding of the early X.

The use of pokeweed as a mitogen did not confer any advantage over PHA, but the result does reinforce the evidence that X inactivation takes place some time early in embryogenesis and earlier than B and T stem cell formation.

Detection of the terminal deletion of Xq on R banded slides had the disadvantage that, although the terminal band showed up very well on the early replicating X, the late replicating X often did not show any bands at all. The number of R banded cells with a terminal deletion showed a similar decrease with BUdR incorporation, as did the fragile site.

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