Technical method

Selection and use of a method for the culture of blood leucocytes to reveal the fra(x) site

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The fragile site on the long arm of the X chromosome was first described in 1969.¹ At the Paris chromosome conference in 1975² it was given the designation fra(x) (q2.8). In the following year Sutherland³ and a number of others described culture methods and culture media which would make the demonstration of the site more reliable.⁴⁻¹¹ In 1975 Turner *et al*¹² described the clinical picture of sex linked mental retardation and estimated the numbers concerned. The constant feature was the mental retardation, sometimes with other clinical abnormalities. The demonstration of the fragile site in the female carriers led to the discovery that some of them were also affected mentally. The early claims for a medium which would allow the chromosome abnormality to be demonstrated with regularity entailed using various proprietary media, particularly TC 199. Later methods stated that the pH of the medium was the important factor, the optimum being 7.6,⁵ which should be attained for the final hours of culture. Finally, the important factor was found to be folic acid. A low concentration of folic acid in the medium made the demonstration of the site in stained chromosomes more reliable. A special medium low in folic acid was produced and this was used in a complete medium which was low in serum, since serum also contains folic acid. The addition of folic acid antagonists such as methotrexate and 5-fluorodeoxyuridine to the medium led to some inhibition of cell growth but greater reliability in the result. The method we adopted was a mixed method since different folic acid antagonists give different numbers of positive cells in the same patient with perhaps a reversed numerical effect in another patient. With this method we believe that false negative results are no longer a problem.

Material and methods

For each case 0.5 ml heparinised venous blood was

placed in each of four sterile universal bottles containing 5 ml culture medium plus 0.1 ml phytohaemagglutinin. The medium in three of the bottles was Gibco 199 with hepes buffer supplemented with 5% pooled human AB serum, 100 µg/ml streptomycin, and 100 units/ml ampicillin. 5-fluorodeoxyuridine was added to one of the cultures (F96) to give a final concentration of 0.6 µg/ml. The medium in the fourth bottle was supplemented with 15% fetal calf serum as well as the human serum and antibiotics and this culture was treated as a normal blood culture in our laboratory. All cultures were incubated at 37°C. After 48 h, 5-fluorodeoxyuridine to give a final concentration of 0.6 µg/ml and methotrexate to give a final concentration of 0.5 µg/ml were added to the second culture (FM) of the first three cultures set up. After a further 21 h 0.1 ml of 0.01% colchicine solution was added to all cultures except F96 and 3 h later they were harvested. F96 was harvested in a similar manner but 24 h later than the other cultures.

For harvesting, the cultures were centrifuged at 100 g for 10 min. The supernatant was discarded, and the cell pellet was then resuspended in 5 ml prewarmed 0.5% KCl solution and incubated at 37°C for 10 min. The tubes were recentrifuged at 100 g for 10 min, the supernatant discarded, and the cell pellet resuspended. The cell suspension was then fixed by slowly adding Carnoy's fixative (3:1, methanol:glacial acetic acid) while agitating the cells in a "whirlimixer." The fixing procedure was repeated twice. Cultures treated with 5-fluorodeoxyuridine and methotrexate tended to need more fixing than normal blood cultures. The cell suspensions were stored at -20°C for at least 1 h before being put on to slides. The cell suspension was centrifuged at 100 g for 10 min, then resuspended in enough fixative to give a slightly cloudy suspension. A clean grease free slide was breathed on in order to create a layer of condensation, then one drop of the cell suspension was immediately dropped onto the centre of the slide, which was then air dried. Where necessary, spreading of the metaphase plates was improved by adding further acetic acid to the fixative or dropping fixative on to the slide after the drop of cell suspension had spread out. Table 1 summarises the culture methods used.

Results

The technique was developed using blood from known carriers of the abnormal X. Subsequently we

Table 1 Summary of methods used

Constituent or step	Bottle			
	1	2 (FM)	3 (F96)	4
Medium 199 + Hepes	5 ml	5 ml	5 ml	5 ml
Phytohaemagglutinin	0.1 ml	0.1 ml	0.1 ml	0.1 ml
Pooled human AB serum	5%	5%	5%	5%
Fetal calf serum	—	—	—	15%
Streptomycin	100 µg/ml	100 µg/ml	100 µg/ml	100 µg/ml
Ampicillin	100 units/ml	100 units/ml	100 units/ml	100 units/ml
5-fluorodeoxyuridine (to final concentration of 0.6 µg/ml)	—	Added at 48 h	Added initially	—
Methotrexate (to final concentration of 0.5 µg/ml)	—	Added at 48 h	—	—
Colchicine (0.01%)	0.1 ml at 69 h	0.1 ml at 69 h	0.1 ml at 93 h	0.1 ml at 69 h
Harvest	At 72 h	At 72 h	At 96 h	At 72 h

Table 2 Results obtained with the four culture methods

Method	No of cases where method gave best results	No of cases where method gave any result
22 affected males		
1	6	15
2	7	13
3	7	14
4	2	10
4 carrier females		
1	1	2
2	0	3
3	2	4
4	1	2

The greatest percentage of cells showing the abnormal X chromosome in any one culture was 75%, the lowest was 4%. The percentage of cells affected does not seem to affect the severity of the handicap.

tested 37 male patients from Normansfield Hospital suffering from undiagnosed mental handicap. In this group seven were found to be carriers of the fragile X. In a group of six boys tested, who were from a special school, two were found to be positive. Table 2 summarises the results from each of the four culture methods used.

Discussion

The development of a reliable technique for demonstrating the fragile X in males suffering from mental handicap is a necessary step to the diagnosis of carrier females and thus to antenatal diagnosis. A few cases of antenatal diagnoses have been reported but there are no informative series. The apparent disappearance of the affected cells in women at the age of about 35 does not make this task easier. The technique is effective in blood cultures and fibroblast cultures. We have found only one young carrier woman, who is married and waiting to start a family. Now that she is aware that she is a carrier she has decided to postpone her first pregnancy in the hope that by that time the method will be shown to be effective. For the present the important step is to test as many young males with

mental handicap as possible.

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