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Heterogeneity in chemical mutagen-induced chromosome damage after G_2 phase exposure to bleomycin, ara-C and gentian violet in cultured lymphocytes of β-thalassaemia traits

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Heterogeneity in chemical mutagen-induced chromosome damage after G_2 phase exposure to bleomycin, ara-C and gentian violet in cultured lymphocytes of β-thalassaemia traits

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Abstract

Chemical mutagen-induced chromosome damage was analysed in cultured peripheral blood lymphocytes from β-thalassaemia traits and healthy individuals. This was prompted by the fact that β-thalassaemia trait is present in 1–17% of different population groups in India. To study mutagen-induced chromosome instability, G_2 lymphocytes were exposed to bleomycin, ara-C or gentian violet in 48-h cultures. Spontaneous chromosome aberration frequencies in lymphocytes from β-thalassaemia traits were found to be in the normal range. In all three clastogen-treated lymphocytes from β-thalassaemia traits, there is a degree of hypersensitivity, when the results are averaged over a number of individuals, but some individuals overlap within the normal range. The heterogeneity in chemical mutagen sensitivity observed in β-thalassaemia traits is discussed in terms of the oxidative damage consequent on the genetic and biochemical features peculiar to the β-thalassaemia trait cell.

Keywords: β-Thalassaemia trait; G_2 lymphocytes; Chromatid aberrations; Bleomycin; Arabinomycin C; Gentian violet

1. Introduction

Earlier we observed a heterogeneity of chromosome damage in lymphocytes from β-thalassaemia traits in the cytokinesis-blocked micronucleus assay and chromosome aberration analysis, after γ-irradiation in G_0 and G_2 phase (Krishnaja and Sharma, 1994). Reports on the increased chromosomal aberrations in lymphocyte cultures from β-thalassaemia homozygotes, the chance finding of a β-thalassaemia trait in an occupational exposure case in our laboratory, as well as the fact that the β-thalassaemia heterozygote frequency encountered in some communities in the Indian population is as high as 17% (Sukumaran and Master, 1974), prompted us to undertake this study. With such a high heterozygote frequency present in certain pockets of the population, the chances of encountering β-thalassaemia traits in radiation-and chemical-related industries do exist and the present study is an attempt to determine sensitivity of β-thalassaemia traits to chemical

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mutagens compared to those of healthy individuals in the general population.

β-Thalassaemia is a common single-gene disorder and it has been estimated that 3% of the world's population carry a β-thalassaemia gene (Kazazian and Boehm, 1988). β-Thalassaemia is a heterogeneous autosomal recessive disease characterised by hypochromic haemolytic anaemia and β-thalassaemia subjects are dependent on blood transfusions to sustain life. More than 90 mutations have been found to cause this disease. In general each ethnic population has its own set of common mutations, together with a variable number of rare ones (Kazazian *et al.*, 1986; Thein *et al.*, 1988; Varawalla *et al.*., 1991). Individuals who carry a single β-thalassaemia gene (β-thalassaemia traits) are essentially normal and carry no intrinsic personal risk, but traits can usually be detected by screening for decreased osmotic red cell fragility, red cell indices that demonstrate a reduced mean corpuscular haemoglobin value, followed by the observation of an elevated haemoglobin A_2 level. β-Thalassaemia is relatively common in India, the β-thalassaemia trait being present in 1–17% of different population groups (Sukumaran and Master, 1974). The aver-

Table 1
The frequencies of chromatid aberrations in bleomycin-, ara C- and gentian violet-treated G_2 lymphocytes from normal subjects and β-thalassaemia traits

	Donor	Chromatid aberrations per cell		Chromatid gaps per cell		Donor	Chromatid aberrations per cell		Chromatid gaps per cell	
		Untreated	Treated	Untreated	Treated		Untreated	Treated	Untreated	Treated
Bleomycin	NS1	0.01	0.15	0.01	0.04	TT1	0.01	0.50	0.01	0.16
5 μg/ml	NS2	0.03	0.15	0.00	0.03	TT2	0.00	0.52	0.01	0.15
Ct.ab/cell *	NS3	0.00	0.27	0.02	0.07	TT3	0.03	0.57	0.00	0.07
	NS4	0.01	0.23	0.00	0.09	TT4	0.02	0.67	0.00	0.03
	NS5	0.02	0.29	0.01	0.06	TT5	0.01	0.74	0.01	0.07
	NS6	0.01	0.30	0.02	0.04	TT6	0.01	0.16	0.01	0.00
	NS7	0.01	0.32	0.01	0.07	TT7	0.01	0.21	0.01	0.04
						TT8	0.01	0.24	0.00	0.01
Ara C	NS1	0.00	0.15	0.02	0.03	TT1	0.02	0.40	0.02	0.11
5×10^{-5} M	NS2	0.02	0.21	0.02	0.09	TT2	0.01	0.40	0.02	0.10
Ct.ab/cell **	NS3	0.01	0.28	0.01	0.09	TT3	0.01	0.54	0.01	0.05
	NS4	0.03	0.34	0.01	0.12	TT4	0.02	0.49	0.00	0.08
	NS5	0.01	0.19	0.02	0.03	TT5	0.01	0.35	0.01	0.03
	NS6	0.02	0.27	0.02	0.02	TT6	0.01	0.34	0.02	0.04
	NS7	0.01	0.19	0.01	0.07	TT7	0.01	0.56	0.01	0.08
	NS8	0.02	0.18	0.01	0.02	TT8	0.03	0.58	0.00	0.06
	NS9	0.01	0.13	0.01	0.03					
	NS10	0.00	0.33	0.01	0.07					
Gentian violet	NS1	0.01	0.05	0.01	0.02	TT1	0.02	0.19	0.01	0.04
1 μg/ml	NS2	0.01	0.03	0.02	0.07	TT2	0.03	0.12	0.01	0.08
Ct.ab/cell	NS3	0.03	0.08	0.00	0.03	TT3	0.00	0.10	0.03	0.10
	NS4	0.00	0.05	0.02	0.04	TT4	0.01	0.07	0.01	0.06
	NS5	0.01	0.07	0.01	0.09	TT5	0.01	0.06	0.01	0.04
	NS6	0.01	0.05	0.02	0.05	TT6	0.02	0.05	0.00	0.01
	NS7	0.02	0.04	0.01	0.02	TT7	0.01	0.05	0.02	0.05
	NS8	0.01	0.05	0.01	0.05	TT8	0.02	0.05	0.01	0.03
	NS9	0.01	0.05	0.01	0.04	TT9	0.01	0.05	0.01	0.07
	NS10	0.01	0.02	0.02	0.08	TT10	0.01	0.05	0.00	0.02
						TT11	0.00	0.02	0.01	0.05

100 metaphases were examined per sample.
* Different from normal values at $p < 0.05$; ** different from normal values at $p < 0.001$.

age incidence of the β-thalassaemia trait in India is 3.3% (Varawalla *et al.*, 1991).

In this report, we present data on bleomycin-, arabinomycin C (ara C)- and gentian violet-induced G_2 chromatid damage in 48-h cultured lymphocytes of β-thalassaemia traits and normals. In all three clastogen-treated groups, there is a certain degree of hypersensitivity when the results are averaged over a number of individuals, but certain individuals overlap within the normal range.

2. Materials and methods

Heparinised blood samples were collected from healthy donors. β-Thalassaemia trait blood samples were obtained by courtesy of the late Dr. P.K. Sukumaran, B.J. Wadia hospital for children, Bombay. Whole blood cultures were initiated within 24 h of collection by a standard procedure of our laboratory (Krishnaja and Sharma, 1991). 4 ml Ham's F10 medium, with 200 mM L-glutamine, 0.5 ml foetal bovine serum (Sigma, USA), 0.1 ml reconstituted PHA (Wellcome Diagnostics, UK) were inoculated with 0.3 ml whole blood. The cultures did not contain any antibiotics. Short-term cytogenetic assays, either 48-h or 72-h cultures using bleomycin-, ara C- and gentian violet-based protocols for G_2 sensitivity, are employed in our laboratory to determine the importance of mutagen-induced chromosome instability in cancer susceptibility. The same protocol is followed here.

Stock solutions of bleomycin (Bleomycin Lundbeak, Amsterdam), ara C (Sigma) and gentian violet (BDH, UK) were prepared in sterile triple-distilled water. Final concentrations in culture were 5 μg/ml bleomycin, 5×10^{-5} M ara C and 1 μg/ml gentian violet. To determine G_2 sensitivity, bleomycin, ara C or gentian violet was added at 45 h, 3 h before culture harvest. Cultures were fixed, after 48-h incubation at 37°C, following a final 3-h treatment with colcemid at a concentration of 0.02 μg/ml. The culture harvest procedure followed the conventional hypotonic KCl treatment fixation with a methanol-acetic acid (3:1) mixture and air-drying on chilled wet slides. Air-dried chromosome preparations were stained in buffered Giemsa.

100 metaphases from each sample were examined for chromosomal aberrations. For the calculation of aberration rates only chromatid breaks and exchanges were considered. Chromatid breaks were considered as single-break events and isochromatid breaks and rare exchanges as two-break events. The chromatid and isochromatid gaps recorded were excluded from the analysis. The data were analysed statistically with Student's *t*-test. The chromatid breaks/cell in subjects from the two groups were compared.

3. Results

The chromatid aberrations per cell and the chromatid gaps per cell observed in untreated as well as bleomycin-, ara C- and gentian violet-treated G_2 lymphocytes from β-thalassaemia traits (TTs) and normal subjects are summarised in Table 1. As reported earlier (Krishnaja and Sharma, 1994), the spontaneous chromosome aberration frequencies in lymphocytes from control TTs were found to be in the normal range. Bleomycin, ara C and gentian violet during G_2 induced mainly chromatid breaks and gaps. However, in all three treated series in normals and TTs the chromatid gap induction was variable.

In bleomycin-treated lymphocytes from seven normal subjects (27–55 years, one female, six males) the chromatid aberrations ranged from 15 to 32, the corresponding values in eight TTs (27–40 years, four females, four males) being 16–74. A high frequency of chromatid aberrations per cell was observed in only five TTs (0.244 vs 0.60, $p < 0.001$) while the incidence of chromatid breaks in lymphocytes from three TTs was similar to that found in metaphase preparations from seven normal subjects. Chromatid exchanges were not observed after bleomycin exposure in the G_2 phase. Statistical comparisons revealed significant differences at the 5% level in the frequencies of chromatid aberrations in the bleomycin-treated lymphocytes from normals versus TTs taken as a group. However, bleomycin-

treated lymphocytes exhibited more multiple aberrations in TTs compared to normals.

In ara C-treated lymphocytes from normal subjects (27–55 years, three females, seven males) the frequency of chromatid aberrations ranged from 13 to 34 and in eight TTs (28–40 years, five females, three males) it was 34–58. Table 1 shows that in ara C-treated lymphocytes, not only the average number of chromatid aberrations, but also the number per individual case clearly exceeds that in lymphocyte cultures of healthy control subjects. A single chromatid exchange each was observed in 1000 metaphases from normal subjects and 800 metaphases from TTs. A highly significant increase relative to that observed in controls was seen in chromatid aberrations per cell in ara C-treated TT G_2 lymphocytes (0.221 vs 0.452, $p < 0.001$). Six TTs showed an increase in the frequency of chromatid aberrations, whereas two TTs exhibited a similar frequency of chromatid aberrations as normals.

No significant difference was observed in the gentian violet response of TT lymphocytes (21–50 years, six females, five males) as a group in comparison to that of healthy donors (28–55 years, one female, nine males), in case of chromatid aberrations per cell. However, in three TTs the chromatid aberrations per cell clearly exceed those in lymphocyte cultures of normal subjects.

4. Discussion

The salient finding of the study was the demonstration of heterogeneity in sensitivity to bleomycin, ara C and gentian violet exposure in G_2 TT lymphocytes. Mention should be made here that experiments were specifically not designed to test the sensitivity to the three chemicals simultaneously in all the TTs studied. However, two TTs showed increased bleomycin-and ara C-induced chromatid aberrations per cell. One TT showed an increase only in the bleomycin-induced chromatid aberrations per cell, whereas the ara C-induced aberration value was in the upper range for normals. Two TTs showed increased gentian violet and ara C-induced chromatid aberrations per cell. On average, although lymphocytes from TTs were significantly more sensitive to chromosome damage by bleomycin and ara C and to a lesser extent by gentian violet than those from normals, a highly variable response was observed. In case of ara C and bleomycin exposure the mean number of break events per cell was twice as high in TTs as in controls, but there was a broad overlap in individual values between the two groups. Further analysis revealed that TTs could be divided into two groups, group I had a higher rate of aberrations compared to normal individuals, whereas group II TTs had a near normal rate of aberrations. Heterogeneity in radiosensitivity of TT lymphocytes after G_0 and G_2 exposure to ^{60}Co γ-rays has been reported earlier (Krishnaja and Sharma, 1994).

The severe cytogenetic toxicity of gentian violet has already been mentioned (Au *et al.*, 1978; Hsu, 1985) and at 5 μg/ml we found extremely sticky and tangled chromosomes, making cytogenetic analysis difficult and inaccurate. Hence the concentration of 1 μg/ml was chosen, with which analysable aberrations could be obtained. Under this protocol, TTs as a group did not show any significant difference, although three TTs exhibited a clear increase in chromatid damage. A significant increase in gentian violet-induced chromosome breakage has been reported in certain constitutional chromosome anomalies and cancer patients (Hsu et al., 1979; Ledbetter et al., 1980).

Ara C was found to induce chromosome rearrangements in G_1 lymphocytes, which were not treated with any mutagen (Kishi, 1987), and the mechanism of ara C-induced G_1 rearrangements was shown to be simply classified into excision repair-dependent and excision repair-independent processes (Kishi and Sekizawa, 1993). In immortalised normal human fibroblast lines, ara C alone did not induce significant levels of aberrations during the last 4 h of the G_2 phase (Mozdarani and Bryant, 1989). Preston (1980) had reported that ara C (5×10^{-5} M) by itself induced 0.15 breaks/cell in the G_2 phase. It should be pointed out, however, that the different phases of the cell cycle are operationally determined, and a residual DNA synthesis can

still be going on in the so-called G_2 stage, thus allowing modifications in the yield of induced aberrations by treatment with DNA synthesis/repair inhibitors (Natarajan and Obe, 1983).

The possibility of genetic heterogeneity among patients with medullary thyroid carcinoma has been suggested on the basis of differential chromosome breakage observed after bleomycin exposure for the last 4 h in 48-h cultures (Tsioupra *et al.*, 1988). Hsu *et al.* (1985) studied bleomycin-induced chromosome breakage in normals and cancer patients and found a continuum of breakage rates, although the distribution of breaks between the two groups was different. Cohen *et al.* (1982) found overlapping values for diepoxybutane-and mitomycin C-induced chromosome aberrations in controls, Fanconi's anaemia patients and heterozygous gene carriers.

Since bleomycins are compounds of high molecular weight, the permeability of the cell membrane to this compound could perhaps play a role in the differences in drug sensitivity (Sidik and Snendon, 1990). Besides metal chelation (mainly Fe^{2+}), the most important feature of bleomycin metabolism is its conversion to the inactive derivative desamidobleomycin by the enzyme bleomycin hydrolase (Povirk and Austin, 1991). Studies have shown a correlation between resistance of certain tumours or cultured cells to bleomycin and their levels of bleomycin hydrolase (Umezawa *et al.*, 1972; Akiyama *et al.*, 1981). The finding of a direct correlation between chromosomal sensitivity to bleomycin and antioxidant enzyme activity, catalases, SOD and POD in mononuclear leukocytes and plasma is in itself interesting and a determination of antioxidant enzyme activities in a given cell population may serve to predict the chromosomal sensitivity to bleomycin (Bolzan *et al.*, 1992). Individuals' ascorbic acid intake could also influence the results of bleomycin-induced chromosome damage (Pohl and Reidy, 1989). As in the case of indirect action of radiation, bleomycin is also known to induce DNA breaks through the production of free radicals (Takeshite *et al.*, 1978). An and Hsie (1992) have provided evidence for the involvement of reactive oxygen species in bleomycin mutagenesis in mammalian cells and its modulation by the intracellular level of SOD activity. With imbalanced β/α ratios of 0.5–0.6 in β-thalassaemia heterozygotes the excess globin chains normally found in thalassaemic red cells generate an increased amount of superoxide radicals and put them at a high risk of oxidative stress (Brunori *et al.*, 1975). Interaction between various factors like O_2 radicals produced by the bleomycin-Fe^{2+}-DNA complex in the nucleus, differences in the intracellular level of antioxidant enzymes, and bleomycin hydrolase, differences in the inhibition/synthesis of proteins responsible for G_2 arrest, can further complicate the mechanisms and pinpointing one or more defects as aetiological factors in the variable response of thalassaemia trait cells is difficult. As discussed in detail in our earlier report in the case of heterogeneity in chromosomal radiosensitivity (Krishnaja and Sharma, 1994), the thesis that the genetic and biochemical features peculiar to thalassaemic trait cells may underlie heterogeneity of chromosome damage in thalassaemia traits is plausible and may present a promising avenue for further research.

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