Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Mutation Research 603 (2006) 41–47
Genetic Toxicology and Environmental Mutagenesis
www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

The role of foetal red blood cells in protecting cultured lymphocytes against diepoxybutane-induced chromosome breaks

Beatriz Porto [a,*], Ricardo Jorge Dinis Oliveira [b], Carla Sousa [b], Jorge Gaspar [c], José Rueff [c], Félix Carvalho [b], Isabel Malheiro [a]

[a] *Laboratory of Cytogenetics, ICBAS—Instituto Ciências Biomédicas Abel Salazar, Universidade do Porto, Largo do Prof, Abel Salazar, no. 2, 4099-003 Porto, Portugal*
[b] *REQUIMTE, Department of Toxicology, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, no. 164, 4050-047 Porto, Portugal*
[c] *Department of Genetics, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Rua da Junqueira, no. 96, 1349-008 Lisboa, Portugal*

Received 1 July 2004; received in revised form 5 September 2005; accepted 27 October 2005
Available online 27 December 2005

Abstract

Diepoxybutane (DEB) is an established mutagen that induces chromosome damage following in vitro treatment of peripheral blood lymphocytes. It is widely used to identify patients with Fanconi Anemia (FA), a clinical situation that is characterized, besides the hypersensitivity to DEB, by an elevated foetal haemoglobin (HbF) content in the peripheral blood. In a previous study, we showed that red blood cells (RBC) from normal individuals can protect cultured lymphocytes against chromosomal breaks induced by DEB and demonstrated the particular role of haemoglobin in the protective effect. In the present work, we studied the influence of RBC extracted from umbilical cord blood of neonates (F cells) on the frequency of DEB-induced chromosome breaks in lymphocyte cultures from normal individuals. Simultaneously, we determined individual *GSTT1* and *GSTM1* genotypes and the activity of Pi-class glutathione *S*-transferase (GSTP), catalase and superoxide dismutase (SOD) in adult and foetal RBC. Our results show that F cells, in comparison with adult RBC, elicit a better protection of cultured lymphocytes from normal individuals against chromosome breaks induced by DEB. Variability in the protective effect among RBC from different individuals was observed; we confirmed that the *GSTT1* genotype modulates this inter-individual variability, but it is not sufficient to explain all of the protective effect of F cells. Our results suggest that the increased protective effect of F cells can be, at least in part, correlated with an increase in the activity of glutathione *S*-transferase, catalase and superoxide dismutase, in particular Cu/Zn SOD, in F cells compared with adult RBC.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Diepoxybutane; Chromosome breaks; Foetal red blood cells (F cells); *GSTT1* and *GSTM1* genotypes; GST, catalase and SOD activities

1. Introduction

Cytogenetic biomarkers are widely used in surveillance studies of human exposure to genotoxic carcinogens. The frequency of cells with chromosomal aberrations (CAs) in cultured peripheral blood lymphocytes is the first biomarker that has shown an

* Corresponding author. Tel.: +351 222062200; fax: +351 222062232.
E-mail address: bporto@icbas.up.pt (B. Porto).

1383-5718/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2005.10.012

association with cancer risk [1,2]. To better understand the cancer predictivity of CAs, it is important to identify factors that modify CA frequency, such as protective components in peripheral blood.

The role of the bi-functional alkylating agent diepoxybutane (DEB) in the induction of CAs in lymphocyte cultures is well established. This effect is used as a diagnostic tool to identify patients with Fanconi Anemia (FA), a chromosome instability syndrome particularly characterized by a hypersensitivity to DNA cross-linking agents, such as DEB, and by an increased predisposition to cancer [3,4]. FA is also characterized by an elevated level of foetal haemoglobin (HgF), which is associated with thrombocytopenia and macrocytosis, and usually precedes the onset of anaemia or neutropenia [5]. However, the clinical importance of this feature has never been directly correlated with the frequency of DEB-induced chromosome damage.

In normal individuals, the response of lymphocytes to DEB-induced CAs is variable. It was already demonstrated that red blood cells (RBC), plasma factors and in particular the glutathione *S*-transferase (GST) gene *GSTT1* (expressed in RBC) are involved in determining the sensitivity of lymphocytes to DEB-induced sister chromatid exchange (SCE) and CAs [6–12]. Recently, we confirmed that RBC from normal individuals can protect cultured lymphocytes against DEB-induced CAs, and that haemoglobin (Hb) is important in this protection [13].

Neefjes et al. [14] demonstrated, by determining erythrocyte GST activity at birth, that neonates are exposed to oxidative stress and experience cell damage as a result of a sudden increase in oxygenation at birth. If, at this time, any protective system against oxidative stress and cell damage is important, the role of foetal RBC (F cells) in protecting against induced chromosomal breaks may be pertinent.

In the present work, we studied the effect of F cells extracted from the umbilical cord blood of neonates, in comparison with adult RBC, on the frequency of DEB-induced chromosome breaks in lymphocyte cultures of normal individuals. The influence of *GSTT1* and *GSTM1* genotypes and of GSTP, catalase and SOD activities of RBC in the protection against DEB-induced chromosome instability was also studied.

2. Materials and methods

2.1. Subjects

Peripheral blood lymphocytes from seven normal and healthy subjects (three males and four females, aged between 31 and 48 years), recruited among blood donors from the Service of Hematology of St. António Hospital were used in this study. None of them had been exposed to drugs. Umbilical cord blood samples from six neonates, routinely collected at the Hospital St. António, were used for RBC isolation.

Informed consent was obtained from all the participants.

2.2. Cells and cell cultures

From each individual, 10 ml of heparinized blood was collected by venipuncture. Samples of blood depleted of RBC by gravity sedimentation (total leukocytes) were used for lymphocyte cultures. As gravity sedimentation does not remove all RBC, and to make sure that leukocyte donor *GSTT1* genotype does not influence the results, gravity sedimentation was redone twice and no residual RBC were left in the sample. RBC suspensions were obtained from whole blood by centrifugation at 2000 rpm and rinsed twice with RPMI medium.

RBC-depleted leukocytes were cultured at a cell concentration of $0.5 \times 10^6\,\text{ml}^{-1}$ in RPMI complete medium supplemented with 15% FCS and antibiotics. The cultures were stimulated with 5 μg/ml of phytohaemagglutinin (PHA, GIBCO, Invitrogen Corporation, USA) and placed in an incubator at 37 °C with a 5% CO2 atmosphere for 72 h. DEB ((±)-1,2:3,4-diepoxybutane, [CAS no. 298-18-0], D-7019 Lot 34H3683, Sigma Chemicals Co, St. Louis), at a final concentration in the medium of 0.05 or 0.1 μg/ml, was added to the cultures 24 h after their initiation, thus exposing cells to the chemical for 48 h. In the appropriate experiments, autologous RBC or F cells were added at an RBC:leukocyte ratio of 100:1.

Since DEB is a suspected carcinogen with unknown risk, appropriate precautions were taken. The cultures were handled using gloves, and all culture procedures and the first part of the harvest were done in a vertical laminar flow hood. Since DEB is rapidly inactivated by concentrated hydrochloric acid (HCl), all disposable culture bottles and pipettes were rinsed with HCl before being discarded.

2.3. Cytogenetic analysis

After 3 days of culture, the cells were harvested after 1 h incubation with colchicine (4 μg/ml) followed by hypotonic treatment with 75 mM KCl and fixation in a 1:3 solution of acetic acid:methanol. Chromosome preparations were made by the air-drying method. For each condition studied, analysis of chromosome aberrations was performed, by one scorer, on 50–100 (mode = 100) Giemsa-stained metaphases observed in coded slides. To avoid bias in cell selection, consecutive metaphases that appeared intact with sufficiently well defined chromosome morphology were selected for study. Each cell was scored for chromosome number and the number and types of structural aberrations. Achromatic areas less than a chromatid in width were scored as gaps while those wider

than a chromatid were scored as breaks. Chromatid exchange configurations, translocations and dicentric and ring chromosomes were scored as rearrangements. Gaps were excluded in the calculation of chromosome aberration frequencies, and each one of the chromosome breaks, chromatid breaks and rearrangements observed were scored as one aberration. The frequency of aberrant cells (with chromosome aberrations) and the number of aberrations per aberrant cell were used in the study.

2.4. DNA extraction

Coded blood samples of all controls were collected into 10-ml heparinized tubes and stored at −20 °C until use. Genomic DNA was obtained from 250 μl of whole blood using a commercially available kit according to the manufacturer's instructions (QIAamp DNA extraction kit; Qiagen, Hilden, Germany). Each DNA sample was stored at −20 °C until analysis.

2.5. GSTM1 and GSTT1 genotyping

GSTM1 and *GSTT1* genotyping for gene deletions were carried out by a multiplex PCR as described by Lin et al. [15] with minor modifications. DNA samples were amplified with the primers: 5′-GAACTCCCTGAAAAGCTAAAGC-3′ (upstream) and 5′-GTTGGGCTCAAATATACGGTGG-3′ (downstream) for *GSTM1*, which produced a 219-bp product in the absence of polymorphic deletion; 5′-TCACCGGATCA-TGGCCAGCA-3′ (upstream) and 5′-TTCCTTACTGGTCCT-CACATCTC-3′ (downstream) for *GSTT1*, which produced a 459-bp product in the absence of polymorphic deletion. The amplification of the albumin gene with the primers 5′-GCCCTCTGCTAACAAGTCCTAC-3′ (upstream) and 5′-GCCCTAAAAAGAAAATCCCCAATC-3′ (downstream) was used as an internal control and produced a 350-bp product. PCR was performed in a final volume of 50 μl, consisting of DNA (0.1 μg), dNTP (0.2 mM each) (Perkin-Elmer), $MgCl_2$ (2.5 mM), each primer (1.0, 0.3 and 0.2 μM for *GSTM1*, *GSTT1* and albumin, respectively), AmplitaqGold polymerase (1.25 units) (Perkin-Elmer), reaction buffer, and 2% DMSO. Amplification was performed with an initial denaturation at 95 °C for 7 min, followed by 35 cycles of amplification performed at 94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min, using a GeneAmp 9600 thermal cycler (Perkin-Elmer Corp). The amplified products were visualized in an ethidium bromide-stained 1.5% agarose gel. All genotype determinations were carried out twice in independent experiments and all the inconclusive samples were reanalysed.

2.6. Enzymatic activity

2.6.1. Catalase

Catalase activity was measured according to the method of Aebi [16] by monitoring the decomposition of H_2O_2 at 240 nm in hemolyzed samples. The enzyme activity was expressed as micromoles of H_2O_2 consumed per minute per milligram of protein.

2.6.2. Pi-class GST isoenzyme

The activity of pi-class GST isoenzymes was assayed by monitoring the formation of GSH conjugate with 1,2-chloro-2-dinitrobenzene (CDNB) at 340 nm [17] in hemolyzed samples. The GST activity was calculated by the appropriate extinction coefficient ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$) and expressed as micromoles per min per milligram of protein.

2.6.3. Superoxide dimutase (SOD)

Copper/zinc superoxide dismutase (Cu/ZnSOD) and manganese superoxide dismutase (MnSOD) were assayed using the method of Flohé and Otting [18] with modifications. A xanthine–xanthine oxidase system was used to generate superoxide radicals (O_2^-) and subsequent reduction of nitroblue tetrazolium (NBT) by O_2^- was monitored at 560 nm during 2 min. Potassium cyanide (2 mM) was used to allow the measurement of MnSOD. Enzyme activity was expressed as units per milligram of protein (1 unit of SOD is defined as the amount of enzyme required to inhibit the rate of NBT reduction by 50%).

2.6.4. GSH

The total glutathione content of RBC was determined by the DTNB-GSSG reductase recycling assay as described before [19]. Briefly, 100 μl of RBC aliquots were precipitated with 100 μl perchloric acid (5% final acid concentration) and centrifuged for 10 min at 13,000 rpm in a refrigerated centrifuge (4 °C). After this time, 100 μl acidic supernatant was neutralized with 100 μl 0.76 M $KHCO_3$ and the sample centrifuged for 1 min at 13,000 rpm. Fresh reagent, containing 0.69 mM NADPH and 4 mM DTNB in 72 mM phosphate buffer, was prepared daily. For the measurement of total glutathione, 100 μl/well of samples, standards or blank were added in duplicate to 96-well microtiter plates, followed by 65 μl/well of the freshly prepared reagent. Plates were then incubated in a thermomax plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA) at 30 °C for 10 min prior to the addition of 40 μl GR per well (10 IU/ml in phosphate buffer). The stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB) was followed for 2 min at 415 nm and compared with a standard curve of GSH.

2.7. Statistical analysis

The Kolmogorov–Smirnov test was used in order to verify the normality of the continuous variables and the Levene test was used to analyse the homogeneity of variances. Since the results obtained by these methods showed that the data distributions were normal and the variances associated were homogeneous, the statistical analysis was performed with SPSS statistical package (version 11) (SPSS inc. Chicago, IL, USA).

Table 1
Effect of added autologous red blood cells (RBC) and umbilical cord blood RBC (F cells) on the frequency of chromosome aberrations in DEB-treated (0.1 μg/ml) lymphocyte cultures from seven normal healthy adult donors

Leukocyte and autologous RBC donor		Cultures of leukocytes + autologous RBC		Cultures of leukocytes + F cells		F cells donor	
Code no.	Genotype	No. of aberrant cells (%)	No. of aberrations per aberrant cell	No. of aberrant cells (%)	No. of aberrations per aberrant cell	Code no.	Genotype
D1	T+M+	24.2	1.13	16.6	1.5	F3	T0M+
D2	T0M+	43.8	1.43	28	1.13	F3	T0M+
D3	T0M+	43.9	1.33	48.8	1.85	F4	T0M0
D4	T0M0	56	1.29	45	1.73	F4	T0M0
D5	T+M+	16	1	9.4	1.2	F5	T+M+
D5	T+M+	16	1	23.1	1.12	F6	T0M0
D6	T+M0	24.4	1.5	12.8	1.27	F5	T+M+
D6	T+M0	24.4	1.5	20.3	1.62	F6	T0M0
D7	T+M+	5.6	1	1	1	F7	T+M0
D7	T+M+	5.6	1	0	0	F8	T+M0
Mean ± standard deviation		30.56 ± 17.86 (a)	1.24 ± 0.20 (b)	20.50 ± 16.54 (c)	1.24 ± 0.52 (d)		

The genotype of genes *GSTT1* and *GSTM1* was determined for each donor. T+: *GSTT1* non-null; T0: *GSTT1* null; M+: *GSTM1* non-null; M0: *GSTM1* null. *t*-test: (a) vs. (c), $p = 0.018$; (b) vs. (d), $p = 0.496$.

3. Results

3.1. Influence of foetal RBC, GSTM1 and GSTT1 on the frequency of chromosome aberrations in DEB-treated lymphocyte cultures from normal individuals

As shown in Table 1, the frequency of aberrant cells was significantly lower in leukocyte cultures to which F cells were added compared with cultures where autologous RBC were added ($p = 0.023$). No significant difference was observed in the number of aberrations per aberrant cell between the two types of culture.

Our results also show that individual *GSTM1* genotypes have no effect on the frequency of DEB-induced aberrant cells, but *GSTT1* genotypes modulate the level of genetic lesions induced by DEB (Table 2). In fact, a significant difference between *GSTT1* 'null' and 'non-null' individuals was observed, both in experiments with adult RBC ($p = 0.002$) and with F cells ($p = 0.006$). This effect was not observed between *GSTM1* 'null' and 'non-null' individuals, both in experiments with adult RBC ($p = 0.208$) and with F cells ($p = 0.292$). However, the *GSTT1* genotype was not sufficient to explain all the protective effect of F cells, since a significant difference between adult RBC and F cells was also observed when experiments with *GSTT1* 'null' and *GSTT1* 'non-null' genotypes were evaluated independently ($p = 0.038$ and 0.037, respectively).

3.2. Comparative study of the enzymatic activities of GSTP, catalase and SOD and the GSH levels between adult RBC and foetal RBC

As shown in Table 3, basal activities of GSTP, catalase and SOD are significantly higher in foetal

Table 2
Influence of individual *GSTT1* and *GSTM1* genotypes on the frequency of DEB-induced chromosome aberrations in lymphocyte cultures

GSTT1 genotype of RBC donor	% aberrant cells in leukocyte cultures where RBC were added (mean ± standard deviation)[a]		
	Leuk + adult RBC	Leuk + F cells	*t*-test
GSTT1 'null'	47.90 ± 7.02	30.30 ± 13.44	$p = 0.038$
GSTT1 'non-null'	17.55 ± 8.88	5.8 ± 6.29	$p = 0.037$
t-test	$p = 0.002$	$p = 0.006$	
GSTM1 'null'	40.20 + 22.34	23.03 + 20.83	$p = 0.178$
GSTM1 'non-null'	32.03 + 16.98	16.70 + 8.09	$p = 0.158$
t-test	$p = 0.208$	$p = 0.292$	

A comparative study of the frequency of aberrant cells between 'null' and 'non-null' genotypes was performed, both in DEB-induced leukocyte cultures where adult red blood cells were added (Leuk + adult RBC) and in leukocyte cultures were F cells were added (Leuk + F cells).
[a] Each mean was determined from the values depicted in Table 1.

Table 3
Total glutathione (GSH) levels and enzymatic activities of glutathione *S*-transferase (GSTP), catalase and superoxide dismutase (total SOD, MnSOD and Cu/ZnSOD) measured in adult red blood cells (RBC) extracted from peripheral blood of seven donors (mean ± standard deviation) and in RBC extracted from umbilical cord blood (F cells) of six donors (mean + standard deviation)

	Total GSH (mean ± S.D.)	Enzyme activity (units/mg protein) (mean ± S.D.)				
		GSTP	Catalase	Total SOD	MnSOD	Cu/ZnSOD
Adult RBC						
Day 0	5.91 ± 1.20	1.59 ± 1.13	59.11 ± 12.11	0.09 ± 0.02	0.05 ± 0.01	0.04 ± 0.03
Day 3, −DEB[a]	4.89 ± 1.33 (n.s.)	2.16 ± 1.63 (n.s.)	80.37 ± 14.33 ($p = 0.005$)	0.38 ± 0.07 ($p < 0.001$)	0.06 ± 0.02 ($p = 0.026$)	0.31 ± 0.07 ($p < 0.001$)
Day 3, +DEB[b]	4.10 ± 0.97 ($p = 0.026$)	2.23 ± 1.46 (n.s.)	77.61 ± 12.61 (n.s.)	0.37 ± 0.09 (n.s.)	0.06 ± 0.02 (n.s.)	0.30 ± 0.09 (n.s.)
Foetal RBC						
Day 0[c]	4.98 ± 1.49 (n.s.)	5.18 ± 2.34 ($p = 0.002$)	78.23 ± 13.84 ($p = 0.011$)	0.37 ± 0.11 ($p < 0.001$)	0.07 ± 0.01 ($p = 0.002$)	0.30 ± 0.11 ($p < 0.001$)
Day 3, +DEB[a]	2.61 ± 1.23 (0.003)	6.00 ± 3.74 (n.s.)	71.64 ± 11.11 (n.s.)	0.30 ± 0.07 (n.s.)	0.06 ± 0.03 (n.s.)	0.23 ± 0.07 (n.s.)

The values were determined at Day 0 (basal activities) and 3 days after being added to non-treated or DEB-treated (0.1 μg/ml) lymphocyte cultures. n.s., not significant.
[a] Level of significance of the difference to basal activity (Day 0).
[b] Level of significance of the difference between adult RBC activities after being added to lymphocyte cultures with and without DEB.
[c] Level of significance of the difference between adult RBC and foetal RBC basal activities (Day 0).

RBC than in adult RBC ($p = 0.002$, 0.011 and <0.001, respectively). Considering the two types of SOD (MnSOD and Cu/ZnSOD) the most significant difference was observed for Cu/ZnSOD (0.04 ± 0.03 versus 0.30 ± 0.13; $p < 0.001$). No significant difference was observed between the basal levels of GSH in adult and foetal RBC.

When adult RBC were added to lymphocyte cultures, a significant increase was observed in their activities of GSTP, catalase and SOD ($p = 0.005$ and <0.001, respectively). Considering the two types of SOD (MnSOD and Cu/ZnSOD) the most significant increase was observed for Cu/ZnSOD, which increased about 7.5 times ($p < 0.001$). The induction of lymphocyte cultures with DEB did not influence significantly the enzymatic activities of GSTP, catalase and SOD, either of adult and foetal RBC. However, a significant decrease of GSH levels was observed ($p = 0.026$ and 0.003, respectively). Interestingly, all the enzymatic activities of adult RBC added to DEB-treated lymphocyte cultures were equal or inferior to the basal activities of foetal RBC.

4. Discussion

DEB is a well-known alkylating agent, whose genotoxic effect has clearly been shown by cytogenetic analysis in DEB-induced lymphocyte cultures. This effect is used for the diagnosis of Fanconi's Anemia, since hypersensitivity to DEB-induced chromosomal breakage is a unique marker for all the FA genotypes so far established [20]. Cellular mechanisms exist that protect lymphocytes from this genotoxic effect. Landi et al. [6] demonstrated that RBC are involved in determining the sensitivity of lymphocytes to DEB-induced sister chromatid exchange. This effect appeared to be mainly mediated by the polymorphism of GSTT1, an erythrocytic detoxification enzyme [8]. Recently we also showed that RBC are involved in determining the sensitivity of lymphocytes to DEB-induced chromosome aberrations [13]. In order to extend this study to different types of RBC, in the present work we investigated the role of F cells, extracted from the umbilical cord blood of neonates, in protecting cultured lymphocytes from normal individuals against DEB-induced chromosome breaks. Our results show that the presence of F cells significantly decreased the frequency of aberrant cells, when compared with the presence of autologous RBC, suggesting that F cells have an increased capacity of protection against DNA damage. There was no change in the number of aberrations inside the cell, which suggests that the DNA repair capacity, and not the RBC protective effect, is influencing this number of aberrations.

Knowing that individual *GSTT1* polymorphism modulates the level of genetic lesions induced by DEB [7–12], in the present study we also analysed the inter-individual variability of *GSTT1* and *GSTM1* genotypes. In fact, concerning the individual genetic polymorphisms evaluated, *GSTT1* is associated with the level of chromosomal damage induced by DEB, the level of DNA damage being significantly lower in 'non-null' individuals, confirming that this enzyme is involved in the detoxification of DEB. However, an inter-individual variation in *GSTT1* was observed both in cultures with autologous RBC and F cells, which, nevertheless, was not sufficient to explain the highly significant variation in the frequency of cells with chromosome breaks between both cultures. So, other factors in F cells may be involved in this DEB detoxification.

Neonates are exposed to oxidative stress and cell damage as a result of a sudden increase in oxygenation, as suggested by studies of erythrocyte GST activity at birth [14]; the increased protective capacity of F cells observed in this study could be an additional aspect in cellular protection against cell damage at birth. To confirm this hypothesis, we studied GSTP (the other form of GST that, besides GSTT1, is expressed in RBC), catalase and SOD basal activities of both adult RBC and F cells, and the same enzymatic activities of both adult and foetal RBC 3 days after being added to non-treated and DEB-treated lymphocyte cultures. In fact, basal activities of these enzymes were significantly higher in foetal RBC than in adult RBC. The most significant difference was observed for SOD, in particular Cu/ZnSOD. We suggest that this increase in enzymatic activities can be, at least in part, responsible for the additional protective effect of F cells. We also observed that the basal GSTP, catalase and SOD activities of adult RBC significantly increased when these cells were added to lymphocyte cultures. Once again, the most significant difference was observed for Cu/ZnSOD; this is in agreement with the fact that lymphoblastoid cell lines tend to exhibit a higher SOD level due to an elevated Cu/ZnSOD activity [21] and reflects a higher protective capacity against the deleterious effects of superoxide radicals. The treatment with DEB did not significantly influence the enzymatic activities of RBC in lymphocyte cultures. However, the significant loss in GSH levels confirms what is already described, i.e., that other forms of GST, in particular GSTT1, are implicated in the process of DEB detoxification through conjugation with GSH. Interestingly, the increase in enzymatic activities of adult RBC added to lymphocyte cultures observed on Day 3 after DEB exposure (Table 3) is equivalent to the basal activities of foetal RBC. This result may suggest that F cells have an elevated enzymatic activity that is important for a sudden need of cellular protection.

All these findings can have important clinical implications, particularly in pathologic situations where increased chromosomal instability is associated with an increase in the frequency of F cells, like in FA, suggesting an improved protective mechanism against DNA damage. Reactivation of HbF synthesis by certain drugs, like erythropoietin and cytostatics such as hydroxyurea, is observed in many patients with sickle cell anaemia and thalassaemia [22–24]; it is known that these drugs can be toxic and potential long-term mutagens, and so the suggested protective effect of F cells in these cases should be further studied and confirmed. Reactivation of HbF synthesis was also described in patients with myelodysplastic syndrome, and its use as a good prognostic parameter was suggested [25]. In conclusion, the importance of our results should be taken into account in studies of pathological situations with an increase in DNA damage associated with an increase in the frequency of F cells in peripheral blood, and so further studies will be performed in the near future.

Acknowledgements

The authors thank the Service of Haematology from the Hospital Geral de St. António for providing blood donor and umbilical cord blood samples. They also thank Dr. Graça Porto for the helpful discussions during the preparation of the manuscript.

References

[1] L. Hagmar, U. Strömberg, S. Bonassi, I.L. Hansteen, L.E. Knudsen, C. Lindholm, H. Norppa, Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts, Cancer Res. 64 (2004) 2258–2263.

[2] H. Norppa, Cytogenetic biomarkers and genetic polymorphisms, Toxicol. Lett. 149 (2004) 309–334.

[3] A.D. Auerbach, B. Adler, R.S.K. Chaganti, Prenatal and postnatal diagnosis and carrier detection of Fanconi anemia by a cytogenetic method, Pediatrics 67 (1981) 128–135.

[4] A.D. Auerbach, A. Rogatko, T.M. Shroeder-Kurth, International Fanconi anemia registry: relation of clinical symptoms to diepoxybutane sensitivity, Blood 73 (1989) 391–396.

[5] A.D. Auerbach, M. Buchwald, H. Joenje, Fanconi anemia, in: B. Vogelstein, K.W. Kinzler (Eds.), The Genetic Basis of Human Cancer, Mc Graw-Hill, New York, 1998, pp. 317–332.

[6] S. Landi, I. Ponzanelli, R. Barale, Effect of red cells and plasma blood in determining individual lymphocytes sensitivity to diepoxybutane assessed by in vitro induced sister chromatid exchanges, Mutat. Res. 348 (1995) 117–123.

[7] H. Norppa, A. Hirvonen, H. Järventaus, M. Uusküla, G. Tasa, A. Ojajärvi, M. Sorsa, Role of GSTT1 and GSTM1 genotypes in determining individual sensitivity to sister chromatid exchange

induction by diepoxybutane in cultured human lymphocytes, Carcinogenesis 16 (1995) 2116–2219.

[8] K. Pelin, A. Hirvonen, H. Norppa, Influence of erythrocyte glutathione *S*-transferase T1 on induction of sister chromatid exchange by diepoxybutane in cultured human lymphocytes, Mutagenesis 11 (1996) 213–215.

[9] S. Landi, I. Ponzanelli, G. Cipollini, G. Frenzilli, A. Luccini, C.P.I. Sbrana, R. Barale, Modulating factors of individual sensitivity to diepoxybutane: chromosome aberrations induced in vitro in human lymphocytes, Mutagenesis 12 (1997) 17–22.

[10] S. Landi, H. Norppa, G. Frenzilli, G. Cipollini, I. Ponzanelli, R. Barale, A. Hirvonen, Individual sensitivity to cytogenetic effects of 1,2:3,4-diepoxybutane in cultured human lymphocytes: influence of glutathione *S*-transferase M1, P1 and T1 genotypes, Pharmacogenetics 8 (1998) 461–471.

[11] K. Schlade-Bartusiak, M. Sasiadek, J. Kozlowska, The influence of GSTM1 and GSTT1 genotypes on the induction of sister chromatid exchanges and chromosome aberrations by 1,2:3,4-diepoxybutane, Mutat. Res. 465 (2000) 69–75.

[12] H. Norppa, Genetic susceptibility, biomarker responses and cancer, Mutat. Res. 544 (2003) 339–348.

[13] B. Porto, L. Chiecchio, J. Gaspar, A. Faber, L. Pinho, J. Rueff, I. Malheiro, Role of haemoglobin in the protection of cultured lymphocytes against diepoxybutane (DEB), assessed by in vitro induced chromosome breaks, Mutat. Res. 536 (2003) 61–67.

[14] V.M. Neefjes, C.T. Evelo, L.G. Baars, C.E. Blanco, Erythrocyte glutathione *S*-transferase as a marker of oxidative stress at birth, Arch. Dis. Child. Fetal Neonatal Ed. 81 (1999) 130–133.

[15] D. Lin, Y. Tang, Q. Peng, S. Lu, C.B. Ambrosone, F.F. Kadublar, Susceptibility to esophageal cancer and genetic polymorphisms in glutatione-*S*-transferase T1, P1 and M1 and cytochrome P450 2E1, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 1013–1018.

[16] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.

[17] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130–7139.

[18] L. Flohé, F. Otting, Superoxide dismutase assays, Methods Enzymol. 105 (1984) 93–104.

[19] M. Carvalho, N. Milhazes, F. Remião, F. Borges, E. Fernandes, T. Monks, F. Amado, F. Carvalho, M.L. Bastos, Hepatotoxicity of 3,4-methylenedioxyamphetamine and R-methyldopamine in isolated rat hepatocytes: formation of glutathione conjugates, Arch. Toxicol. 78 (2004) 16–24.

[20] M. Levitus, M.A. Rooimans, J. Steltenpool, N.F. Cool, A.B. Oostra, C.G. Mathew, M.E. Hoatlin, Q. Waisfisz, F. Arwert, J.P. de Winter, H. Joenje, Heterogeneity in Fanconi anemia: evidence for 2 new genetic subtypes, Blood 103 (2004) 2498–2503.

[21] K.H. Lee, S. Abe, Y. Yanabe, I. Matsuda, M.C. Yoshida, Superoxide dismutase activity and chromosome damage in cultured chromosome instability syndrome cells, Mutat. Res. 244 (1990) 251–256.

[22] D. Loukopoulos, New therapies for the haemoglobinopathies, J. Intern. Med. Suppl. 740 (1997) 43–48.

[23] B.R. Saxon, D. Rees, N.F. Olivieri, Regression of extramedullary haemopoiesis and augmentation of fetal haemoglobin concentration during hydroxyurea therapy in beta thalassaemia, Br. J. Haematol. 101 (1998) 416–419.

[24] M. de Montalembert, Hydroxyurea and other agents stimulating synthesis of fetal haemoglobin, Pathol. Biol. 47 (1999) 55–58.

[25] D. Reinhardt, D. Haase, C. Schoch, S. Wollenweber, E. Hinkelmann, W.v. Heyden, G. Lentini, B. Wormann, W. Schroter, A. Pekrun, Hemoglobin F in myelodysplastic syndrome, Ann. Hematol. 76 (1998) 135–138.