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CYTOGENETIC DIEPOXYBUTANE SENSITIVITY IN SERBIAN CHILDREN WITH FANCONI ANEMIA

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Abstract – Fanconi anemia (FA) is an inherited disorder with aplastic anemia, cancer susceptibility, and hypersensitivity to alkylating agents such as diepoxybutane (DEB). The DEB test is used to screen for FA among patients with bone marrow failure syndromes (BMFS). From February of 2004 to May of 2006, 29 children with BMFS were diagnosed and treated at the Mother and Child Health Care Institute of Serbia (MCHIS). In the examined group, five out of 29 patients (17.2%) were found to have increased DEB-induced chromosome breakage (0.58-2.15 vs. 0.00-0.20 breaks/cell; $p<0.001$) with no overlap. Our results suggest the importance of this analysis for differential diagnosis and adequate therapy of FA among patients with BMFS.

Key words: Fanconi anemia, diepoxybutane, aplastic anemia, marrow failure

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INTRODUCTION

Bone marrow failure syndromes (BMFS) are disorders of hematopoietic stem cells that can lead to peripheral pancytopenia and marrow hypoplasia, when it is called aplastic anemia (AA) (B a g b y *et al.,* 2003). Aplastic anemia can be inherited or acquired. The main causes of inherited AA are congenital in nature, as in the case of Fanconi anemia (FA) (A l t e r, 1995).

Fanconi anemia is a rare autosomal recessive condition with familial aplastic anemia, short stature, developmental anomalies, and increased cancer susceptibility (B a g b y *et al.,* 2003), as well as chromosomal hypersensitivity to cross linking agents [cisplatin, mitomycin C (MMC), diepoxybutane (DEB) and melphalan] (A u e r b a c h *et al.,* 1976; A u e r b a c h *et al.,* 2001; J o e n j e *et al.,* 2001). The clastogenic effect of DNA cross linking agents such as DEB is manifested as increased chromosomal instability of FA cells, which provides a unique marker for the FA genotype (S a s a k i *et al.,* 1973; A u e r b a c h *et al.,* 1976).

In view of the reported 20% to 30% occurence of FA in cases of AA (A l t e r, 1995; W i n d a s s *et al.,* 1987), we conducted a discriminant analysis of DEB-induced chromosomal breakage (A u e r b a c h *et al.,* 1989) in children with bone marrow failure syndromes as part of screening for FA.

PATIENTS AND METHODS

Patients

From February of 2004 to May of 2006, 29 children with BMFS were diagnosed and treated at the Department of Hematology of MCHIS.

Patients were selected for DEB-induced chromosomal breakage studies on the basis of congenital abnormalities, aplastic anemia, and other hematological indicators known to be connected with FA, as well as family screening. Sensitivity and chromosomal instability of peripheral blood lymphocyte cultures, induced by DEB were the main factors for dividing patients into two groups: DEB-sensitive (DEB+, FA) and DEB-insensitive (DEB-, non-FA) patients.

DEB test

The DEB test was carried out according to methods described in the literature (A u e r b a c h *et al*., 1981; A u e r b a c h *et al*., 1989; A u e r b a c h *et al*., 2005) with minor modifications. Diepoxybutane- treated and control peripheral lymphocyte cultures of the patients, were prepared in the same conditions and at the same time as their counterpart cultures of healthy persons (family members). Addition of DEB to the blood cultures took place after 48 hours of cultivation at a final concentration of 0.1 μg/mL (A u e r b a c h *et al*., 1989; A u e r - b a c h *et al*., 2005) and the cells were exposed to 24 hours of cross-linking action in the dark. After 70 hours of cultivation, colcemid was added (2.5 μg/mL) for a further 2 hours and cultures were harvested by standard procedures for chromosome preparation. Gimsa solution staining was applied (A u e r b a c h *et al*., 2005). One hundred metaphases from the mutagen-treated culture and from the control untreated culture were analyzed for chromosome number and for the number and types of structural chromosome aberrations. Ten metaphases were karyotyped according to international nomenclature (ISCN, 2005). Chromosome breaks, chromatid breaks, and acentric fragments were scored as one break. Dicentric chromosomes, ring chromosomes, and radial figures were scored as two events (A u e r b a c h *et al*., 1989; W e g n e r *et al*., 1999). The evaluation parameters were: percentage of aberrant metaphases, breaks per cell, types of aberrations and breaks per aberrant cell.

Discriminant analysis

The chi square test was used for identification of significant difference between the examined cultures of patients (W e g n e r *et al*., 1999) and healthy persons. The cut-off values for discrimination between the DEB+ group and the DEB- group were as indicated previously (A u e r b a c h *et al*., 1989).

RESULTS

The results on DEB sensitivity showed that five patients in the group of 29 examined children were hypersensitive to DEB with a variety of chromosome and chromatid types of breaks and interchanges (Fig. 1). The maximal percentage of DEB- induced aberrant cells was 72.22% (patient No. 1[1]), while the mean value (mean ± S.D.) for the DEB+ group was 45.84%±16.11% (Tables 1 and 2). The mean value of DEB-induced chromosome breaks/cell for DEB+ patients was 1.17±0.65, with a minimal value of 0.58 breaks/cell (patient No. 5) and maximal value of 2.15 breaks/cell (patient No. 1) (Table 1). The range of breaks per DEB-induced aberrant cell was 1.41 – 3.06, while the mean value was 2.47±0.75.

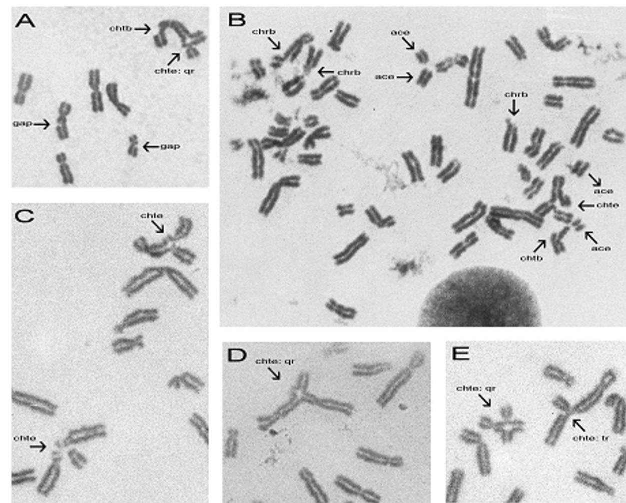

Fig.1. Diepoxybutane-induced chromosomal aberrations in patients with Fanconi anemia. A, B, C, D and E: partial metaphases of FA patients. Chrb: chromosome break, ace: acentric fragment, chtb: chromatid break: chtb, chte: chromatid exchange, tr: triradial, qr: quadriradial.

Table 1. Chromosomal breakage results in the group of DEB- sensitive patients.

Patient	Spontaneous breaks/cell	Cell with spontaneous breaks (%)	DEB induced breaks/cell	Cell with induced breaks (%)	DEB induced breaks/aberrant cell
1.	0,01	1,00	2,15	72,22	2,97
2.	0,07	5,00	1,50	49,00	3,06
3.	0,00	0,00	0,95	32,00	2,97
4.	0,08	8,00	0,68	35,00	1,94
5.	0,27	18,00	0,58	41,00	1,41
Mean	0,09	6,40	1,17	45,84	2,47
S.D.	0,11	7,23	0,65	16,11	0,75
Range	0,00 – 0,27	0 – 18	0,58 – 2,15	32 – 72,22	1,41 – 3,06

Patients with no hypersensitivity to DEB (DEB-) had mean percentage of induced aberrant cells of 1.45%±2.87% (range of 0.00-12.00) and mean breaks/cell value of 0.02±0.05 (range of 0.00-0.20) (Table 2). In the control group the mean percentage of DEB-induced aberrant cells was 0.43%±1.03% (range of 0.00-5.00), while the mean value of breaks per cell was 0.01±0.03 (range of 0.00-0.17) (Table 2). Statistical analysis showed significant difference between the DEB+

[1] Diepoxybutane- induced chromosome breakage was analyzed in 54 metaphases because of the presence of a bad mitotic index.

Table 2. Chromosome breakage results in patients with bone marrow failure syndromes.

Parametrizations	Group	N	Mean	Median	S.D.	Minimal value	Maximal value
Spontaneous breaks/cell	DEB+	5	0,09	0,07	0,11	0,00	0,27
	DEB-	24	0,01	0,00	0,02	0,00	0,07
	Control	30	0,0003	0,00	0,0019	0,00	0,01
Cells with spontaneous breaks (%)	DEB+	5	6,40	5,00	7,23	0,00	18,00
	DEB-	24	0,64	0,00	1,81	0,00	7,00
	Control	30	0,04	0,00	0,19	0,00	1,00
DEB induced breaks/cell	DEB+	5	1,17	0,95	0,65	0,58	2,15
	DEB-	24	0,02	0,00	0,05	0,00	0,09
	Control	30	0,01	0,00	0,03	0,00	0,17
Cells with induced breaks (%)	DEB+	5	45,84	41,00	16,11	32,00	72,22
	DEB-	24	1,45	0,00	2,87	0,00	12,00
	Control	30	0,43	0,00	1,03	0,00	5,00
Induced breaks/aberrant cell	DEB+	5	2,47	2,97	0,75	1,41	3,06
	DEB-	24	0,46	0,00	0,63	0,00	1,67
	Control	30	0,36	0,00	0,77	0,00	3,40

and DEB- groups (Mann-Whitney test: $p<0.0001$) (Table 2) and no overlapping.

Spontaneous breakage was increased in four DEB+ patients (Table 1: pat. Nos. 1, 2, 4, and 5), but one of them (patient No. 3) had no spontaneous breakage. Thus, the mean percentage of spontaneously aberrant cells was 6.40%± 7.23% (range of 0.00-18.00), while the mean value of uninduced breaks/cell for the DEB+ group was 0.09±0.11 (range 0.00-0.27) (Table 1). Three of the DEB- patients had increased spontaneous breakage, the mean values being: 0.64%±1.81% for the percentage of aberrant cells (range of 0.00-7.00) and 0.01± 0.02 for breaks/cell (range of 0.00-0.07) (Table 2). Hence, it was impossible to distinguish of these two groups on the basis of spontaneous breakage because of obvious overlap. The control group had spontaneous chromosomal instability similar to that in the DEB- group, with a mean percentage of aberrant cells of 0.04%±0.19% (range of 0.00-1.00) and a mean value of breaks/cell of 0.003±0.019 (range of 0.00-0.01).

DISCUSSION

Fanconi anemia is a rare autosomal recessive disease, the most common of inherited BMFS, with developmental defects (morphometric, skeletal, renal, and genital anomalies), short stature, and cutaneous abnormalities (cafe-au-lait spots) (Alter, 2005; Bagby *et al.,* 2003; Young, 1995). There is considerable variation of the pattern of clinical manifestations in this disorder, as well as genetic heterogeneity, things that make diagnosis more difficult (Alter, 1996; Dokal, 1998; Esmer *et al.,* 2003; Liu *et al.,* 1994; Najean *et al.,* 1976). Because the FA phenotype overlaps with that of a variety of inherited and acquired aplastic anemias, in children with pancytopenia or thrombocytopenia and hypoplastic bone marrow, a diagnosis of FA must be taken into in consideration (Bagby *et al.,* 2003; Kook *et al.,* 1998).

Fanconi anemia is one of the chromosome instability and DNA repair disorders that are characterized by chromosomal fragility and increased cellular hypersensitivity to specific mutagenic agents and radiation (Auerbach *et al.,* 1976; Tischkowitz *et al.,* 2003). This group of disorders includes ataxia-telangiectasia (AT), Nijmegen breakage syndrome (NBS), Bloom syndrome (BS), xeroderma pigmentosum (XP), Cockayne syndrome (CS), Werner syndrome (WS), trichothiodystrophy syndrome (TTD), Rothmund-Thompson syndrome (RTS), and ICF (immunodeficiency, centromeric heterochromatin instability, and facial anomalies) syndrome. Spontaneous chromosomal breakage is their common marker, so FA cannot be distinguished by this feature. However, hypersensitivity of FA cells to the cytotoxic and clastogenic effects of DNA cross-linking agents like DEB and MMC provides a unique cellular marker that is used to distinguish FA from other chromosomal breakage syndromes (Wegner *et al.,* 1999).

In our study, five (17.2%) cases out of 29 were diagnosed as FA on the basis of DEB induced chromosomal breakage studies. This incidence of FA patients among Serbian children with AA may be attributable to the small number of patients evaluated for FA (Cho *et al.,* 1997; Kook *et al.,* 1998). It could not be the true incidence of FA in Serbian patients with pediatric AA because not all AA patients underwent DEB screening.

A study of the International Fanconi's Anemia Registry (IFAR) showed that the range of spontaneous chromosome breakage in the FA group of 104 patients (0.02-1.90 breaks/cell with a mean of 0.27) overlapped with the range found in the non-FA group of 224 patients (0.00-0.12 breaks/cell with a mean of 0.02) (Auerbach *et al.,* 1989). The unreliability of using the base line of chromosome breakage in differential diagnosis of FA (Auerbach *et al.,* 1989; Kook *et al.,* 1998) was confirmed in the present study. The range of spontaneous breaks per cell in the DEB+ (FA) group was 0.00 to 0.27 with a mean 0.09±0.11, and no significant difference was found in relation to the range of spontaneous breaks/cell (0.00 to 0.01.) in the DEB- (non-FA) group.

According to the IFAR report the mean percentage

of cells with DEB- induced breaks in the FA (DEB+) group was 85.15±1.99 ($p<0.01$), while in the non-FA (DEB-) group it was significantly different- 5.12±0.28 ($p<0.001$) (Auerbach *et al.,* 1989). Similarly, the mean of DEB- induced breaks per cell in FA (DEB+) patients was 8.96±0.448 ($p<0.001$), in contrast to the non-FA (DEB-) group, in which the mean was 0.06±0.004 ($p<0.001$) (Auerbach *et al.,* 1989). There was no range overlap between the two groups. In the present study the mean percentage of cells with DEB induced breaks in FA (DEB+) patients was 45.84%±16.11% ($p<0.01$), while in non-FA (DEB-) patients it was significantly lower 1.45%±2.87% ($p<0.001$). Similarly, FA (DEB+) patients were significantly distinguishable from non-FA (DEB-) patients on the basis of chromosomal breakage (1.17±0.65 vs 0.02±0.05 breaks/cell). The discrepancy in the mean percentage of cells with breaks and mean breaks per cell between the IFAR study and the current work is possibly attributable to size of the sample, modification of the test, and variable sensitivity to DEB among particular FA complementation groups (Kook *et al.,* 1998); or to somatic mosaicism (Gregory *et al.,* 2001). Our results are in line with the study of Kook *et al.* (1998) reporting mean breaks/cell values of 1.95±1.35 in the FA (DEB+) group and 0.02±0.03 in the non-FA (DEB-) group.

Screening by the DEB test is screening for patients with FA cell hypersensitivity and chromosome fragility, which contributes to the final diagnosis of FA. It is an important step in establishing the FA genotype, which is complicated by the existence of 11 complementation groups and a number of mutations that have to be examined in every DEB- sensitive patient.

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ЦИТОГЕНЕТИЧКА СЕНЗИТИВНОСТ НА ДИЕПОКСИБУТАН КОД ДЕЦЕ СА ФАНКОНИЈЕВОМ АНЕМИЈОМ ИЗ СРБИЈЕ

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Фанконијева анемија (ФА) је наследно обољење са апластичном анемијом, предиспозицијом за канцер и хиперсензитивношћу на кластогене као диепоксибутан (ДЕБ). ДЕБ тест је искоришћен за скрининг ФА код пацијената са аплазијом коштане сржи (АКС).

У Институту за здравствену заштиту мајке и детета Србије је у периоду од фебруара 2004. године до маја 2006. године дијагностиковано 29 деце са АКС.

У испитиваној групи код 5/29 пацијената (17.2%) је нађена повећана хромозомска нестабилност индукована ДЕБ-ом (0.58-2.15 vs. 0.00-0.20 прекида по ћелији; p<0.001).

Наши резултати указују на значај ове анализе у диференцијалној дијагнози и адекватној терапији ФА у групи пацијената са АКС.