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Role of haemoglobin in the protection of cultured lymphocytes against diepoxybutane (DEB), assessed by in vitro induced chromosome breakage

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Abstract

Diepoxybutane (DEB) is an alkylating agent that can be used to assess chromosome instability in repair-deficient subjects. Previous authors investigated the role of red blood cells (RBC) in determining individual susceptibility to DEB in normal healthy donors, and demonstrated that a polymorphic enzyme in RBC, Glutathione *S*-transferase T1 (GSTT1), is involved in DEB detoxification. In the present work we studied the influence of individual *GSTM1* and *GSTT1* genotypes and the presence of RBC on the frequency of DEB-induced chromosome breakage in lymphocyte cultures from normal individuals and, in particular, the influence of isolated components of RBC: RBC membranes, RBC lysate, and haemoglobin. Our results confirm that individual *GSTT1* genotypes modulate the level of genetic lesions induced by DEB; however, this effect was not sufficient to explain the highly significant variation in chromosome breakage between whole blood and RBC-depleted cultures. We showed that RBC can protect cultured lymphocytes against chromosome breakage induced by DEB and we demonstrated the particular role of haemoglobin in the protective effect.

Keywords: Diepoxybutane; Chromosome breakage; Red blood cells; Haemoglobin

1. Introduction

Diepoxybutane (DEB) is a bifunctional alkylating agent that can induce chromosome breakage in lymphocyte cultures. This effect can be used to discriminate patients with Fanconi anaemia (FA), i.e. one of the cancer-prone chromosome instability syndromes that is characterized by a hypersensitivity to DNA cross-linking agents, such as DEB [1,2]. In fact, the low clastogenic effect of a nontoxic concentration of DEB in normal individuals, and even in other chromosome instability syndromes (for example, *ataxia telangiectasia*, *xeroderma pigmentosum* and Bloom's syndrome), is highly increased in FA patients [1–5]. Even so, the response of lymphocytes to the induction of sister chromatid exchanges (SCE) and chromosome

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aberrations (CA) by DEB at low doses can be variable among normal individuals. In previous studies [6–9], it was shown that a subgroup of normal donors is characterized by higher frequencies of DEB-induced SCE and CA than other donors. Consequently, donors can be divided into two distinct patterns of cytogenetic responsiveness: low-respondents and high-respondents. It was suggested that this bimodal distribution could be due to a factor responsible for the "sensitive" phenotype. Landi et al. [10] demonstrated that red blood cells (RBC) and plasma factors are involved in determining the sensitivity of lymphocytes to DEB-induced SCE and CA. Norppa et al. [8] described the role of the *GSTT1* gene (expressed in RBC) in determining the individual sensitivity to DEB. More recently it was confirmed that the presence of the *GSTT1* gene reduces the relative sensitivity of the lymphocytes to the induction of SCE and CA by DEB [11–14]. However, although most of the variation in SCE frequency can be explained by *GSTT1* genotype, other factors than GSTT1 may be important in determining DEB sensitivity measured by CA.

In order to further investigate possible specific factors that can contribute to modulation of the genotoxicity induced by DEB, in the present work we studied the influence of individual *GSTM1* and *GSTT1* genotypes and the presence of RBC on the frequency of DEB-induced chromosome breakage in lymphocyte cultures from normal individuals and, in particular, the influence of isolated components of RBC: RBC membranes, RBC lysate, and haemoglobin.

2. Materials and methods

2.1. Subjects

The study group consisted of 20 normal healthy blood donors (11 males and 9 females) with an age range from 23 to 46 years. None of the controls had been exposed to drugs. All procedures were done with the informed consent of the participants.

2.2. Cells and cell cultures

From each individual 10 ml of heparinized blood was collected by venipuncture. Samples of whole blood and blood depleted of RBC by gravity sedimentation (total leukocytes) were used in cultures. RBC suspensions were obtained from whole blood after centrifugation at 2000 rpm and rinsed twice with RPMI medium. RBC membrane and lysate suspensions were obtained as follows: sterile distilled H_2O was added to packed RBC (1 ml RBC/2 ml H_2O); this suspension was refrigerated (−4 °C) during 15 min; after centrifugation (1400 rpm, 10 min), the lysate (the supernatant containing haemoglobin) was RBC-depleted from the membranes (the pellet, rinsed twice with lysis solution). Lysate and membrane suspensions were reconstituted with RPMI to a final concentration equal to the initial RBC concentration.

Whole blood (0.5 ml) or RBC-depleted leukocytes (at the same cell concentration as determined in the 0.5 ml of whole blood) were cultured in RPMI complete medium supplemented with 15% FCS and antibiotics. Cultures were stimulated with 5 μg/ml of PHA and placed in an incubator at 37 °C with a 5% CO_2 atmosphere for 72 h. DEB ((±)-1,2:3,4-diepoxybutane, [298-18-0], D-7019 Lot 34H3683, Sigma), 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. Since DEB is a suspected carcinogen with unknown risk, appropriate precautions were taken. Cultures were handled using gloves, and all work was done in a vertical laminar flow hood (for culture procedures and the first part of the harvest procedure). Since DEB is rapidly inactivated by concentrated hydrochloric acid (HCl), all disposable culture bottles and pipettes were rinsed with HCl before being discarded.

In the appropriate experiments, autologous or heterologous RBC were added at a RBC-leukocyte ratio of 100:1, RBC membranes and RBC lysate were added at the same concentration as RBC, and purified ferrous haemoglobin (Hg-A_0, Sigma) was added at an initial concentration of 10 mg/ml RBC.

2.3. Cytogenetic analysis

After 3 days of culture, cells were harvested after 1 h incubation with colchicine (4 μg/ml) followed by hypotonic treatment with 75 mM KCl and fixed in a 1:3 solution of acetic acid:methanol. Chromosome preparations were made following standard methods.

Analysis was performed on 50–100 (mode = 100) Giemsa-stained metaphases from each preparation.

Only when the mitotic index was very low and the number of breaks was high, a minimum of 25 metaphases was counted. To avoid bias in cell selection, consecutive metaphases which appeared unbroken and with sufficient well-defined chromosome morphology were selected for study. Each cell was scored for chromosome number and the number and types of structural abnormalities. Achromatic areas less than a chromatid in width were scored as gaps while those more than a chromatid in width were scored as breaks. Chromatid exchange configurations (triradials and quadriradials), translocations, dicentrics and ring chromosomes were scored as rearrangements. Gaps were excluded in the calculation of chromosome breakage frequencies, and rearrangements were scored as two breaks.

2.4. DNA extraction

Blood samples from 12 individuals 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. Genotyping

GSTM1 and *GSTT1* genotyping for gene deletions was carried out by a multiplex PCR as described by Lin et al. [15] with the modifications described by Teixeira et al. [16]. All the genotype determinations were carried out twice in independent experiments and all the inconclusive samples were reanalyzed.

2.6. 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 analyze the homogeneity of variances. Since the results obtained by these methods showed that the data distributions were normal and the associated variances homogeneous, the statistical analysis was performed with SPSS statistical package (version 11) (SPSS, Inc., Chicago, IL).

3. Results

3.1. Influence of RBC, GSTM1 and GSTT1 on the frequency of chromosome breakage in DEB-treated lymphocyte cultures

The presence of chromosome breaks in a series of DEB-treated lymphocyte cultures from each of 12 individuals was determined, and a comparison was made between different types of cultures: whole blood cultures, cultures of RBC-depleted leukocytes, cultures of RBC-depleted leukocytes to which autologous RBC were added and cultures of RBC-depleted leukocytes to which heterologous RBC were added. As shown in Table 1, the frequency of cells with breaks was significantly higher in cultures without RBC (RBC-depleted leukocytes) than in whole blood cultures ($P = 5.29\text{E-}05$). No significant difference was observed in the frequency of cells with breaks between whole blood cultures and cultures of RBC-depleted leukocytes where autologous RBC were added ($P = 0.3371$). However, when heterologous RBC were added a significant decrease in the frequency of breaks was observed ($P = 0.0069$).

Our results also show that individual *GSTM1* genotypes have no effect on the frequency of DEB-induced chromosome breaks, but *GSTT1* genotypes modulate the level of genetic lesions induced by DEB. In fact, in the experiments depicted in Table 1, a significant difference between *GSTT1* 'null' and 'non-null' individuals was observed in experiments with whole blood (7.35 ± 1.85 versus 4.79 ± 1.85, respectively; $P = 0.016$) and in leukocytes supplemented with autologous RBC (7.83 ± 1.84 versus 4.16 ± 1.88, respectively; $P = 0.008$), but this effect was not observed in the experiments carried out in cultured leukocytes (26.63 ± 19.27 versus 33.93 ± 12.92, respectively; $P = 0.448$) and in cultured leukocytes in the presence of heterologous RBC (3.13 ± 0.85 versus 3.89 ± 2.15, respectively; $P = 0.518$); it is important to note that all the heterologous RBC used were from *GSTT1* 'non-null' individuals. Despite the observed effect of *GSTT1* genotype in the modulation of genetic damage induced by DEB, a significantly higher frequency of chromosome breaks was observed in RBC-depleted cultures (31.49 ± 14.89) compared with whole blood cultures from *GSTT1* null individuals (7.35 ± 1.85)

Table 1
Chromosome breakage in DEB-treated (0.05 μg/ml) peripheral blood lymphocytes from 12 normal healthy donors: cultures of whole blood (a), RBC-depleted leukocytes (b), RBC-depleted leukocytes to which autologous red blood cells (RBC) were added (c) and RBC-depleted leukocytes to which heterologous RBC were added (d)

Donor		Frequency of cells with chromosome breaks (%)			
Code no.	Genotype	Whole blood (a)	Leukocytes (b)	Leukocytes + aut RBC (c)	Leukocytes + het RBC (d)
1	M0T0	9.4	24.5	6.0	3.0
2	M+T0	8.0	7.4	10.3	4.0
3	M+T0	7.0	53.3	8.0	3.5
4	M+T0	5.0	21.3	7.0	2.0
5	M0T+	6.0	24.5	6.0	6.0
6	M0T+	5.0	32.1	3.0	1.1
7	M0T+	3.3	48.7	1.0	4.0
8	M+T+	6.0	44.4	5.0	3.2
9	M+T+	6.0	30.6	5.0	4.0
10	M+T+	5.0	47.6	3.0	3.0
11	M+T+	4.0	33.0	4.0	2.0
12	M+T+	3.0	10.5	6.3	7.8
Mean		5.64 ± 1.87	31.49 ± 14.85	5.38 ± 2.49	3.63 ± 1.81

The genotype of genes *GSTT1* and *GSTM1* was determined for each donor. M+: *GSTM1* non-null; M0: *GSTM1* null; T+: *GSTT1* non-null; T0: *GSTT1* null. *t*-test: (a) vs. (b), $P = 5.29E\text{-}05$; (a) vs. (c), $P = 0.3371$; (a) vs. (d), $P = 0.0069$.

($P = 0.0048$). So, other factors may also be involved in DEB detoxification.

In order to know if RBC concentration had an influence on the frequency of DEB-induced chromosome breaks, a serial study was performed of lymphocyte cultures from one individual with different concentrations of RBC (Table 2). Our results show that, for the same leukocyte concentration, there was a decrease in the frequency of chromosome breaks proportional to the increase in RBC concentration. In fact, the number of cells with breaks obtained at the DEB dose of 0.05 μg/ml perfectly regresses on the reciprocal of the amount of RBC added to the incubation mixture (slope coefficient $P = 0.0009$, $r^2 = 0.9837$).

The effect of the addition of old RBC was also tested in DEB-treated lymphocyte cultures from two normal individuals (blood donors 14 and 15). It was observed that, while in whole blood cultures the frequency of cells with breaks was 4% (in both individuals), in cultures of RBC-depleted leukocytes where old RBC (aged during 4 weeks) were added that frequency increased to 44.1% in one individual and to 11% in the other.

Table 2
Influence of red blood cell (RBC) concentration on chromosome breakage in DEB-treated peripheral blood lymphocyte cultures from one normal healthy donor (no. 13)

Cell cultures	DEB: 0.05 μg/ml		DEB: 0.1 μg/ml	
	No. of cells with breaks (%)	No. of breaks per cell	No. of cells with breaks (%)	No of breaks per cell
WB (0.25 ml RBC)	0	0.00	3	0.04
LEUK + 0.2 ml RBC	2	0.02	13	0.30
LEUK + 0.1 ml RBC	4	0.07	11	0.16
LEUK + 0.05 ml RBC	9	0.12	20	0.32
LEUK + 0.025 ml RBC	26	0.32	50	1.21
LEUK (0.3 ml LEUK)	27	0.36	No dividing cells	No dividing cells

All cultures have the same leukocyte concentration. WB: whole blood cultures; LEUK: cultures of RBC-depleted leukocytes.

Table 3
Chromosome breakage in DEB-treated (0.05 μg/ml) peripheral blood lymphocytes from three normal healthy donors: cultures of whole blood (a), RBC-depleted leukocytes (b), RBC-depleted leukocytes to which isolated membranes of red blood cells (RBC) were added (c) and RBC-depleted leukocytes to which RBC lysate was added (d)

Donor no.	Frequency of cells with chromosome breaks (%)			
	Whole blood (a)	Leukocytes (b)	Leukocytes + RBC membrane (c)	Leukocytes + RBC lysate (d)
16	11.1	49.0	42.9	13.0
17	8.7	58.0	56.0	7.4
18	4.0	28.0	27.0	4.0
Mean	7.93 ± 3.61	45.00 ± 15.39	41.97 ± 14.52	8.13 ± 4.54

t-test: (a) vs. (b), $P = 0.018467023$; (c) vs. (d), $P = 0.0237399$; (a) vs. (c), $P = 0.416024825$; (b) vs. (d), $P = 0.095663364$.

3.2. Influence of isolated components of RBC on the frequency of chromosome breakage in DEB-treated lymphocyte cultures

In order to ascertain if isolated components of RBC had an influence on the frequency of DEB-induced chromosome breaks, several experiments involving blood components were carried out, as follows.

In a first experiment we determined the frequency of cells with breaks in a series of DEB-treated lymphocyte cultures from each of three individuals, and a comparison was made between the different types of culture: whole blood cultures, cultures of RBC-depleted leukocytes, cultures of RBC-depleted leukocytes to which isolated RBC membranes were added and cultures of RBC-depleted leukocytes to which RBC lysate was added. As shown in Table 3, the frequency of cells with breaks was significantly higher in cultures without RBC (RBC-depleted leukocytes) than in whole blood cultures ($P = 0.018467$). This result corroborates the data shown in Table 1. As to the isolated blood components, the results show that the frequency of cells with breaks was significantly higher in leukocyte cultures where RBC membranes were added compared with leukocyte cultures where RBC lysate was added ($P = 0.023740$). Besides, we observed that there was no significant difference between the frequency of cells with breaks in cultures of whole blood and cultures of leukocytes were RBC lysate was added ($P = 0.416025$). Accordingly, there was no significant difference between the frequency of cells with breaks in cultures of RBC-depleted leukocytes and cultures of leukocytes were RBC membranes were added ($P = 0.095663$).

In order to evaluate if, in the RBC lysate, the haemoglobin could influence the frequency of chromosome breaks in DEB-treated lymphocyte cultures, a second set of experiments was performed, with the following types of cultures: whole blood cultures, cultures of RBC-depleted leukocytes, and cultures of RBC-depleted leukocytes to which haemoglobin (Hb) was added. As shown in Table 4, the addition of Hb to RBC-depleted leukocyte cultures of both donors 19 and 20 resulted in a decrease in chromosome breakage similar to that observed in whole blood cultures; besides, for donor 20 this decrease was proportional to Hb concentration, i.e. the frequency of cells with breaks and the number of breaks per cell were inversely proportional to the RBC concentration.

Table 4
Chromosome breakage in DEB-treated (0.05 μg/ml) peripheral blood lymphocytes from two normal healthy donors: cultures of whole blood (WB), RBC-depleted leukocytes (LEUK), and RBC-depleted leukocytes to which haemoglobin (Hb) was added

Donor no.	Cell cultures	No. of cells with breaks (%)	No. of breaks per cell
19	WB	9	0.10
	LEUK	34	0.57
	LEUK + Hb (10 mg/ml RBC)	19	0.30
20	WB	18	0.20
	LEUK	28	0.44
	LEUK + Hb (5 mg/ml RBC)	16	0.24
	LEUK + Hb (10 mg/ml RBC)	14	0.22
	LEUK + Hb (20 mg/ml RBC)	11	0.14

4. Discussion

The response of lymphocytes to the genotoxic effects of DEB has been extensively studied, not only because this drug can be used to discriminate patients with Fanconi anemia but also because there is a marked inter-individual variability in DEB responsiveness among normal individuals. The results obtained in the present study confirm and extend the data obtained by Landi et al. [10] on the effect of RBC in determining the sensitivity of an individual's lymphocytes to DEB. In fact, the presence of RBC decreased the frequency of chromosome breakage in DEB-treated whole blood cultures from normal donors, when compared with DEB-treated cultures of RBC-depleted leukocytes. Furthermore, when isolated components of RBC were added to the cultures of RBC-depleted leukocytes, we observed that the addition of Hb, and not membranes, resulted in a decrease of chromosome breakage similar to that observed in whole blood cultures.

It is known that RBC contain complex enzyme systems that are involved in the protection of its main components, Hb and membrane, against oxidative stress [17–19]. RBC are also capable of metabolizing certain drugs and chemicals. In fact, they contain high concentrations of reduced glutathione and glutathione transferases, which participate in the local detoxification of various drugs and chemicals which could damage Hb and membranes [20–22]. This property of RBC has been used as a source of metabolic activation in in vitro toxicology studies [23]. In the present study the results suggest that RBC, besides its involvement in the protection of its main components, are involved in the protection of lymphocytes against DEB toxicity, and possibly other cell systems not yet studied.

When we tested the role of isolated RBC components in the protection of cultured lymphocytes against DEB we observed that membranes alone can not protect lymphocytes in the same way that RBC protect. Conversely, haemoglobin alone has a protective role similar to that of RBC. The role of haemoglobin in the protection of lymphocytes against SCE induced by styrene was already suggested by Norppa et al. [24]. They showed that the induction of SCE by styrene in whole blood lymphocyte cultures of several donors was dependent on the presence of RBC, and it was suggested that this activation is the result of a conversion of styrene into styrene 7,8-oxide by oxy-haemoglobin. In the present work we showed the role of haemoglobin in the protection of lymphocytes against chromosomal breakage induced by DEB. In fact, the observations described in Tables 2 and 4 suggest that the protection effect of haemoglobin is a function of its concentration. Therefore, the haemoglobin concentration plays a definite and quantifiable role and the amount should be carefully taken into account when estimating the clastogenic activity of DEB at the individual level. All these findings have important practical implications, since the large inter-individual differences in RBC and haemoglobin concentrations or types can modulate the response of lymphocytes to the toxic effect of DEB. Patients with Fanconi anemia, who have their diagnosis based on a DEB-test for chromosomal breakage detection, are characterized by a high level of fetal haemoglobin, and so the specific role of this type of haemoglobin in the protection of FA patients against toxicity will be further studied.

In the present study, a significant inter-individual variation in the experiments conducted in the presence of RBC, concerning the levels of chromosomal damage, was observed for each donor, suggesting that individual factors could be associated with a differential modulation of the levels of DNA damage after exposure to DEB. Concerning the individual genetic polymorphisms evaluated, *GSTT1* is the only one associated with the level of chromosomal damage induced by DEB, the level of DNA damage observed being significantly lower in 'non-null' individuals, suggesting that this enzyme could be involved in the detoxification of DEB. The fact that *GSTT1* is not expressed in human lymphocytes [25] can explain the absence of effects of *GSTT1* on DEB genotoxicity in RBC-depleted cultures, when compared with the significant effect observed in whole blood cultures. The fact that all heterologous RBC added to RBC-depleted cultures were from *GSTT1* 'non-null' individuals can also explain the significant decrease in chromosome breakage in those cultures compared with whole blood cultures where *GSTT1* 'null' individuals are included. However, the inter-individual variation observed in these experiments is not sufficient to explain the highly significant variation in chromosome breakage between whole blood and RBC-depleted cultures. So, other

factors may be involved in DEB detoxification, and the role of haemoglobin was demonstrated in this study.

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