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BLEOMYCIN-INDUCED CHROMOSOMAL DAMAGE IN TUBEROUS SCLEROSIS

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Summary To investigate the possible involvement of mutagen-induced chromosomal instability in tuberous sclerosis the blood lymphocytes obtained from eleven patients with this disease and eleven healthy controls of comparable age and sex were exposed to bleomycin *in vitro* during the late S and G_2 phases of the cell cycle. Neither the spontaneous aberration yields nor the bleomycin-induced chromosomal sensitivity differed between the two groups. The chromosomal distribution of 578 and 478 induced breaks in patients and controls, respectively, was similar. Thus, bleomycin-induced G_2 chromosomal hypersensitivity in lymphocytes of patients with tuberous sclerosis is not an intrinsic feature of this hereditary disease.

Key Words chromosomal aberrations, tuberous sclerosis, bleomycins, mutation

INTRODUCTION

Tuberous sclerosis (TS) is an autosomal dominant neurocutaneous syndrome characterized by hamartomatous growth in multisystems. It is recognized as a significant cause of infantile spasms and developmental handicap (Gomez, 1988). It has been reported that some of the cultured cell strains derived from patients with TS show hypersensitivity to the lethal effect of gamma-irradiation and to the cytotoxic chemical, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Paterson *et al.*, 1982; Scudiero *et al.*, 1981; Hayashi *et al.*, 1985). However, Ohno and Takeshita (1984) found that for the endpoints of colony forming efficiency, attachment ability, and population doubling time after treatment with MNNG, ethylmethane sulfonate, mitomycin C (MMC), and 4-nitroquinoline-*N*-oxid (4NQO) TS fibroblasts were within the range exhibited by normal cells.

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We have analyzed chromosomal damage in the cultured TS and control lymphocytes after bleomycin (BLM) treatment *in vitro* during the late S and G_2 phases of the cell cycle.

MATERIALS AND METHODS

Patients and controls. Eleven unrelated patients with TS and eleven control individuals were involved in the study. All TS patients met the minimal criteria for the disease according to Gomez (1988). Controls had no symptoms suggesting a disorder associated with chromosomal instability (absence of neurological and haematological disease, frequent infections or immunodeficiency, malformations, or signs of premature ageing). No controls acknowledged a history of chemotherapy or radiation exposure and detailed family history revealed no excess of cancer or spontaneous abortions among their first and second degree relatives. The ages of the patients and controls were similar (Table 1), ranging from 2.0 to 25.1 years and from 1.7 to 31.1 years, respectively.

Cultures. 3.0 ml of freshly drawn venous blood in phenol-free heparin was cultured in 40.0 ml of serum free medium, GPM-2 Sevac (Institute of Sera and Vaccines, Czechoslovakia), containing RPMI 1640 medium, lyophilized growth-promoting proteins of bovine serum GPBoS Sevac (Institute of Sera and Vaccines, Czechoslovakia) 5 g/liter; lactalbuminhydrolysate 1 g/liter; and natrium bicarbonate, 0.85 g/liter. The medium was supplemented with 1% (v/v) phytohaemagglutinin HA 15 (Wellcome), penicillin (50 UI/ml), and streptomycin (0.05 mg/ml). The serum free medium, GPM-2 Sevac, was used from one batch without diluting to ensure good reproducibility. All the samples were incubated for 72 hr in the dark at 37°C in a close atmospheric system, *i.e.*, sealed culture vessels.

After 68 hr of culture, the lymphocytes were exposed to bleomycin (Bleocin, Nippon Kayaku, Inc.) at a final concentration of 30 μg/ml for the last 4 hr of culture. Before cell harvest, colcemid (0.1 μg/ml) was given for 1.5 hr. Untreated samples received colcemid only. Both BLM-exposed and unexposed lymphocytes were treated in hypotonic solution (0.075 M potassium chloride) for 20 min at 37°C and fixed in methanol: glacial acetic acid (3:1, v/v). The suspensions were finally dropped on to slides and air dried. The slides were Giemsa (Merck) stained, and some were G-banded to determine the exact locations of break points recorded previously in Giemsa-stained metaphases.

Scoring aberrations. Chromosome analyses were made on coded preparations. Only well spread metaphases were evaluated. One hundred mitotic cells were examined per person in the BLM-treated samples, in untreated controls 50 metaphase spreads were evaluated to ascertain spontaneous yields. Gaps were not enumerated. Pulverized cells (here defined as cells with more than 7 break events) were disregarded in the final computation, but their frequency was recorded. The following criteria were used to distinguish gaps and breaks: (i) when the length of the achromatic region was equal to or shorter than the width of the chromatid,

the lesion was considered a gap; when the achromatic segment was longer than the width of the chromatid, the lesion was regarded as a break (Chatham Bars Inn Workshop Conference, 1971), (ii) if a lesion was a gap according to the previous definition and if there was a clear misalignment of the chromatid distal to centromere it was counted as a break (Harnden and Klinger, 1985).

For the calculation of aberration frequencies, chromatid breaks were considered as single break events and the rare chromatid exchanges or chromosome type aberrations, mostly isochromatid breaks, as two break events. The frequency of breakage was then expressed as the number of break events per cell. The break events were assigned to the chromosomes or chromosome group and the chromosome arms. The number of breaks which could not be classified with certainty was not included in the distribution analysis (Table 2). To compare the aberration yields between the two groups, we used the unpaired Student's *t*-test. The distribution of aberration types was evaluated by the chi-square test.

RESULTS

The number of aberrant cells in untreated lymphocytes of controls and TS patients was 8 (1.5%) and 9 (1.6%), respectively. The difference was not statistically significant ($p > 0.05$). The BLM-induced chromosomes damage in TS patients and healthy controls is shown in Table 1. There were no significant differences between the frequency of aberrant cells, pulverizations, chromatid breaks, isochromatid breaks, or chromatid exchanges. The number of break events per cell, ranging from 0.31 to 0.93 in the patients and from 0.43 to 1.16 in controls, was similar. Thus, BLM-induced chromosomal instability in the late S and G_2 lymphocytes of patients with TS does not seem to be an intrinsic feature of this disease.

The distribution of breaks on chromosomes 1–3 and chromosome groups B-F/G is documented in Table 2. We could not find any difference between the groups studied.

DISCUSSION

It has repeatedly been reported that in the lymphocytes of some patients with malignant tumors there is an increase in number of BLM-induced chromosomal aberrations compared with the normal healthy population (Hsu *et al.*, 1985; Cherry and Hsu, 1983; Hsu *et al.*, 1989; Vorechovsky and Zaloudik, 1989a). A predisposition to site- or tissue-specific oncogenesis could also be associated with the changes in the distribution of induced chromosomal damage in the peripheral lymphocytes, as has been shown for testicular cancer (Vorechovsky and Zaloudik, 1989a) and for neuroblastoma (Vernole *et al.*, 1989). It is likely that chromosomal sensitivity to mutagens plays an important role in carcinogenesis of organs and tissues that have direct contact with the external environment, such as respiratory, digestive, and integumentary systems (Hsu *et al.*, 1989). Furthermore, a similar

Table 1. Bleomycin-induced chromosomal damage.

Patients with TS	Age (years)	Number of cells analyzed	Number of cells with aberrations	Number of				Break events per cell
				pvz	ctb	csb	cte	
1	16.5	100	45	7	57	2	0	0.66
2	6.0	100	29	1	41	0	0	0.41
3	21.1	100	35	3	60	1	0	0.64
4	2.5	100	23	2	28	1	0	0.31
5	8.3	100	30	2	41	4	0	0.50
6	8.3	100	41	3	66	6	0	0.80
7	5.3	100	49	13	75	3	0	0.93
8	17.5	100	37	10	39	2	1	0.50
9	12.0	100	50	5	75	5	0	0.90
10	25.1	100	54	7	84	1	0	0.93
11	2.0	100	54	9	79	1	0	0.89
Mean (±S.D.)	11.3 (±7.4)	100	40.6 (±10.2)	5.6 (±3.7)	58.6 (±18.1)	2.4 (±1.8)	0.1	0.69 (±0.21)

Control group	Age (years)	Number of cells analyzed	Number of cells with aberrations	Number of				Break events per cell
				pvz	ctb	csb	cte	
1	17.5	100	37	6	51	1	1	0.59
2	21.5	100	43	6	54	4	1	0.68
3	6.0	100	34	3	40	1	0	0.43
4	12.1	100	67	12	90	5	1	1.16
5	1.7	100	42	10	44	3	0	0.56
6	12.5	100	40	4	60	4	0	0.71
7	4.1	100	30	4	36	2	0	0.42
8	31.1	100	39	6	48	1	0	0.53
9	11.5	100	31	3	42	0	0	0.43
10	8.3	100	32	2	44	2	0	0.49
11	16.8	100	31	3	50	0	0	0.52
Mean (±S.D.)	13.0 (±8.1)	100	38.7 (±10.0)	5.4 (±3.0)	50.8 (±14.0)	2.1 (±1.6)	0.3	0.59 (±0.20)
Signif. (*t*-test)	NS		NS	NS	NS	NS	NS	NS

Note: NS, not statistically significant ($p<0.05$); S.D., standard deviation; pvz, the cells with more than 7 break events; ctb, chromatid break; csb, chromosome-type aberration; cte, chromatid exchange.

Table 2. Distribution of bleomycin-induced breaks in individual chromosomes or in chromosome groups.

	Chromosome or chromosome group								Total number of located breaks	Number of unclassified ctb
	1	2	3	B	C	D	E	F/G		
Patients with TS	34	55	13	71	298	56	47	4	578	67
Control persons	27	49	13	73	236	47	31	2	478	86

increase in number of chromosomal aberrations was also found in some heritable cancer-prone conditions after X-irradiation or BLM treatment *in vitro* (Parshad *et al.*, 1983; Sanford *et al.*, 1987; Vorechovsky *et al.*, 1989b). It appears that this increased chromosomal damage results from deficient mechanisms of DNA repair (Gantt *et al.*, 1987). However, our results with TS patients show that neither chromosomal hypersensitivity to this mutagen nor distributional changes of located aberrations seem to be associated with TS under these experimental conditions.

The analysis of sister chromatid exchanges (SCE), chromosome aberrations, and cell proliferation kinetics did not reveal any difference between TS and normal lymphocytes in non-treated or MNNG-treated samples (Ieshima *et al.*, 1984). The spontaneous and MMC-induced SCE frequencies were also found to be within the normal range in TS patients (Iijima *et al.*, 1985). The effect of gamma radiation on the induction of SCE and chromosome aberrations in unstimulated TS lymphocytes has recently been reported by Iijima *et al.* (1988) who found no difference between SCE frequencies of TS lymphocytes and those of control lymphocytes, but chromosome aberrations in TS lymphocytes were significantly higher than those in the normal controls at a dose of 4 Gy, but not at lower doses. It seems likely that as high a dose as possible should be used in studies of this kind with the aim of stressing the system and unmasking any genomic instability (but the dose should also be compatible with obtaining sufficient number of mitotic cells for analysis). Discrimination between ataxia-telangiectasia and normal chorion villi cultures was better with gamma radiation than with BLM (Llerena *et al.*, 1989), and these results were in agreement with those obtained by other authors (Zampetti-Bosseler and Scott, 1985; Taylor *et al.*, 1979).

Yoshida *et al.* (1985) examined the formation and rejoining rates of X-ray-induced DNA single-strand breaks in radiosensitive and radioresistant fibroblast lines from patients with TS and from normal individuals, using the alkaline elution method. Although they found no differences between these cell lines in the frequency of DNA single strand breaks directly produced by X-irradiation at any dose up to 7.5 Gy, a kinetic analysis of these breaks indicated an increased rate of rejoining in the initial fast repair component when compared with normal fibroblast lines. This abnormality might be related to basic defect in TS.

To minimize the proportion of the cells exposed in the S phase of the cell cycle we shortened the time of exposure to BLM from 5 to 4 hr, which we had used pre-

viously (Vorechovsky *et al.*, 1989b). We obtained a comparable number chromosome-type aberrations (4.0% in the patients and 4.1% in controls, the difference being negligible). The data reported by Al-Achkar (1988) do suggest that even in our system used in this study a considerable number of the evaluated mitotic cells was exposed during the S phase. It is not likely that the observed numbers of chromosome-type aberrations in BLM-treated samples could be explained by the background levels, they are at least partly due to the exposure during the S phase. The evaluation bias of the rearranged chromosomes might also be involved.

The complex medium GPM-2 was useful for lowering the variability in the number of break events per cell in repeated samples and improved the reproducibility of the results. The identification of all the factors responsible for experimental variability is required in such assays. Some of the important factors have been identified by Sanford *et al.* (1989) in their studies of chromosomal radiosensitivity.

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