

## Differential expression of the ICF (immunodeficiency, centromeric heterochromatin, facial anomalies) mutation in lymphocytes and fibroblasts

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**SUMMARY** Fibroblasts from a patient with ICF syndrome were grown in the presence of excess of nucleotides, in media with different amounts of folic acid, and with caffeine in an attempt to induce the chromosomal anomalies observed in lymphocytes. We induced despiralisation and breakages in the centromeric heterochromatin of chromosomes 1 and 16 but not associations and multibranching. We suggest that the absence of the major chromosomal anomalies in fibroblasts from patients with ICF might be the result of both a longer G<sub>2</sub> in these cells and differential patterns of interphase heterochromatin associations in the two tissues.

The ICF syndrome is characterised by immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9, and 16, and facial anomalies.<sup>1</sup>

In five of the six patients reported so far, obvious chromosomal anomalies were found only in lymphocytes, and in a case recently observed by Turleau *et al* (1989, personal communication) centromeric instability was not seen in fibroblasts either. Chromosome stretching, breakage, and arm duplications of chromosome 1 were seen in fibroblasts only in the case of Howard *et al.*<sup>2</sup>

In a previous paper<sup>1</sup> we postulated that the chromosomal abnormalities in the ICF syndrome might be the result of a mutation interfering with the time of duplication of part of the centromeric heterochromatin and, as a consequence, of its condensation cycle.

Delayed chromatin condensation caused by late and incomplete replication is believed to control the expression of some fragile sites in human chromosomes.<sup>3</sup>

As in the ICF syndrome, fragile sites are not usually seen in cultured fibroblasts unless they are induced by experimental conditions.

We tried to induce in the fibroblasts of the patient described previously<sup>1</sup> the chromosomal lesions typical of her lymphocytes, using agents or media known to interfere with DNA synthesis and to

increase fragile site expression: excess of nucleotides,<sup>4</sup> culture media deficient in folic acid,<sup>5</sup> and caffeine.<sup>6</sup>

In addition we present some further clinical data on the patient.

### Clinical data

At the age of five and a half years the girl (fig 1) was readmitted to hospital for pulmonary infection and on this occasion a new series of laboratory examinations was performed with the following results: IgA 10, IgG 295, IgM 10 mg×100 ml (normal values for age, IgA 93±27, IgG 929±228, IgM 56±18). These tests were performed before treatment with immunoglobulins. Percentage reduction of B lymphocyte count was SIg 1% (normal 8±4%) with total lymphocyte count 3220/mm<sup>3</sup> (normal >1500). A defect of chemotaxis of the polymorphonuclear neutrophil (PMNn) was investigated by means of a 'skin chamber'<sup>7</sup>: 19 510 000 cells/cm<sup>2</sup>/24 h with PMNn 90.3% (normal 63 635 000±8 252 958 PMNn >90%). Cell mediated immunity, phagocytosis, and O<sub>2</sub> production of PMNn was normal. HLA: A10, A28, B8, B27, Cw1, Cw2.

Since the last examination the girl has failed to thrive and her height is now 102 cm (3rd to 10th centile) and weight 14.6 kg (<3rd centile). She has severe chronic bronchitis with bronchiectasis throughout the lower lobes and purulent bronchorrhoea levels above 10 ml/24 h; she has maxillary



FIG 1 *The patient at five and a half years.*

sinusitis and conductive bradycardia with tympanic damage from previous otitis.

#### Materials and methods

The effect of different concentrations of folic acid

in the culture medium was studied by growing fibroblasts in Ham's F10 (1.3 mg/l folic acid) supplemented with 20% bovine serum, in TC 199 (0.01 mg/l folic acid) supplemented with 5% bovine serum for 48 hours, and in MEM without folic acid (MEM-FA) with 5% bovine serum for 48 hours.

Excess of nucleotides was studied by growing fibroblasts in medium F10 in the presence of thymidine at concentrations of 600 and 300 µg/ml, continuously for 24 hours (experiments 1 and 2), or for 17 hours followed by seven hours in TC 199 after removal of excess of thymidine (experiments 3 and 4). Similarly, adenosine 5'-triphosphate (500 µg/ml) was added for 24 hours (experiment 5) or for 17 hours+seven hours in TC 199 after removal of the excess adenosine (experiment 6).

The effect of caffeine was studied by adding 2.2 mmol/l of the substance for six hours to cells grown in F10 and in MEM-FA (experiments 7 and 8).

All cultures were exposed to colcemid (0.02 µg/ml) for the last three hours.

Chromosome preparations in all the experiments were stained with DA-DAPI.

The length of G<sub>2</sub> in fibroblasts was determined with a pulse of 20 minutes of tritiated thymidine (Amersham, specific activity 5 µCi/mmol/l) at a concentration of 1 µCi/ml and then processing the cultures after three, four, five, six, seven, and eight hours in medium without <sup>3</sup>H-TdR. Colcemid (0.02 µg/ml) was added for the last 30 minutes. The proportions of labelled metaphases were scored after autoradiography with NTB2 Kodak emulsion.

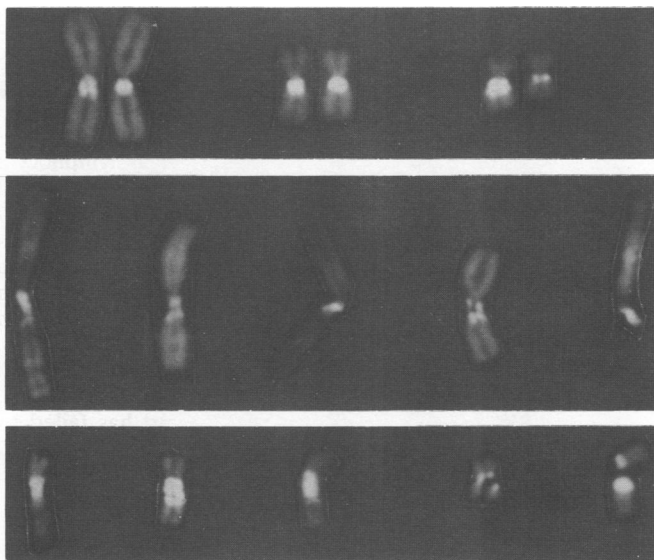


FIG 2 (a) Chromosomes 1, 9, and 16 with normal appearance of the centromeric regions from patient's fibroblast cultures. Chromosomes 1 (b) and 16 (c) with various degrees of stretching up to breakage. (DA-DAPI staining.)

## Results

The most striking chromosomal abnormalities found in ICF lymphocyte cultures (multibranch configurations and associations of chromosomes 1, 9, and 16) were not induced in fibroblasts by any of the experiments. However, we found various degrees of despiralisation of the centromeric heterochromatin of chromosomes 1 and 16, which is the most frequent anomaly in lymphocytes.

In some metaphases these chromosomes appeared stretched up to breakage of one or both chromatids (fig 2). Chromosome 9 was less frequently stretched and was never broken.

To ascertain the possible presence of these minor anomalies, we re-examined the cultured fibroblasts obtained at the first examination of the patient, which in our previous paper<sup>1</sup> were reported to have no abnormalities.

We found five cells with despiralisation of chromosomes 1 or 16 or both among the 91 cells analysed (5.5%), a frequency not significantly different from that found in control fibroblasts (2.0%).

Table 1 shows the results obtained in the patient and control fibroblasts grown in media with different

amounts of folic acid: Ham's F10, TC 199, and MEM-FA.

The frequency of anomalies in the patient's fibroblasts increased to 74% in cells grown in MEM-FA and to 68% in TC 199 compared with 6.8% and 20.6%, respectively, in control fibroblasts. The differences between the number of cells with anomalies in the patient and in the control are highly significant.

The results obtained with an excess of thymidine are shown in table 2. With the highest dose (600 µg/ml, experiment 1) only a few cells in mitosis were found in both the patient and control cultures and the difference between the number of cells with anomalies was not significant. With 600 µg/ml followed by addition of TC 199 (experiments 3 and 4) and with a lower dose (300 µg/ml, experiment 2), the differences between patient and control cells are significant.

In all the experiments chromosome 1 was the most frequently involved in despiralisation, followed by chromosomes 16 and 9.

An excess of adenosine (table 2, experiment 5) produced no significant difference in the induction of anomalies between patient and control. After culture in medium free of folic acid (experiment 6) the frequencies of anomalies in the two systems

TABLE 1 *Effects of different concentrations of folic acid in the culture medium on ICF fibroblasts.*

Culture medium		Total cells analysed	Total cells with anomalies of 1, 9, and 16 (%)	% abnormal chromosomes*		
				1	9	16
Ham's F10	Proband	91	5 (5.4)	3.2	—	1.0
	Control	100	2 (2.0)	0.5	0.5	—
TC 199	Proband	76	52 (68.4)	36.0	4.6	18.4
	Control	29	6 (20.6)	12.0	—	—
MEM-FA	Proband	54	40 (74.0)	41.6	1.8	7.4
	Control	44	3 (6.8)	3.4	1.1	—

\*In this and in the following tables the frequencies of abnormal chromosomes 1, 9, and 16 are calculated on the total number of these chromosomes in the cells analysed.

TABLE 2 *Effects of an excess of thymidine and adenosine on ICF fibroblasts.*

Experiment		Total cells analysed	Total cells with anomalies of 1, 9, and 16 (%)	% abnormal chromosomes*		
				1	9	16
1 TdR 600 µg/ml	Proband	26	12 (46.1)	23.0	1.9	7.6
	Control	13	3 (23.0)	15.3	3.8	—
2 TdR 300 µg/ml	Proband	39	19 (48.7)	33.3	—	3.8
	Control	14	1 (7.1)	3.5	—	—
3 TdR 600 µg/ml+TC 199	Proband	55	34 (61.8)	40.0	—	5.4
	Control	41	7 (17.0)	7.3	—	1.2
4 TdR 300 µg/ml+TC 199	Proband	57	32 (56.1)	37.6	1.7	10.5
	Control	36	1 (2.7)	1.0	—	—
5 ATP 500 µg/ml	Proband	90	39 (43.3)	25.5	1.6	4.4
	Control	91	37 (40.6)	19.7	3.8	1
6 ATP 500 µg/ml+TC 199	Proband	99	27 (27.2)	15.1	—	4.5
	Control	89	9 (10.1)	3.9	—	—

\*See table 1.

TABLE 3 Effects of caffeine on ICF fibroblasts.

Experiment		Total cells analysed	Total cells with anomalies of 1, 9, and 16 (%)	% abnormal chromosomes*		
				1	9	16
7 Caffeine in Ham's F10	Proband	100	30 (30.0)	18.0	1.0	7.5
	Control	101	8 (7.9)	3.9	—	—
8 Caffeine in MEM-FA	Proband	48	39 (81.2)	50.0	2.0	9.0
	Control	71	15 (21.1)	7.7	2.8	—

\*See table 1.

were reduced, but were significantly higher in the patient's cells.

Caffeine added to F10 medium for the last six hours of culture (table 3, experiment 7) resulted in a higher frequency of anomalies in the patient than in the control, although the overall frequency of the patient's cells with anomalies was lower than that in cultures treated with an excess of nucleotides.

If cells were grown for the same period of time in MEM-FA plus caffeine (experiment 8) the frequency of cells with anomalies was further increased both in the patient and in the control (81% and 21%, respectively).

The mean length of  $G_2$  measured in fibroblasts from the patient and from a control did not differ significantly at five and a half hours and slightly more than five hours respectively.

## Discussion

Excess of thymidine is known to result in a decrease of deoxycytidine (dCTP) and folic acid deficiency in a reduction of thymidine monophosphate (dTMP).<sup>8</sup>

Both these conditions, leading to alterations of the nucleotide pools, increased the frequency of chromosome anomalies in the ICF patient's fibroblasts when compared with those from a control.

These results could be interpreted as suggested by Laird *et al*<sup>3</sup> to explain the expression of fragile sites: the mutation present in the ICF syndrome could result in a delay in duplication of the late replicating pericentromeric regions of chromosomes 1, 9, and 16. In this situation any disturbance in the availability of nucleotides further shifts duplication of these regions towards  $G_2$ , leading to their incomplete condensation. The observed increase of chromosome anomalies in ICF fibroblasts treated with caffeine, which is supposed to shorten the  $G_2$  period,<sup>9</sup> seems to support this hypothesis.

Caffeine is known to induce a marked increase in the expression of fragile sites if used in combination with other inducers, such as FUdR<sup>6</sup> or aphidicolin,<sup>10</sup> or in cultures deprived of thymidine.<sup>11</sup> In our experiments, adding caffeine to cells grown in a folic

acid free medium, we obtained the maximum frequency (81%) of ICF fibroblasts with anomalies.

A longer  $G_2$  in fibroblasts than in lymphocytes is the probable reason for the observed lower expression of ICF chromosome abnormalities in the former tissue, in which decondensation must somehow be induced.

Because it was impossible to estimate directly the length of  $G_2$  in ICF lymphocytes, we compared the duration of  $G_2$  in the patient's fibroblasts (5.5 hours) and in normal fibroblasts (5.2 hours) with the time of 2.3 to 3.5 hours reported for normal lymphocytes.<sup>12</sup> The length of  $G_2$  in fibroblasts, both normal and from the ICF patient, was longer than in normal lymphocytes.

The frequency of anomalies seen in chromosome 9 was similar to that found in controls, which could be the result of a different composition of its centromeric heterochromatin. Recently two repetitive DNA clones have been shown to be related to the centromeric heterochromatin of chromosomes 1, 9, and 16: clone pHuR195, isolated from satellite DNA II, is specific for chromosome 16 but, at low stringency conditions, hybridises also with chromosome 1, while satellite III related clone pHuR98 hybridises with chromosome 9 only.<sup>13</sup> This indicates a similarity in the centromeric heterochromatin of chromosomes 1 and 16, while that of chromosome 9 shows a different composition.

Heterogeneity of the centromeric heterochromatin of the three chromosomes was already indicated by the different behaviour of chromosomes 1 and 16 in comparison with 9 when stained with DAPI/actinomycin D<sup>14</sup> or G11.<sup>15</sup>

We can therefore assume that the mutation in ICF mainly affects specific sequences of chromosomes 1 and 16. The absence in ICF fibroblasts of the most striking chromosome anomalies (multibranching and association with fusion of centromeric heterochromatin) and the failure to induce these abnormalities could be because the interphase association patterns of centromeric heterochromatin of chromosomes 1, 9, and 16 are different in lymphocytes and fibroblasts. These chromosomes are known to asso-

ciate in lymphocyte interphase,<sup>16</sup> while no data are available, to our knowledge, for fibroblasts. Preliminary observations on the association of positive DA-DAPI bodies in interphase nuclei in the two tissues indicate a high number of cells without chromocentres in fibroblasts, while in lymphocytes the DA-DAPI positive bodies varied between two and eight with the majority of cells showing four bodies (data not shown).

Variations in the pattern of heterochromatin association in different tissues has been reported<sup>17</sup> in the mouse.

In conclusion, we think that the mutation responsible for the ICF syndrome affects primarily the duplication time of at least part of the centromeric heterochromatin of chromosomes 1 and 16.

The variation of expression at the chromosomal level of this defect, that is, decondensation, in different tissues depends on the length of G<sub>2</sub>. In fact, the frequency of anomalies in fibroblasts was increased by shifting DNA duplications towards G<sub>2</sub>. In turn, different patterns of interphase heterochromatin association in lymphocytes and fibroblasts can lead to the formation of the more complex anomalies in the former tissue.

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