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Cell cycle analysis in the diagnosis of Fanconi's anemia

Previous studies have demonstrated a cell cycle disturbance in Fanconi's anemia (FA), with a G2 block. Different methods for diagnosis of FA by flow cytometry have been proposed. We describe here a new, highly sensitive and specific approach that utilizes cell cycle analysis after incubation with phytohemagglutinin and melphalan.

Sir,

The diagnosis of Fanconi's anemia (FA) is classically founded on the demonstration of an increased chromosomal sensitivity to alkylating agents, such as diepoxybutane (DEB).[1,2] Previous studies have demonstrated the possibility of diagnosing FA by flow cytometric analysis, showing a block of cell cycling in the G2 phase.[3-5] However, the experimental conditions of the test have not been clearly defined and different methods have been proposed. We evaluated different concentrations of a readily available alkylating drug, melphalan, in the flow cytometric diagnosis of FA. Nineteen controls, 13 patients diagnosed as or suspected of having FA (Table 1), 15 FA parents and 27 patients with various non-FA cytopenias were studied after having given informed consent. Peripheral blood mononuclear cells (PBMCs) were incubated (10^6/mL) for 72 h in IMDM plus 10% FCS and 1% phytohemagglutinin (PHA) in the presence or not of melphalan (Alkeran, GlaxoWellcome) 0.01, 0.05, 0.1, 0.5, 1, 2 µg/mL (added 24 h after the initiation of culture). PBMCs were then treated with an automated DNA staining kit (DNA-prep, Coulter). The PI fluorescence of individual nuclei was measured by a flow cytometer Epics-XL2 (Coulter). The results were analyzed by Multicycle software (Phoenix Flow Systems). Percentages of cells in the G2 phase were compared by Wilcoxon's test. Specificity and sensitivity were calculated using the MiniStat program.[6]

In ten patients with a positive DEB test and various expressions of cytopenia and physical abnormalities

Table 1. Clinical and laboratory data of 10 FA patients. Cell cycle is evaluated after exposure to melphalan 0.1 and 0.5 µg/mL. Three cases (a, b, c), suspected to be FA (DEB test negative or border-line, G2 block), are shown at the bottom.

	Age (yrs)	*Hematologic features*	*Physical abnormalities*	*DEB test*	*%G2 0.1 µg/mL*	*%G2 0.5 µg/mL*
1	6	thrombocytopenia	facies, kidney ectopia, vesicoureteral reflux, skin hyperpigmentation	pos	27	31
2	6	pancytopenia	facies, short stature, left kidney hypoplasia, right kidney ectopia and dysplasia with vesicoureteral reflux, atrial septal defect, hip dysplasia, deafness	pos	40	38
3	8	neutropenia	microcephaly, short stature, thumb adduction, dental hypoplasia	pos	29	54
4	6	normal	facies, microcephaly, short stature, hypertelorism	pos	46	52
5	6	normal	facies, short stature, vesicoureteral reflux, left kidney hypoplasia, *café-au-lait* spots	pos	30	68
6	8	pancytopenia	facies, short stature, right kidney hypoplasia	pos	–	50
7	12	pancytopenia	–	pos	–	60
8	10	pancytopenia	facies, *café-au-lait* spots, hexadactyly	pos	41	54
9	7	thrombocytopenia	horseshoe kidney, duodenal stenosis, hip dysplasia	pos	37	31
10	16	pancytopenia	facies	pos	64	62
a	5	thrombocytopenia	growth retardation, kidney ectopia, cryptorchidism	neg	26	39
b	11	pancytopenia	thumb defect	border-line	61	39
c	1	normal	atrial septal defect, kidney ectopia with vesicoureteral reflux, double right thumb	border-line	39	60

DEB test: pos = cells with chromosome breaks >45%; border-line = cells with chromosome breaks >11<45%; neg = cells with chromosome breaks <11%

Table 2. Percentage of cells in G2/M phase in controls, FA patients, various non-FA cytopenias (6 acquired aplastic anemia, 2 Diamond-Blackfan anemia, 10 acquired neutropenia, 2 MDS, 2 acquired erythroblastopenia, 5 acquired thrombocytopenia) and FA parents. Flow cytometry analysis was performed after exposure to PHA alone or in the presence of 0.01, 0.05, 0.1, 0.5, 1, 2 µg/mL melphalan.

	PHA	*0.01*	*0.05*	*0.1*	*0.5*	*1*	*2*
controls n = 19	8±3 (1-13)	6±4 (0-10)	8±3 (0-14)	9±4 (0-13)	19±8 (9-39)	20±9 (2-32)	21±16 (0-40)
FA n = 10	16±7 (7-30)	21±8 (14-33)	29±5 (23-38)	39±12 (27-64)	50±12 (31-68)	49±17 (36-68)	21±22 (6-37)
FA parents n = 15	6±3 (0-10)	7±3 (0-11)	8±4 (0-13)	10±4 (5-17)	20±6 (12-32)	16±12 (3-32)	16±23 (0-58)
Cytopenias n = 27	6±3 (0-13)	6±4 (0-10)	7±5 (0-14)	9±5 (0-19)	19±8 (0-35)	16±12 (0-34)	5±10 (0-31)

(diagnosed as FA) we observed a significant (p<0.001) accumulation of cells in G2 phase in the following experimental conditions: PHA, PHA+melphalan 0.01, 0.05, 0.1, 0.5 µg/mL (Table 2). Results yielded with melphalan 0.1, 0.05 and 0.01 µg/mL discriminated well between controls and FA patients; establishing a G2% cut-off of 21; the sensitivity and specificity of the test performed with melphalan 0.1 µg/mL was 100%. G2 peak was usually observed with melphalan 0.5 µg/mL. The percentage of G2 cells did not correlate with the presence of pancytopenia or the severity of malformations. No significant difference in the percentage of cells in G2 phase was observed between controls and non-FA cytopenias, nor between controls and FA parents (heterozygous). We observed a normalization of G2 phase when patient 7 developed an acute myeloid leukemia. G2 phase also normalized in patient 10 during G-CSF treatment.[7] In three patients (*a, b, c*), all with a phenotype suspicion of FA, we observed significant G2 block, although the DEB test was negative or border line. Patient *c* is a first-degree cousin of patient #10 (DEB positive). At present, a complete mutation analysis of the FA genes is not available.[8] Our data may support a suggestion that cell cycle analysis is more sensitive than the DEB test. Moreover, the possibility of a negative DEB test in FA has been described.[9,10]

In conclusion, cell cycle analysis after exposure to PHA plus melphalan is a simple and rapid test, specific and probably more sensitive than DEB. Since only molecular diagnosis could confirm our hypothesis, for now we suggest a period of using both tests to diagnose Fanconi's anemia.

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Key words

Fanconi's anemia, cell cycle analysis, DEB test.

Contributions and Acknowledgments

FT and UR conceived and designed the study. FT wrote the paper and, with NC, carried out the colony assays and cytofluorimetric analyses. LL discussed and analyzed data. GP performed the DEB tests. UR, PS and FT are the clinicians involved in following the patients. We thank Prof. Giuseppe Basso for his help in flow cytometric analysis.

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Near-tetraploid acute myeloid leukemia after allogeneic bone marrow transplantation

Tetraploidy and near-tetraploidy are infrequently observed in acute myeloid leukemia (AML).[1-7] Several cases have been reported in patients treated for other cancers[4,8] or after autologous bone marrow trans-