*Journal of Medical Genetics*, 1983, **20**, 404–407

# Chromosomal breakage in normal and fragile X subjects using low folate culture conditions *

M VEKEMANS, B POPOVICH, D ROSENBLATT, AND P MONROE

*From the Departments of Pediatrics and Pathology, MRC Genetics Group, and Centre for Human Genetics, McGill University, Montreal, Canada.*

SUMMARY To investigate whether the fragile X syndrome is associated with a generalised chromosomal instability, we compared the frequency and distribution of chromosomal breakage in lymphocytes grown in low folate medium from normal subjects and from patients with the syndrome. Although low folate conditions increased the rate of chromosome breakage, no difference in frequency or distribution of chromosomal breakage was found between the two groups. This suggests that the fragile X syndrome is not associated with a generalised chromosome instability expressed in folate deficient medium and assessed in terms of chromosomal breakage.

The initial observation by Lubs[1] that a fragile site on the long arm of the X chromosome is associated with one form of X linked mental retardation has recently been confirmed by numerous laboratories.[2–6] However, using the identification criteria and culture conditions originally described by Sutherland,[7] quite variable frequencies of expression (ranging from 2 to 56% in affected hemizygous males and 0 to 28% in obligate heterozygous females) have been reported. These low folate culture conditions have also been shown to result in the variable expression of several autosomal fragile sites.[7] The nature and the mechanism of the factors underlying this variability of expression of fragile sites remain unknown.

Because low folate conditions have been shown to increase the expression of chromosome breakage,[8 9] we compared the frequency and distribution of chromosomal breakage in low folate conditions in normal subjects and patients with the fragile X syndrome. This study specifically addresses the possibility of a general chromosomal instability in cells of patients with the fragile X syndrome.[10]

## Materials and methods

Microcultures of phytohaemagglutinin stimulated peripheral lymphocytes were established from 10 normal healthy adults (five males and five females) and three patients with the fragile X syndrome. Heparinised blood (0·3 ml) was inoculated into Eagle's minimum essential medium (5 ml) lacking folic acid but supplemented with non-essential amino-acids, bicarbonate, 0·1 mmol/l L-methionine, and 10% fetal calf serum, which had been previously dialysed at 4°C for 24 hours against two changes of a 10-fold volume of 0·9% NaCl. After 94 hours' incubation at 37°C, colcemid (0·05 μg/ml final concentration) was added to the culture medium 90 minutes before harvesting. The cells were treated with 0·075 mol/l KCl for 12 minutes and fixed in 3:1 methanol:acetic acid, followed by two changes of fixative. Chromosome preparations were made by placing drops of cells, suspended in fixative, onto precleaned microscope slides. Slides were initially stained with 4% Giemsa in buffered solution (pH 6·86). Chromosome preparations were screened for all chromosomal breakage with both chromatid and chromosomal breaks each being scored as one break. A total of 200 metaphases was scored per person and they were then destained and Giemsa-trypsin banded. The location of all breaks was then determined according to the classification of the International System for Human Cytogenetic Nomenclature.[11] The expected distribution of breakpoints was based on chromosome length (Series D, ISCN 1978).

## Results

The mean frequency of breaks per metaphase (mean ± SEM) in normal males and females was

*This is publication No 83028 from the McGill University Montreal Children's Research Institute.

Received for publication 26 April 1983.
Accepted for publication 11 May 1983.

TABLE 1 *Frequency and distribution of breaks in control subjects and patients with the fragile X syndrome.*

| | *No of breaks* | *Control males* | | *Control females* | | *Fragile X males* | |
|---|---|---|---|---|---|---|---|
| | | *No of cells* | | *No of cells* | | *No of cells* | |
| | | *Observed* | *Expected* | *Observed* | *Expected* | *Observed* | *Expected* |
| | 0 | 675 | 677·10 | 743 | 736·57 | 387 | 282·50 |
| | 1 | 212 | 203·13 | 228 | 235·70 | 106 | 110·93 |
| | 2 | 21 | 30·47 | 28 | 37·11 | 13 | 16·08 |
| | 3 | 5 | 3·05 | 5 | 4·02 | 1 | 1·55 |
| | 4 | 1 | 0·23 | 4 | 0·32 | 2 | 0·11 |
| | 5 | 1 | 0·01 | 1 | 0·02 | 1 | 0·01 |
| Mean | | 0·30 | | 0·32 | | 0·29 | |
| SEM | | 0·02 | | 0·02 | | 0·03 | |
| SD | | 0·57 | | 0·61 | | 0·60 | |
| $\chi^2$ (df) | | 1·38 (1) | | 0·60 (1) | | 0·30 (1) | |

TABLE 2 *Chromosome distribution of breaks in control subjects and patients with the fragile X syndrome.*

| *Chromosomal region* | *Males* | | *Females* | | *Fragile X* | |
|---|---|---|---|---|---|---|
| | *No of breaks* | | *No of breaks* | | *No of breaks* | |
| | *Observed* | *Expected* | *Observed* | *Expected* | *Observed* | *Expected* |
| 1p | 18 | 10·49 | 20 | 11·59 | 9 | 5·67 |
| q | 10 | 11·20 | 7 | 12·38 | 3 | 6·06 |
| 2p | 9 | 8·09 | 6 | 8·94 | 2 | 4·37 |
| q | 13 | 12·52 | 19 | 13·84 | 9 | 6·78 |
| 3p | 43 | 8·24* | 52 | 9·11* | 28 | 4·46* |
| q | 11 | 9·31 | 17 | 10·29 | 3 | 5·03 |
| 4p | 0 | 4·71 | 3 | 5·20 | 0 | 2·55 |
| q | 19 | 11·48 | 17 | 12·69 | 4 | 6·21 |
| 5p | 2 | 4·57 | 4 | 5·05 | 1 | 2·47 |
| q | 2 | 11·06 | 6 | 12·22 | 7 | 5·98 |
| 6p | 3 | 5·92 | 6 | 6·54 | 2 | 3·20 |
| q | 41 | 9·24* | 28 | 10·22* | 15 | 5·00* |
| 7p | 4 | 5·38 | 3 | 5·94 | 4 | 2·91 |
| q | 12 | 8·40 | 9 | 9·28 | 10 | 4·54 |
| 8p | 0 | 4·32 | 0 | 4·77 | 1 | 2·34 |
| q | 3 | 8·35 | 5 | 9·23 | 1 | 4·51 |
| 9p | 4 | 4·37 | 3 | 4·83 | 0 | 2·36 |
| q | 2 | 7·97 | 1 | 8·80 | 2 | 4·31 |
| 10p | 1 | 4·01 | 3 | 4·43 | 2 | 2·17 |
| q | 1 | 7·79 | 2 | 8·61 | 10 | 4·21 |
| 11p | 1 | 4·76 | 0 | 5·26 | 1 | 2·57 |
| q | 4 | 7·09 | 6 | 7·83 | 3 | 3·84 |
| 12p | 0 | 3·61 | 4 | 3·99 | 2 | 1·95 |
| q | 8 | 8·37 | 12 | 9·24 | 2 | 4·53 |
| 13p | 0 | 1·64 | 0 | 1·81 | 0 | 0·89 |
| q | 6 | 7·97 | 1 | 8·81 | 1 | 4·31 |
| 14p | 0 | 1·71 | 0 | 1·89 | 0 | 0·93 |
| q | 11 | 7·44 | 14 | 8·22 | 1 | 4·02 |
| 15p | 0 | 1·80 | 0 | 1·99 | 0 | 0·98 |
| q | 1 | 7·09 | 2 | 7·84 | 3 | 3·83 |
| 16p | 0 | 3·57 | 2 | 3·94 | 0 | 1·93 |
| q | 17 | 5·07* | 21 | 5·60* | 11 | 2·74* |
| 17p | 1 | 2·83 | 0 | 3·13 | 0 | 1·53 |
| q | 1 | 5·52 | 1 | 6·10 | 1 | 2·99 |
| 18p | 0 | 2·33 | 1 | 2·57 | 0 | 1·26 |
| q | 0 | 5·20 | 2 | 5·75 | 1 | 2·81 |
| 19p | 1 | 3·19 | 0 | 3·53 | 0 | 1·73 |
| q | 1 | 3·67 | 2 | 4·05 | 0 | 1·98 |
| 20p | 2 | 2·99 | 0 | 3·30 | 0 | 1·62 |
| q | 3 | 3·59 | 1 | 3·97 | 0 | 1·94 |
| 21p | 0 | 1·51 | 0 | 1·67 | 0 | 0·82 |
| q | 2 | 3·37 | 3 | 3·73 | 0 | 1·82 |
| 22p | 0 | 1·60 | 0 | 1·77 | 0 | 0·86 |
| q | 0 | 3·64 | 1 | 4·02 | 0 | 1·98 |
| Total | 257 | 256·98 | 284 | 283·97 | 139 | 138·99 |

*Probability of $\chi^2$ test is significant ($p<0·05$) after correction for Bonferroni inequality.

TABLE 3 *Frequency of chromosomal breakage at specific sites in control subjects and patients with the fragile X syndrome.*

| Chromosomal region | Males | | Females | | Fragile X | |
|---|---|---|---|---|---|---|
| | No of breaks | | No of breaks | | No of breaks | |
| | Observed | Expected | Observed | Expected | Observed | Expected |
| 3p1 | 39 | 4·08* | 42 | 4·51* | 25 | 2·21* |
| 3p2 | 4 | 4·16 | 10 | 4·60 | 3 | 2·25 |
| 6q1 | 1 | 4·42 | 0 | 4·88 | 1 | 2·39 |
| 6q2 | 40 | 4·82* | 28 | 5·34* | 14 | 2·61* |
| 16q1 | 0 | 2·96 | 0 | 3·27 | 0 | 1·60 |
| 16q2 | 17 | 2·11* | 21 | 2·33* | 11 | 1·14* |

*Probability of $\chi^2$ test is significant ($p<0·05$) after correction for Bonferroni inequality.

$0·30 \pm 0·02$ and $0·32 \pm 0·02$, respectively (table 1). In patients with the fragile X syndrome the mean frequency of breaks per metaphase was $0·29 \pm 0·03$. This mean frequency does not differ significantly from the mean frequency of breaks per metaphase in normal males, and the distribution in the three groups did not deviate significantly from a Poisson distribution (table 1). This suggests that the distribution of breaks per metaphase is random.

In the three groups, after correction for Bonferroni inequality,[12] an excess of breaks was observed in chromosomes 3, 6, and 16 (table 2). In particular there was a clustering of breaks in chromosomal regions 3p1, 6q2, and 16q2 (table 3). Most of the breaks within the regions 6q2 and 16q2 were located next to the telomere in band 6q26 and 16q22 respectively, giving the chromosomes a 'satellite' appearance. The distribution of 'satellite 6q' and 'satellite 16q' among normal males and females was heterogeneous ($p<0·001$). The significance of the inter-individual heterogeneity in the frequency of these two autosomal sites remains unclear.

## Discussion

The mean frequency of breaks per metaphase observed in lymphocytes from normal subjects or patients with the fragile X syndrome when grown in folic acid deficient medium was 0·30. This rate is significantly (six times) higher than the mean rate (0·05) usually found in our laboratory and in published reports for cells cultured in non-folate deficient medium.[13] It is interesting to note that similar frequencies to ours have been observed previously both in peripheral blood leucocytes[9] and direct marrow preparations taken from patients with folic acid (and vitamin $B_{12}$) deficiency.[14]

Our study also demonstrates that, using a folate deficient medium, the in vitro breakage rate in patients with the fragile X syndrome is not higher than in normal subjects. This suggests that the fragile X syndrome is not associated with a general chromosomal instability expressed in folate deficient medium and assessed in terms of chromosome breakage. The observed distribution of breaks also varies significantly from those observed in other culture or genetic conditions. For example, after correcting for Bonferroni inequality,[12] all excess of breaks is found in chromosomes 3, 7, and 14 in standard culture conditions,[13] whereas in Fanconi's anaemia[15] this excess is limited to chromosome 1.

The clustering of the breaks on specific autosomal regions (3q14, 6q26, and 16q22) is very different from a random distribution in both patients with the fragile X syndrome and normal subjects. This is another argument in favour of a specific effect of the folate deficient medium on chromosomes.[8 16] On the other hand, it emphasises the importance of banding all preparations when cells are grown in folate deficient medium because, as suggested by others,[6 17 18] it is easy to misclassify a 6q26 as a fragile X. Our data also demonstrated that the expression of this break is heterogeneous among persons and that the frequency of expression can be quite high. For example, in one notable male control, the frequency of 6q26 breakage was 11%. However, it remains to be determined whether this variability of expression is genetically controlled.

With respect to the fragile X syndrome, we have previously shown that there is in normal females a background level of breakage on the long arm of the X chromosome which could be interpreted as a fragile X.[19] This raises the question of false positive cytogenetic results in the diagnosis of the fragile X syndrome. Relevant to this question are the recent findings of hemizygous males with the fragile X but with normal intelligence.[20–23] In normal males, however, we found no breakage on the long arm of the X chromosome and other studies have confirmed this finding.[6 17 24 25] This may perhaps reflect the fact

that in males, 200 metaphases carry 200 rather than 400 X chromosomes. In normal females, it is not certain whether the chromosomal lesions we found on the X chromosome meet the criteria for the definition of fragile sites.[4] We therefore suggest the term 'pseudo-fragile X' to designate these lesions on the X chromosome in the absence of any familial evidence.

Thus it is clear that the expression of the fragile site on the X chromosome is a quantitative phenomenon and that every laboratory must set up its own control standards. We have ourselves suggested that a frequency above or equal to 1% in males and 2% in females may indicate a positive diagnosis.[19]

In conclusion, our study indicated that folic acid deficient medium significantly increases the expression of spontaneous chromosome breakage and demonstrated that the fragile X syndrome is not associated with a general chromosomal instability expressed spontaneously and assessed in terms of chromosome breakage.

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Correspondence and requests for reprints to Dr M Vekemans, Department of Pathology, The Montreal Children's Hospital, 2300 Tupper, Montreal, Quebec H3H 1P3, Canada.