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FUdR Induction of the X Chromosome Fragile Site: Evidence for the Mechanism of Folic Acid and Thymidine Inhibition

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SUMMARY

Experiments designed to illuminate the mechanism by which folic acid and thymidine inhibit expression of the Xq28 fragile site in human lymphocytes are described. The fragile site is induced by 5-fluorodeoxyuridine (FUdR), a potent inhibitor of thymidylate synthetase, in the presence of otherwise inhibiting concentrations of folic acid but not in the presence of thymidine. These results indicate that the fragile site is expressed because of depletion of deoxythymidine monophosphate (dTMP) available for DNA synthesis.

INTRODUCTION

The association between a fragile site on the long arm of the X chromosome (fragile X) and one important form of X-linked mental retardation has recently been reported by a number of laboratories [1–6], and the fragile X appears to be a reliable indicator of a clinically recognizable syndrome. The exact location of the fragile site has not been determined, for example, in prophase-banded chromosomes. However, it is either at or near the interface of bands Xq27 and Xq28 [3, 6] and, in accordance with the Paris Conference nomenclature, will be identified here as Xq28.

Studies of this syndrome have been greatly facilitated by the observations of Sutherland [7] that lymphocytes from affected individuals must be cultured in medium deficient in folic acid and thymidine for expression of the fragile X. A high pH of the medium and a reduced serum component were also reported to increase the proportion of cells expressing the fragile X. These conditions were also found to

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be necessary for the expression of certain other autosomal fragile sites that are apparently without phenotypic effects in the heterozygote. Based on these observations, three possible mechanisms for the appearance of fragile sites at specific chromosomal locations were suggested: despiralization of the chromatids in late G_2 or early S; viral modification of the DNA; and an alteration of DNA synthesis due to a reduced supply of dTMP arising during a state of folic acid deficiency.

Our observations supported the findings that folic acid and thymidine have an inhibiting effect on expression of the X-chromosome fragile site. The present studies were designed to clarify the mechanism by which the absence of folic acid and thymidine cause the appearance of the Xq28 fragile site.

Folic acid is involved in a number of one-carbon transfer reactions, including those involved in amino acid, purine, and pyrimidine metabolism. However, the observation that thymidine as well as folic acid inhibited expression of the fragile X suggested that the role of folic acid in dTMP biosynthesis was involved. The inhibiting effect of folic acid on expression of the fragile X can be negated by FUdR, a potent inhibitor of thymidylate synthetase. However, the inhibiting effect of thymidine is not negated by FUdR. These results indicate that the X-chromosome fragile site is expressed because of depletion of dTMP available for DNA synthesis.

MATERIALS AND METHODS

Heparinized blood samples from mentally retarded males known to have the fragile X and from normal male controls were obtained for this study. Blood was cultured by conventional whole blood microculture technique for 96 hrs in modified medium F-10 (Gibco, Grand Island, N.Y.) without folic acid, thymidine, or hypoxanthine ("M" medium), supplemented with 5% fetal bovine serum. The culture medium was initially adjusted to pH 7.7 at room temperature, and no further adjustments were made since in preliminary experiments using M medium, no significant difference was seen in the proportion of cells expressing the fragile X over the range pH 7.1–7.7. FUdR, 5-bromodeoxyuridine (BrdU) (Sigma, St. Louis, Mo.), folic acid, and thymidine (Gibco) were dissolved in PBS and added at the beginning of the culture period, unless otherwise indicated in the tables. In the initial experiments, the final concentrations of folic acid and thymidine were chosen to correspond to those normally found in F-10 medium.

Air-dried slides were either stained in orcein or Q-banded, and whenever possible, at least 50 cells were scored by each of two observers for the fragile X and other chromosome or chromatid aberrations including gaps, breaks, and exchanges. A chromatid or isochromatid gap at the distal end of the long arm of a medium-sized chromosome was scored as positive for the fragile X in nonbanded preparations. All cultures were coded before harvesting, and each experiment included a control; thus all observations were made without knowledge of the status of the individual or treatment of the culture.

RESULTS

The fragile X was demonstrable in 16%–38% of the lymphocytes from the seven affected males studied when cultured in medium lacking folic acid and thymidine (tables 1–3). The addition to the medium of 3 μM folic acid completely inhibited the expression of the fragile X in all affected males. The addition of thymidine at 4.1 μM (1 mg/liter) reduced the proportion of cells expressing the fragile X to about one-half (tables 1 and 2). Increasing the thymidine concentration to 20 μM almost completely inhibited fragile X expression (table 2).

TABLE 1

EFFECT OF FUdR AND BrdU ON FRAGILE X EXPRESSION

TREATMENT					B III 19 AFFECTED ♂		P II 6 AFFECTED ♂		P II 4 AFFECTED ♂		D220 control ♂	
Folic acid (3 μM)	Thymidine (4.1 μM)	FUdR (0.1 μM)	BrdU (10 μM)		fra X/ cells*	cs. + ct.ab. /cells†	fra X/ cells	cs. + ct.ab. /cells	fra X/ cells	cs. + ct.ab. /cells	fra X/ cells	cs. + ct.ab. /cells
−	−	−	−	.....	29/150	5/150	39/160	5/160	31/155	17/155	1/180	16/180
+	−	−	−	.....	0/100	1/100	2/134	4/134	0/100	0/100	0/100	1/100
−	+	−	−	.....	10/100	1/100	9/100	0/100	4/86	2/86	0/100	1/100
+	+	−	−	.....	0/100	0/100	0/75	1/75	0/100	0/100	0/81	0/81
−	−	+	−	.....	7/53	4/53	13/100	5/100	9/92	3/92	0/69	10/69
+	−	+	−	.....	14/85	3/85	19/84	3/84	12/72	2/72	2/35	2/35
−	+	+	−	.....	6/100	3/100	6/100	1/100	5/100	1/100	0/100	0/100
+	+	+	−	.....	6/100	2/100	11/100	1/100	8/100	7/100	0/90	1/90
−	−	−	+	.....	11/100	2/100	3/75	0/75	0/67	1/67	0/60	0/60

* fra X/cells = no. X chromosomes in which the fragile site was seen/total cells scored.
† cs. + ct.ab./cells = no. chromosome and chromatid abnormalities, that is, chromatid gaps, isochromatid gaps, and breaks/total cells scored.

TABLE 2

FUdR INDUCTION OF FRAGILE X

TREATMENT			D294 AFFECTED ♂		D295 AFFECTED ♂		D293 CONTROL ♂	
Thymidine (μM)	Folic acid (μM)	FUdR (μM)	fra X/ cells*	cs. + ct.ab. /cells†	fra X/ cells	cs. + ct.ab. /cells	fra X/ cells	cs. + ct.ab. /cells
0	0	0	20/100	5/100	18/100	2/100	0/100	10/100
0	3	0	0/100	1/100	0/100	2/100	0/100	0/100
0	9‡	0.1	24/100	7/100	27/100	5/100	0/100	12/100
0	3	0.1	31/100	18/100	16/52 (31%)	4/52	0/100	25/100
0	3	0.5	33/100	12/100	13/80 (16%)	3/80	0/100	24/100
4.1	0	0	13/100	2/100	8/100	2/100	0/100	0/100
4.1	0	0.1	32/100	4/100	8/100	5/100	0/100	7/100
4.1	0	0.5	26/100	8/100	16/100	6/100	0/100	8/100
4.1	3	0.1	24/100	6/100	11/100	1/100	0/100	5/100
4.1	3	0.5	14/100	7/100	26/100	4/100	0/100	11/100
20	0	0	0/100	0/100	1/100	0/100	0/100	0/100
20	0	0.1	3/100	1/100	1/100	1/100	0/100	1/100
20	0	0.5	4/100	1/100	3/100	2/100	0/100	0/100
20	3	0.1	6/100	1/100	7/100	1/100	0/100	2/100
20	3	0.5	3/100	0/100	0/100	2/100	0/100	1/100

* fra X/cells = no. X chromosomes in which the fragile site was seen/total cells scored.
† cs. + ct.ab./cells = cells = no. chromosome and chromatid abnormalities, that is, chromatid gaps, isochromatid gaps, and breaks/total cells scored.
‡ 3 μM folic acid added to cultures at 96, 48, and 24 hrs prior to harvest.

TABLE 3

FUdR INDUCTION OF FRAGILE X AT VARIOUS TIMES PRIOR TO HARVEST

TREATMENT		EXPERIMENT 1						EXPERIMENT 2			
		DIII-24 AFFECTED ♂		C IV-6 AFFECTED ♂		D233 CONTROL ♂		D262 AFFECTED ♂		D260 CONTROL ♂	
Folic acid (3 μM; 96 hrs)	FUdR exposure (0.1 μM; hrs prior to harvest)	fra X/ cells*	cs. + ct.ab. /cells†	fra X/ cells	cs. + ct.ab. /cells	fra X/ cells‡	cs. + ct.ab. /cells	fra X/ cells	cs. + ct.ab. /cells	fra X/ cells	cs. + ct.ab. /cells
–	–	16/100	11/100	27/100	12/100	4/100	16/100	38/100	4/100	0/100	1/100
+	–	0/100	0/100	1/100	0/100	0/100	0/100	0/100	0/100	0/100	2/100
+	96	13/100	9/100	19/100	10/100	0/100	18/100	...	...	...	...
+	72	9/100	15/100	19/100	10/100	5/100	14/100	...	...	...	...
+	48	18/100	11/100	23/100	13/100	0/100	17/100	...	...	...	...
+	24	10/100	10/100	32/100	9/100	0/100	8/100	...	...	...	...
+	18	10/100	6/100	16/100	10/100	0/100	6/100	18/100	9/100	0/100	5/100
+	12	8/100	7/100	13/100	3/100	0/100	3/100	9/100	5/100	0/100	9/100
+	9	...	...	...	...	...	...	5/100	2/100	0/100	2/100
+	6	...	...	...	...	...	...	0/63	0/63	0/100	2/100
+	3	...	...	...	...	...	...	0/100	1/100	0/100	1/100

* fra X/cells = no. X chromosomes in which the fragile site was seen/total cells scored.
† cs. + ct.ab./cells = no. chromosome and chromatid abnormalities, that is, chromatid gaps, isochromatid gaps, and breaks/total cells scored.
‡ See text for discussion.

The inhibiting effect of folic acid was totally negated by the addition of 0.1–0.5 μM FUdR to the culture medium (tables 1–3). As shown in table 3, FUdR was effective in inducing expression of the fragile X when added at various times up to 9 hrs before harvesting but in an apparent declining proportion of cells after the final 24 hrs of culture. The fragile X was not induced by FUdR during the last 6 hrs of culture, that is, during the late S-period, in cells from the one male studied. In contrast to the situation with folic acid, FUdR had no appreciable effect on the inhibition of the fragile X by thymidine when the two compounds were added simultaneously. Two concentrations of FUdR and thymidine were tested to ensure that results were not merely a result of competition as substrates for thymidine kinase.

To determine if the inducing effect of FUdR on the fragile X might be due to a low level of direct incorporation into DNA subsequently inducing a gap at Xq28, BrdU, another halogenated thymidine analog that is more readily incorporated into the DNA but which is not an inhibitor of thymidylate synthetase [8], was added to cultures incubated in glass tubes in the dark from three affected males and one control (table 1). In contrast to FUdR, BrdU reduced, rather than increased, the frequency of the fragile X in all three affected males.

As shown in tables 1–3, the frequency of other chromosome and chromatid aberrations is increased when lymphocytes are cultured using conditions that demonstrate the fragile X. As is the case with expression of the fragile X, the addition of folic acid or thymidine inhibits the appearance of these aberrations, while FUdR induces them when thymidine is limited or absent from the medium. This suggests a common mechanism for the expression of the fragile X and these other aberrations that appear throughout the genome. However, the frequency of these other aberrations is not noticeably different, regardless of culture conditions, between affected males and normal controls, indicating that a generalized breakage phenomenon or chromosome instability is not responsible for the appearance of the fragile X in affected males.

One of 180 cells from control D220 (table 1) and four of 100 cells from control D233 (table 2) were scored as positive for the fragile X in nonbanded preparations. Because the fragile X is rarely seen in unaffected males under conditions in our laboratory, 200 Q-banded cells from each of the individuals in table 2 were scored for the fragile X. The control male D233 was found to have a fragile site at the distal end of 6q, which closely resembled the fragile X on orcein-stained preparations. Control D233 was the only individual in this study demonstrating the 6q fragile site.

DISCUSSION

These results with cultured lymphocytes confirm that folic acid and thymidine inhibit the expression of the fragile site on the X chromosome, and further show that the inhibiting effect of folic acid, but not that of thymidine, can be negated by addition of FUdR to the culture medium.

This may be explained by the fact that FUdR is intracellularly converted by thymidine kinase to 5-fluorodeoxyuridine monophosphate (FdUMP), which in turn is a potent inhibitor of thymidylate synthetase [9, 10] (fig. 1). In the absence of

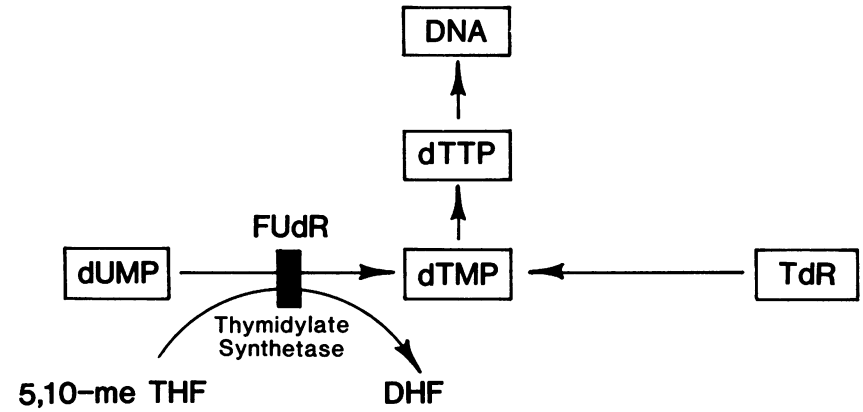

FIG. 1.—Pathways of dTTP biosynthesis and inhibition by FUdR. *5,10-meTHF* = N^5,N^{10}-methylene tetrahydrofolate; *DHF* = dihydrofolate; *TdR* = thymidine; *dUMP* = deoxyuridine monophosphate; *dTMP* = deoxythymidine monophosphate; *dTTP* = deoxythymidine triphosphate; *FUdR* = 5-fluoro-deoxyuridine.

exogenous thymidine, the pool of dTMP is depleted, thus arresting DNA synthesis. Exogenous thymidine bypasses this block by conversion to dTMP as catalyzed by thymidine kinase in the "salvage pathway." In this study, added folic acid is available for participation in other one-carbon transfer reactions involving amino acid and purine metabolism, making it unlikely that these reactions are important in the mechanism of fragile X expression.

These observations support the hypothesis that the fragile X is expressed by limiting the dTMP pool and, thus, the deoxythymidine triphosphate (dTTP) pool available for DNA synthesis [7]. This explanation may also apply to other fragile sites, both certain ones that are clearly inherited and those chromosome and chromatid aberrations that occur throughout the genome under conditions of folic acid and thymidine deficiency. The data do not support the notion that there is a deficiency in thymidylate synthetase in individuals with the fragile X since inhibition of this enzyme does not result in fragile X expression in control males.

Exactly how depletion of the dTMP pool results in a site-specific gap or fragile site is not known. These sites may represent sections of thymine-rich DNA that does not fully complete synthesis, thus leaving gaps, when the dTTP pool is restricted [7]. Alternatively, the fidelity of DNA replication at the Xq fragile site may be very sensitive to the normal relative pool sizes of the pyrimidine nucleotides resulting in base mispairing when these pool sizes are perturbed. While the details of such imbalances are still being evaluated, the indirect inhibition of thymidylate synthetase by methotrexate in a human lymphoid cell line has been shown to result in an increase in misincorporation of uracil into the DNA, possibly producing double-strand breaks due to futile DNA repair when dTTP is limited [11]. In addition, Chinese hamster ovary cell mutants deficient in dTTP synthesis have been shown to have greatly increased ratios of deoxycytosine triphosphate (dCTP):dTTP and increased rates of site-specific mutation at two of four loci tested [12].

It is not clear why the fragile X is expressed in only a proportion of lymphocytes or why it is not consistently demonstrated in fibroblasts despite repeated attempts in various laboratories [4, 13, 14]. Using medium lacking folic acid and thymidine with reduced serum or dialyzed serum, and employing various harvesting techniques, we have observed the fragile X in fibroblasts from both affected males and carrier females, but usually in less than 1% of the cells. This low frequency is markedly increased, for example to 8/26 cells in one trial, by growing cells in M medium plus 5% fetal bovine serum and treatment with 0.1 μM FUdR for 48 hrs prior to harvest. However, FUdR at this concentration is very cytotoxic to human fibroblasts, making this procedure presently unsuitable for diagnostic purposes. Further methodological improvements in the use of FUdR or other inhibitors of DNA synthesis may result in a reliable technique for demonstrating the fragile X in cultured fibroblasts.

ADDENDUM

Since submission of this manuscript, we have found that the cytotoxic effect of FUdR on fibroblasts can be greatly reduced, while still inducing expression of the Xq fragile site, by culturing the cells in medium without thymidine but with normal concentrations of folic acid and 15%–20% fetal bovine serum. Using this medium and treating fibroblasts with 0.05 μM or 0.1 μM FUdR for 24 or 48 hrs prior to harvest has resulted in preparations with large numbers of dividing cells in which the fragile X can readily be seen. Thus far, the fragile X has been seen in 20% and 25% of the fibroblasts from two hemizygous males (compared with 11% and 12% of their lymphocytes) and 19% of the fibroblasts from a heterozygous female (compared with 12% of her lymphocytes).

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Grant applicants should submit, *no later than April 15, 1981*, a written application for support in *triplicate* containing all of the following information: 1) name, age, highest degree awarded, present location, and position; 2) other sources of funds which will be used to defray travel and meeting expenses; and 3) details of planned participation in 6th International Congress (i.e., workshop to which applicant has been invited including abstract of presentation, or abstract of poster to be presented). Such applications, which will be reviewed by committee, should be sent to: Dr. Leon Rosenberg, Yale University School of Medicine, Department of Human Genetics, 333 Cedar St., P.O. Box 3333, New Haven, CT 06510. All applicants will be notified of the outcome of their submission by *June 1. Please note:* Some NIH institutes will consider requests from recipients of research and training grants for reallocation of funds into the foreign travel category for attendance at this meeting. Please seek further information from appropriate institute authorities.