5-fluoro-2'-deoxyuridine induction of the fragile site on Xq28 associated with X linked mental retardation

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SUMMARY 5-fluoro-2'-deoxyuridine (FUdR) was found to be highly effective in inducing the heritable fragile site on Xq28 associated with mental retardation. Lymphocytes from two affected males manifested the fragile site in 30 to 40% of the mitoses when grown in the presence of FUdR.

This observation suggests that depletion of the dTMP pool via thymidylate synthetase inhibition is responsible for the expression of the heritable fragile site on Xq28.

The expression of the fragile site on the X chromosome (Xq27 or 28, hereafter called Xq28),¹ associated with one type of X linked mental retardation,²⁻¹⁴ has been shown to be highly dependent on the culturing conditions.^{15 16} Apart from a slightly alkaline pH optimum and a low serum content (5%), the main factor necessary for the expression of the fragile site on the X is the culture medium's deficiency of folic acid and thymidine. The fragile site is expressed in medium 199 which is relatively deficient in folic acid (0.01 mg/l), whereas in McCoy's medium, which is rich in folic acid (10 mg/l), no fragile site can be seen. Also, the folic acid antagonist methotrexate (MTX) enhances the expression.

The nature of the above mentioned inducers and inhibitors suggests that the process of expression could be operating during DNA synthesis, as discussed by Sutherland,¹⁶ the critical step being the conversion of uridine monophosphate (dUMP) to thymidine monophosphate (dTMP) catalysed by the enzyme thymidylate synthetase. This reaction requires as co-enzyme the folic acid derivative 5,10-methylene tetrahydrofolate ($\text{CH}_2 = \text{FAH}_4$) which is converted to dihydrofolate (FAH_2) (fig 1).

Accordingly the known inducers of the fragile site, folic acid deficiency and MTX, will limit the amount of $\text{CH}_2 = \text{FAH}_4$, thereby limiting the pool of dTMP required for DNA synthesis, whereas the known inhibitors, folic acid and thymidine, will increase the pool of dTMP available for incorporation into DNA.

FIG 1 Schematic representation of the reaction catalysed by thymidylate synthetase. MTX is a competitive inhibitor of dihydrofolate reductase, thereby limiting the pool of $\text{CH}_2 = \text{FAH}_4$. See text for further explanation.

In preliminary experiments we have tested whether 5-fluoro-2'-deoxyuridine (FUdR) could mimic the effect of folic acid deficiency on the expression of the fragile site on the X chromosome. The rationale is that FUdR in mammalian cells is converted to fluoro-deoxyuridine monophosphate (dFUMP), which is a strong and specific inhibitor of thymidylate synthetase (fig 1).^{17 18}

FIG 2 The X and Y chromosome from case No 495-80, Q banding (top) and Giemsa staining. Left: from a metaphase cultured in Hepes buffered medium 199 (pH 7.6) + 5% FCS. Right: from a metaphase cultured in McCoy's 5A medium + 20% serum + 0.4 $\mu\text{mol/l}$ FUDR. Arrows show the fragile site on Xq28.

Materials and methods

Lymphocytes from two unrelated mentally retarded males, who had been found to have the fragile site on the X chromosome in a previous investigation (unpublished results), were grown for 3 or 4 days as full blood cultures in the following media:

- (1) Hepes buffered medium 199 (Gibco) supplemented with 5% inactivated fetal calf serum (FCS) (Gibco), pH 7.6 to 7.7.¹⁶
- (2) Bicarbonate buffered McCoy's 5A medium supplemented with 20% pooled human serum (pH not measured).

FUDR (Sigma) was added to a final concentration of 0.4 $\mu\text{mol/l}$ when the cultures were started. The cells were grown in the presence of FUDR throughout the culturing period. Colcemid was added for the last 1½ hours. Hypotonic treatment, fixation, and slide preparation was by routine chromosomal methods. Slides were stained with quinacrine mustard and randomly chosen metaphases from each culture were examined by fluorescence microscope (Leitz Orthoplan). A metaphase was scored as positive when a fragile site at Xq28 could be identified (fig 2).

Results

The results are summarised in the table.

When the lymphocytes were grown in medium 199 + 5% FCS, the addition of FUDR did not significantly alter the percentage of positive mitoses. In both subjects we found 30 to 40% positive cells.

We have never observed the fragile site on Xq28 in lymphocytes grown in McCoy's medium + 20%

TABLE Percentage of cells showing the fragile site on Xq28 (positive cells) when cultured in medium 199 and McCoy's 5A medium. (FUDR: 0.4 $\mu\text{mol/l}$ throughout the culturing period.)

Case No	Medium	Culture time (days)	No of cells examined	% positive cells
495-80	199	3	60	44
	199	4	60	27
	199 + FUDR	4	50	44
	McCoy's + FUDR	4	50	30
532-80	199	3	60	45
	199	4	60	44
	199 + FUDR	4	50	34
	McCoy's + FUDR	4	100	43
	McCoy's	3	100	0

serum. A striking contrast to this was seen when FUDR was added. Then the percentage of positive cells in both subjects were in the same range as in the cultures grown in medium 199.

We found no morphological differences between the appearance of the fragile sites induced by either folic acid deficiency or FUDR (fig 2). Apart from the fragile site on the X chromosome, other non-randomly situated gaps and breaks were frequently observed in the FUDR treated cultures. This was also the case when cells were cultured in folic acid deficient medium (unpublished results).

Discussion

It is interesting to note that in the folic acid deficient medium 199 the addition of FUDR did not increase the frequency of cells expressing the fragile site, whereas in the folic acid rich medium (McCoy's) the effect of FUDR was evident. One possible explanation for this is that in the presence of $\text{CH}_2 = \text{FAH}_4$ a marked enhancement of the binding of dFUMP to thymidylate synthetase occurs.¹⁸ In practical terms this could mean that in order to get the optimal inhibiting activity of FUDR the medium should be rich in folic acid. This could simplify the detection of fragile sites considerably, since most chromosomal culturing media have a higher folic acid content than medium 199.

In this preliminary observation we have not defined the optimal concentration or culturing conditions for the FUDR induced fragile site on Xq28. It may well be that the optimal condition varies between subjects and between different cell types, in view of the great variability in the expression of this fragile site reported by all investigators. In particular, severe difficulties are associated with the detection of the fragile site in female carriers (for a review see Sutherland¹⁹) and in fibroblasts.²⁰ Only by combining folic acid deficient culturing conditions with special in situ hypotonic and fixation

techniques has the fragile site on Xq28 been shown to exist in chromosomes derived from fibroblasts.²¹

We are at present investigating whether FUDR in appropriate concentrations will provide a simple and reliable tool for the detection of the fragile site on Xq28 in affected males, in carrier females, and in fibroblasts and amniotic fluid cells. Also, the other known heritable fragile sites dependent on the folic acid concentration in the tissue culture medium might be inducible with FUDR. We have not been able to investigate this for those heritable fragile sites dependent on folic acid concentration classified by Sutherland,¹⁶ but we have found that the heritable fragile site on 12q13 (which was not included in Sutherland's work) is expressed in folic acid deficient medium and by FUDR.²²

The expression of the fragile site on Xq28 by FUDR alone strongly suggests that the culturing conditions for increasing the frequency hitherto used operates via the same mechanism: depletion of the dTMP pool via thymidylate synthetase inhibition leading to impaired DNA synthesis

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