The fragile X syndrome in a large family. I Cytogenetic and clinical investigations

H VEENEMA*, J P M GERAEDTS†, G C BEVERSTOCK‡, AND
P L PEARSON‡

From *the Clinical Genetics Centre, Leiden; †the Department of Genetics and Cell Biology, Maastricht; and
‡the Department of Human Genetics, Leiden, The Netherlands.

SUMMARY Cytogenetic and clinical investigations were performed in 85 members of a large family, in which 18 males and seven females were mentally retarded. In the male patients the fragile site Xq27 was found in 6 to 44% (mean 22.5%) of peripheral blood lymphocytes. One non-retarded male expressed the cytogenetic abnormality in 6% of his cells. In 21 females the fra(X) was found in 3 to 28% (mean 8.7%) of their cells. Two obligate carriers did not express the fragile site. A significant difference in expression between the seven retarded (mean 16.7%) and seven non-retarded female carriers of corresponding age (mean 6.3%) was found ($\alpha=0.01$). No significant correlation between expression and age could be established, either in males or in females. The cytogenetic results appeared to be consistent.

To avoid false positives, a cut-off point was chosen: males were considered to be fra(X) negative if no more than one in 100 cells showed the abnormality; for females the cut-off point was two in 100 cells. Segregation analysis did not detect significant deviations from the expected ratios.

The putative presence of a transmitting male is discussed. The results of recombinant DNA analysis will be published elsewhere. Clinical investigations confirmed the findings of others. CT scans showed an enlargement of the ventricular system that exceeded the expected age changes.

Since the detection of the fragile site at band q27 of the X chromosome,^{1,2} cytogenetic analysis of lymphocytes is indispensable for diagnosing non-specific mental retardation. Folic acid deficiency is associated with the expression of the cytogenetic abnormality,³ depletion of thymidilic acid probably being the underlying cause of the phenomenon.⁴ However, the frequency of expression varies greatly and rarely exceeds 50%, even in hemizygous males.⁵ Complete lack of expression is often found in obligate carriers.^{6,7} Low folate conditions, on the other hand, can cause a considerable increase in the rate of non-specific telomeric changes which are morphologically similar to the fragile site.⁸ A cut-off point has to be chosen to avoid false positives, but this increases the risk of false negative estimates. This causes difficulties in genetic counselling.

The relationship between the cytogenetic abnormality and its phenotypic expression is even more complex. The X chromosomal nature of the abnor-

mality implies that all males showing the fragile site should have an abnormal phenotype, but the fra(X) has been demonstrated in normal non-retarded men.^{9,10} Moreover, several families have been reported in which the fragile X has presumably been transmitted by normal men to their carrier daughters.¹¹⁻¹⁷ Incomplete evidence exists about the cytogenetic expression in such males. A 79% penetrance of mental impairment in males has been found by Sherman *et al*¹⁷ using segregation analysis of 206 pedigrees. Approximately half of the females could be traced by mental impairment or fra(X) expression or both.

Incomplete cytogenetic expression, particularly in females, has been explained in various ways. A negative effect of aging on the frequency of the marker has been reported by some authors^{6,18-20} and refuted by others.^{21,22} Studies of the correlation between X inactivation and fra(X) expression also produced controversial results.²³⁻²⁵ Sometimes familial characteristics have been assumed to play a role, as in some families the patients express the

marker in a lower percentage than in others.²⁶ An inverse correlation has consistently been found in carriers between expression of the marker X and intelligence.^{6 18 20 23 27} However, little is known about the phenotypic differences, since complete

intelligence testing has only been performed in a minority of cases.

Reports concerning the families in which a normal man presumably passed the fragile site to his daughters are intriguing. The women in these

FIG 1 Pedigree of the fra(X) family. All subjects in generations II and III, except for III.15, are dead. All numbered subjects without brackets have been investigated cytogenetically.

exceptional sibships are rarely if ever mentally retarded,^{7 28 29} while in general 30% of carrier women are mentally deficient.^{6 21} Pembrey *et al*²⁹ explained this phenomenon by formulating the hypothesis that a premutation is present in these carriers which generates the definite defect at crossing over during meiosis. Recombinant DNA investigations are needed to clarify this question and also to improve the detection of cytogenetically non-expressing carriers.

To elucidate some of the problems we performed cytogenetic investigations on a large family. These were followed by psychological studies of intelligence level and intelligence profile.³⁰ Finally we performed linkage studies³¹ with a number of DNA probes which detect DNA sequence polymorphisms at the end of the long arm of the X chromosome. In this paper we report the cytogenetic and clinical results.

Materials and methods

The family study was undertaken after the fra(X) syndrome was diagnosed in seven brothers. All family members related in the female line were asked for their cooperation and cytogenetic investigations were performed on a total of 85 persons. Eighteen men and seven women had not been able to finish primary education or to maintain themselves independently and were therefore considered to be mentally retarded (fig 1).

Chromosomal investigations were performed by culturing peripheral blood lymphocytes for 72 hours in medium 199 with 5% fetal calf serum, buffered with HEPES buffer. Giemsa banding was performed by standard techniques to confirm involvement of the X chromosome. At least 50 cells were examined from each family member. We considered women to be negative if no more than one in 50 cells expressed the fragile site (unless they were obligate carriers). If only one in 50 cells was fra(X) positive in men, then a further 50 cells were analysed. Only if another fragile X was found was the result considered to be positive. This cut-off point to avoid false positives lies only slightly above the limit for non-specific telomeric changes estimated by Steinbach *et al*.³²

A number of clinical investigations were performed to compare the phenotypic expression in this family with that in published reports. Testicular volumes were calculated according to Cantu *et al*³³ from the formula $\frac{\pi}{6} \times l \times w^2$ in which l =length and w =width of the testis.

The family study was extended after it appeared that 16 fra(X) positive subjects were descendants of a man (III.12) who was said to have been normal.

None of his sons, but several of his daughters, expressed the fragile site. Either he had transmitted the abnormality to his daughters or he had married a carrier woman. Both possibilities had to be considered as we discovered that his wife's sister also had a fra(X) positive daughter (IV.52) with three fra(X)

TABLE 1 Cytogenetic results in males.

Fra(X) positive men (n=19)			Fra(X) negative men (n=19)		
Pedigree No	Age (y)	% fra(X)	Pedigree No	Age (y)	Fra(X)
IV. 1*	75	26	IV. 38	65	-
2	74	28	46H	70	-
7	66	19	47	56	-
8*	64	19	51	47	-
9	63	30	56	71	-
10†	62	6	57	56	-
11	59	40	58H	82	-
12	58	28	62	53	-
55	64	9	V. 53	45	-
63	52	9	64	25	-
68	60	14	68	16	1/100
V. 45	19	44	76	43	1/100
57	29	16	77H	55	-
58	37	26	VI. 110	23	-
63	30	42	121	21	-
66	30	18	122	19	-
67	26	34	152	26	-
69	33	6	153	25	-
79	27	14	161	17	-

*Dead.

†Non-retarded.

H=husband.

TABLE 2 Cytogenetic results in females.

Fra(X) positive women (n=21)			Fra(X) negative women (n=26)		
Pedigree No	Age (y)	% fra(X)	Pedigree No	Age (y)	Fra(X)
IV. 4	71	8	III. 15	88	-
24	59	28	IV. 40	63	-
41	62	6	42	52	-
46	66	9	48†	60	2/100
49	50	9	50†	49	-
52	54	5	60	66	-
58	74	20	61	59	-
59	68	18	64	78	-
67*	70	10	V. 51	42	-
V. 54	43	4	60	37	-
55	41	4	71	36	-
56*	38	20	73	44	-
59*	32	21	75	36	-
61*	31	14	78	39	-
62*	23	24	80	42	2/100
65*	20	22	81	38	2/100
70*	30	6	82	30	2/100
72	26	3	VI. 111	21	-
74	53	8	131	9	-
77	50	26	142	26	-
VI. 132	8	8	151	27	-
			162	13	-
			171	22	-
			172	17	-
			181	20	-
			191	20	-

*Mentally retarded. †Obligate carrier.

positive children. Another possibility, proposed by Pembrey *et al.*,²⁹ is the existence of a recombination enhancing factor in both women, who could both have been married to a non-expressing hemizygous man.

Results

The cytogenetic results in 38 males and 37 females are summarised in tables 1 and 2. Eighteen males expressing the marker were mentally retarded. One man expressing the fragile site in 6% of his cells finished primary and secondary education, lived independently, and was a foreman in a factory. He did not have the long face with large ears that was seen in his seven retarded brothers (fig 2). Karyotyping was repeated in IV.10 as well as in six other persons at an interval of more than a year and consistent results were obtained (table 3).

In 16 sibships we ascertained the number of normal and fra(X) persons, the sex ratio, and the mental status (table 4). Three men and six women were dead but 34 males and 36 females could be investigated cytogenetically. Sixteen men were found to be normal and 18 to be fra(X) positive. Fifteen women were fra(X) negative and 21 positive. These figures do not differ significantly from the expected segregation ratio. Seven carrier females appeared to be mentally retarded (33%).

Large differences in the size of the sibships existed in successive generations. In generation IV the mean size of a sibship was 10.25, in generation V it was 3.85, and in generation VI only 2.16. No differences in reproductive fitness between normal women and non-retarded carriers were found in two generations. The mean number of children of parents in generation IV was 3.66 in normal females and 3.30 in carriers. In the next generation it was 2.25 and 2.20, respectively (table 5). Neither retarded men nor retarded women produced any offspring.

We also studied the relation between expression of the marker and age (fig 3). In males, no influence of age on the frequency of expression could be

TABLE 3 Consistency of the cytogenetic results.

Pedigree No	Percentage fragile sites	
	First test	Second test
IV. 1	26	25
2	20	28
8	19	20
9	30	25
10	6	6
V. 63	18	14
67	34	50

FIG 2 The seven mentally retarded brothers. (Reprinted with permission of the editors of *Tijdschr Kindergeneesk.*)

TABLE 4 Segregation in 16 sibships.

Sibship No	No of males		N	Fra(X) positive	MR	No of females		N	Fra(X) positive	MR
	Alive	Dead				Alive	Dead			
IV.1-13	12	3	1(?)	8	7	1			1	
38-51	3		3			11	4	2	5	
55-63	5		3	2	2	4		2	2	
64-68	1			1	1	4	2	1	1	1
V.53-59	3		1	2	2	4			4	2
60-62						3		1	2	2
63-65	2		1	1	1	1			1	1
66-68	3		1	2	2					
69-73	1			1	1	4		2	2	1
74-77	1		1			3		1	2	
78-79	1			1	1	1		1		
VI.110-111	1		1			1		1		
121-122	2		2							
131-132						2		1	1	
151-153	2		2			1		1		
171-172						2		2		
Total (16 sibships)	37	3	16	18	17	42	6	15	21	7

established. In females, a slight negative correlation was found ($r=-0.0346$) which disappeared if separate calculations were made for non-retarded carriers ($r=0.0004$) and retarded carriers ($r=0.0009$). Since a negative correlation is known to exist between the frequency of expression and IQ, and as the mean age of the retarded carriers was lower than that of non-retarded carriers (35 versus 52 years), this may explain the phenomenon.

The mean frequency of expression in seven retarded carriers was 16.7%. In seven non-retarded carriers of about the same age (the mean ages being 35 and 42 years, respectively), the mean percentage

TABLE 5 Number of children of carriers and normal females per generation.

Carriers		Normal females	
Pedigree No	No of children	Pedigree No	No of children
IV. 4	2	IV.40	1
24	3	60	5
41	1	61	5
46	7		
48	3		
49	3		
50	3		
52	5		
58	4		
59	2		
Mean	3.30		3.66
V.54	2	V.60	3
55	2	71	2
72	2	73	2
74	3	75	2
77	2	78	3
		80	2
		81	2
		82	2
Mean	2.20		2.25

FIG 3 The relationship between expression of the fragile site and age in males and females.

was 6.3%. This difference is highly significant: $\alpha=0.01$ on Wilcoxon's test (table 6).

The clinical picture in this family corresponds with that in other published reports. Sixteen out of 18 retarded fra(X) positive males and five out of seven retarded carrier females showed the typical long face with prominent chin and large ears (fig 4). Non-retarded carrier females did not show facial characteristics by which they could be distinguished from their fra(X) negative sibs.

Testicular volumes were calculated in nine men and were found to exceed the 90th centile in four of them.^{34 35} In a fifth man, the enlargement of the left testicle may have been compensatory for the removal of the right one in his youth (table 7). A testicular biopsy performed in IV.8 did not show any abnormality. Estimation of growth hormones, LH, FSH, and testosterone in these men produced normal results and excretion of amino acids or glucuronic acid was within normal limits.

TABLE 6 Expression in retarded and non-retarded carriers of corresponding mean age (Wilcoxon's test).

Retarded carriers				Non-retarded carriers			
Rank	No	Age (y)	% fra(X)	Rank	No	Age (y)	% fra(X)
9	70	10		6	71	8	
11	38	20		2.5	43	4	
12	32	21		2.5	41	4	
10	31	14		1	26	3	
14	23	24		6	53	8	
13	20	22		8	50	9	
4	30	6		6	8	8	
73	Mean 35	Mean 16.7		32	Mean 42	Mean 6.3	

TABLE 7 Testicular volume.

Pedigree No	Right testis (ml)	Left testis (ml)
IV. 1	33.02	32.48
2	22.48	15.46
7	19.16	14.95
8	—	38.22
9	32.27	29.28
11	31.71	31.85
12	37.58	42.80
55	19.71	16.62
63	21.60	18.95

CT scans of the skull were performed in IV.7, IV.8, and IV.12. In all of them, enlargement of the ventricular system that exceeded the expected age alterations was found (fig 5).

Discussion

Recent reports present contradictory data about the segregation of the fragile X site. Sherman *et al*³⁴ calculated a 20% deficit of affected males compared with the expected segregation ratio. Fryns³⁶ and Tejada *et al*³⁷ reported a surplus of retarded males with the fragile site in their families, while others found the expected number of 50% retarded sons.³³ In our kindred the number of observed male patients in 16 sibships was in agreement with the expected number. The number of carriers surpassed the number of normal women but did not differ significantly from the expected number (table 4). Thus, the segregation data were in agreement with X linked inheritance. The existence of three kinds of hemizygous male, namely the fra(X) positive

FIG 4 A normal woman (right) with her retarded fra(X) positive brother and sister.

FIG 5 CT scan from IV.9 showing dilated ventricular spaces and cortical atrophy in excess of age related changes.

retarded and non-retarded males and the phenotypically and cytogenetically normal males who can transmit the abnormality, is sufficient reason for an occasional disturbance of the segregation data.

We observed phenotypic expression in 17 out of 18 marker positive males (94.4%). The paucity of reports about fra(X) positive normal men seems to confirm this high penetrance of mental deficiency in fra(X) positive males.

A striking peculiarity was the low percentage of cells with the fragile site in the fra(X) positive normal man (IV.10) compared with his seven retarded brothers (6% to, on average, 27%). Differences in frequency of expression between non-retarded and retarded carriers have been connected with lyonisation.¹⁸ However, it is difficult to see how the degree of inactivation of the X chromosome could determine the visibility of the fragile site. Hypotheses to explain this have not yet been confirmed and, moreover, could not apply to the phenomenon in men.

Neither in the male IV.10 nor in the carrier females could the differences in expression be explained by a negative correlation between age and the expression of the marker. Familial characteristics do not apply as all belong to the same kindred. In this connection, the women in sibship IV.38 to IV.51 are very interesting. Assuming that the abnormality has been passed to them by their normal father, they could be assigned to the group of exceptional carriers who rarely, if ever, are mentally retarded. Only four out of seven of these carriers expressed the fragile site in 9, 9, 6, and 2% of their cells, respectively. These appear to be much lower percentages than found in other members of the same family. Such a low expression would be in agreement with other reports about such exceptional families.³⁹ The assumption that all seven women in this sibship are carriers is strengthened by the fact that IV.40 has a severely retarded grandson (not available for investigation).

If these females are omitted from the segregation analysis in table 4 the ratio of normal women to carriers becomes 13:16, which is not significantly different from one. If all are counted as carriers, the ratio becomes 13:23 which is still in agreement with the expected ratio in X linked inheritance ($p > 0.05$, Student's *t* test). A full explanation for the irregularities in the inheritance of the fragile X syndrome will only emerge from a better understanding of the fragile site itself.

Further data about the fragile X syndrome in this family, emerging from psychological investigations, appear in the following paper. Linkage studies of DNA sequence polymorphisms will be reported at a later date.

We thank Dr F Eulderink for investigating the testicular biopsy and Dr A J M Pelckmans for making available the CT scans. We are grateful to Mrs N Zvelebil and G Korthof for the cytogenetic analyses and to Mrs I van Leeuwen for her genealogical support and her help in organising the contacts with the family.

References

- Harvey J, Judge C, Weiner S. Familial X-linked mental retardation with an X-chromosome abnormality. *J Med Genet* 1977;14:46-50.
- Sutherland GR. Heritable fragile sites on human chromosomes. Demonstration of their dependence on the type of tissue culture medium. *Science* 1977;197:265-6.
- Barbi G, Steinbach P, Vogel W. Nonrandom distribution of metotrexate-induced aberrations on human chromosomes. Detection of further folic acid sensitive fragile sites. *Hum Genet* 1984;68:290-4.
- Gardiner GB, Wenger SL, Steele MW. In vitro reversal of fragile-X expression by exogenous thymidine. *Clin Genet* 1984;25:135-9.
- Cantù ES, Jacobs PA. Fragile X expression: relationship to the cell cycle. *Hum Genet* 1984;67:99-102.
- Chudley AE, Knoll J, Gerrard JW, Shepel L, McGahey E, Anderson J. Fragile (X) X-linked mental retardation. Relationship between age and intelligence and the frequency of expression of fragile (X)(q27). *Am J Med Genet* 1983;14:699-712.
- Sherman SL, Jacobs PA, Morton NE, et al. Further segregation analysis of the fragile X syndrome with special reference to transmitting males. *Hum Genet* 1985;69:289-99.
- Steinbach P, Barbi G, Boller T. On the frequency of telomeric chromosomal changes induced by culture conditions suitable for fragile X expression. *Hum Genet* 1982;61:160-2.
- Dakar MG, Chidiac P, Fear CN, Berry AC. Fragile X in normal male: a cautionary tale. *Lancet* 1981;i:780.
- Webb GC, Rogers JG, Pitt DB, Halliday J, Theobald T. Transmission of fra (X)(q27) site from a male. *Lancet* 1981;ii:1231-2.
- Camerino G, Mattei MG, Mattei JF, Jaye M, Mandel JL. Close linkage of fragile-X mental retardation syndrome to haemophilia B and transmission through a normal male. *Nature* 1983;306:701-4.
- Fryns JP, Van den Berghe H. Transmission of fragile (X)(q27) from normal male(s). *Hum Genet* 1982;61:262-3.
- Gardner RJM, Smart RD, Cornell JM, Merckel LM, Beighton P. The fragile X chromosome in a large Indian kindred. *Clin Genet* 1983;23:311-7.
- Jacobs PA, Mayer M, Matsura J, Rhoads F, Yee SC. A cytogenetic study of a population of mentally retarded males with special reference to the marker (X) syndrome. *Hum Genet* 1983;63:139-48.
- Martin JP, Bell J. A pedigree of mental defect showing sex linkage. *J Neurol Psychiatry* 1943;6:154-7.
- Nielsen KB, Tommerup N, Poulsen H, Mikkelsen N. X-linked mental retardation with fragile X. A pedigree showing transmission by apparently unaffected males and partial expression in female carriers. *Hum Genet* 1981;59:23-5.
- Rhoads FA, Oglesby AC, Mayer M, Jacobs PA. Marker syndrome in an Oriental family with probable transmission by normal male. *Am J Hum Genet* 1982;12:205-17.
- Jacobs PA, Glover TW, Mayer M, et al. X-linked mental retardation: a study of seven families. *Am J Med Genet* 1980;7:471-89.
- Turner G, Daniel A, Frost M. X-linked mental retardation

- macro-orchidism and the Xq27 in carriers of X-linked mental retardation. *J Pediatr* 1980;**96**:837-41.
- ²⁰ Howard-Peebles PN. Fragile sites in human chromosomes. II. Demonstration of the fragile site Xq27 in carriers of X-linked mental retardation. *Am J Med Genet* 1980;**7**:497-501.
- ²¹ Turner G, Brookwell R, Daniel A, Selikowitz M, Zilibowitz M. Heterozygous expression of X-linked mental retardation and X-chromosome marker fra(X)(q27). *N Engl J Med* 1980;**303**:662-4.
- ²² Uchida IA, Joyce EM. Activity of the fragile X in heterozygous carriers. *Am J Hum Genet* 1982;**34**:286-93.
- ²³ Opitz JM, Sutherland GR. Conference report. International Workshop on the fragile X and X-linked mental retardation. *Am J Med Genet* 1984;**17**:5-94.
- ²⁴ Uchida IA, Freeman VCP, Jamro H, Partington MW, Soltan HC. Additional evidence for fragile X activity in heterozygous carriers. *Am J Hum Genet* 1983;**35**:861-8.
- ²⁵ Carpenter NJ, Leichtman LG, Say B. X-linked mental retardation. *Am J Dis Child* 1982;**136**:392-8.
- ²⁶ Soudek D, Partington MW, Lawson JS. The fragile X syndrome. I. Familial variation in the proportion of lymphocytes with the fragile site in males. *Am J Med Genet* 1984;**17**:241-52.
- ²⁷ Kähkönen M, Leisti J, Wilska M, Varonen S. Marker X-associated mental retardation. A study of 150 retarded males. *Clin Genet* 1983;**23**:397-404.
- ²⁸ Davies KE, Mattei MG, Mattei JF, *et al.* Linkage studies of X-linked mental retardation: high frequency of recombination in the telomeric region of the human X-chromosome. *Hum Genet* 1985;**70**:249-55.
- ²⁹ Pembrey ME, Winter R, Davies KE. A premutation that generates a defect at crossing-over explains the inheritance of fragile (X) mental retardation. *Am J Med Genet* 1985;**21**:709-17.
- ³⁰ Veenema H, Veenema T, Geraedts JPM. The fragile X syndrome in a large family. II. Psychological investigations. *J Med Genet* 1987;**24**:32-8.
- ³¹ Veenema H, Carpenter NJ, Bakker E, Hofker MH, Millington Ward A, Pearson PL. The fragile X syndrome in a large family. III. Investigations on linkage of flanking DNA markers with the fragile site Xq27. Submitted for publication.
- ³² Steinbach P, Barbi G, Böller T. On the frequency of telomeric chromosomal changes induced by culture conditions suitable for fragile X expression. *Hum Genet* 1982;**61**:160-2.
- ³³ Cantù JM, Scaglia HE, Medina M, *et al.* Inherited congenital normofunctional testicular hyperplasia and mental deficiency. *Hum Genet* 1976;**33**:23-33.
- ³⁴ Farkas LG. Basic morphological data of external genitals in 177 healthy central European men. *Am J Phys Anthropol* 1972;**34**:325-8.
- ³⁵ Zackmann M, Prader A, Kind HP, Häftiger M, Budliger H. Testicular volume during adolescence. Cross-sectional and longitudinal studies. *Helv Paediatr Acta* 1974;**29**:61-72.
- ³⁶ Fryns JP. The fragile X syndrome. A study of 83 families. *Clin Genet* 1984;**26**:497-528.
- ³⁷ Tejada I, Nicolas H, Cheruy C, Boué J. Study of a large sibship with mentally retarded males and females carrying fragile X. *Eur Soc Hum Genet Symp Madrid, Spain*, 29 Sept-2 Oct 1982:124.
- ³⁸ Howard-Peebles PN, Stoddard GR, Mims MG. Familial X-linked mental retardation, verbal disability and marker X-chromosomes. *Am J Hum Genet* 1979;**31**:214-22.
- ³⁹ Mattei JF, Mattei MG, Aumeras C, Auger M, Giraud F. X-linked mental retardation with the fragile X. A study of 15 families. *Hum Genet* 1981;**59**:281-9.

Correspondence and requests for reprints to Dr H Veenema, Instituut voor Anthropogenetica, PO Box 9503, 2300 RA Leiden, The Netherlands.