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Efficiency of a ^{252}Cf source in normal or in B-l0 enriched lymphocytes evaluated by SCGE assay, classical cytogenetics and FISH technique

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Abstract Biological effectiveness of a californium-252 source was evaluated after irradiations *in vitro* of normal or pre-treated cells with compound enriched in the B-10 ion ($Na_2{}^{10}B_{12}H_{11}SH$ also known as BSH) in order to check the possibility of any enhancement effect due to the process of boron neutron capture. Peripheral blood lymphocytes were used as a model for human cells. Human blood samples or isolated lymphocytes were irradiated with the isotopic source of ^{252}Cf, at the Faculty of Physics and Nuclear Techniques at the University of Mining and Metallurgy, Kraków (both the neutron source and the samples were placed in an "infinite" polyethylene block). DNA and chromosomal damage were studied to compare the biological effectiveness of irradiation. Single cell gel electrophoresis also known as the Comet assay was done to investigate the DNA damage. Classical cytogenetic analysis was applied to assess the frequencies of unstable aberrations (dicentrics, rings and acentric fragments). To evaluate the frequencies of stable aberrations the fluorescence in situ hybridisation (FISH) with probes for chromosomes 1, 4 (14.3% of the whole genome) was performed. Linear (or close to linear) increases with radiation doses were observed for the DNA damage and aberration frequencies in lymphocytes both untreated or pre-treated with BSH. Levels of translocations evaluated for the whole genome were comparable with the frequencies of dicentrics and rings. No significant differences were detected due to radiation dose in the frequencies of sister chromatid exchanges (SCE) detected in the second mitosis. Statistically no significant differences were observed in various biological end-points between normal or boron pre-treated cells.

Key words BNC reaction • BSH • ^{252}Cf • DNA and chromosomal damage • FISH • lymphocytes

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Introduction

Neutrons were applied for tumour therapy almost as soon as the neutron was discovered, and in spite of the long period since the first patient was treated, there is still no general agreement on the place of neutrons in radiotherapy [20]. Neutrons lose their energy either by elastic or inelastic collision with nuclei of the absorbing material. In elastic scattering, the greatest energy loss occurs in the case of collisions with nuclei of similar mass. Therefore, in biological tissues during elastic collisions neutrons transfer most of their energy to hydrogen nuclei; recoil protons, which result from this interaction, produce an ionisation like any heavy charged particles. Most of inelastic interactions occur with nuclei other than hydrogen. Energetic charged particles biologically very effective (e.g. proton or α particles) often are ejected from nuclei excited by inelastic interaction [20]. Clinical trials are underway with the use of low energy neutrons for cancer therapy based on the neutron capture reaction (NC). The most interesting nuclear reaction for the boron neutron capture therapy is $^{10}B(n, \alpha)\,^{7}Li$. Cross section of this reaction for thermal neutrons is one of the highest known, so that it is why fission neutrons are the most frequently applied beams for BNCT. A neutron spectrum similar to the energy of fission neutrons has the californium-252 source [20].

In this study, peripheral blood lymphocytes were used to evaluate in vitro the effectiveness of radiation from the ^{252}Cf source in

Table 1a. Dose rates estimated for samples placed at the point 2 cm from the ^{252}Cf source yielding 1×10^7 neutrons (both source and samples placed in "infinite" polyethylene block).

	Quantity					
	Flux density	Convertion factor k_n	H = knF	Q	D = H/Q [mGy/h]	
Type of radiation	(cm^{-1} s^{-1})	($mSv \times cm^2 \times s$)h	[MSv/h]	[Sv/Gy]	whole blood	isolated lymphocytes
Fast neutrons	4×10^5	1.26×10^{-3}	500	25	22.28	41.9
Thermal neutrons	2×10^5	0.04×10^{-3}	8	4	2.2	4.1
Gamma rays	–	–	–	–	14.96	28.9

Table 1b. Basic parameters taken for the evaluation of the dose rates of fast and thermal neutrons from the Cf-252 source, calculated with the application of the MCNP software. Dose rates estimated for human lymphocytes (volume ≈ 0.0622 cm^3) and whole blood samples (volume ≈ 1.2 cm^3) placed at the point 2 cm from the ^{252}Cf source yielding 1×10^7 neutrons (both source and samples placed in "infinite" polyethylene block).

	Type of radiation			
Sample	Neutrons [mGy/h ± SD]	Gamma rays [mGy/h ± SD]	Induced gamma rays [mGy/h ± SD]	Total [mGy/h ± SD]
Isolated lymphocytes	41.95 ± 1.2	27.8 ± 1.0	1.15 ± 0.13	70.1 ± 1.6
Whole blood	22.28 ± 1.0	14.09 ± 0.8	0.87 ± 0.06	37.25 ± 1.3

normal cells or pretreated with a compound enriched in the B-10 ion. Three various methods were applied at a molecular and cellular level to detect any damage induced. The alkaline version of SCGE assay was performed to detect DNA strand breakage. Classic cytogenetic and flu-orescence *in situ* hybridisation (FISH) analyses were carried out to evaluate chromosome aberration frequencies in the first mitotic division (dicentrics, rings, translocations). Cells in the second and third mitotic division were screened to evaluate sister chromatid exchange (SCE) frequencies and any potential influence of radiation on cell cycle kinetics.

Materials and methods

Lymphocytes preparation and BSH pretreatment

Blood was collected by venepuncture from a healthy male donor. Samples of heparinized whole blood to study cytogenetic damage or isolated lymphocytes for the DNA damage analysis were used for irradiation.

Lymphocytes were separated using an Histopaque separation medium. The cells were washed in RPMI 1640 supplemented with a 15% foetal calf serum, and suspended in the same medium at room temperature.

To study the influence of BNC reaction, half of the blood samples or isolated lymphocytes were pretreated with mercaptoborane $Na_2B_{12}H_{11}SH$ (BSH; final BSH concentration 31.8 μg/ml, i.e. 18.9 ppm of boron-10) supplied by Centronics Limited, Croydon, UK. The treatment with BSH was done at 37°C, one hour before irradiation. 2 ml samples of blood or 0.5 ml of lymphocytes (approx. 100 000 cells) were irradiated at room temperature in eppendorfs; one sample untreated and pretreated with BSH at the same time.

Irradiation

^{252}Cf neutrons

Irradiation was done with the ^{252}Cf source (4.5 mCi – May 1998) of the Faculty of Physics and Nuclear Techniques of the University of Mining and Metallurgy in Kraków. For irradiation the eppendorfs were placed in a polyethylene chamber filled with distilled water to obtain a uniform distribution of fast and low energy neutrons. The distance of the end of eppendorf samples from the radiation source was 2 cm which (after regarding different spread of lymphocytes in the whole blood and in the medium) gave dose-rates of fast neutrons 37.24 and 70.1 mGy/h for the whole blood and isolated lymphocytes, respectively. Thermal neutrons dose-rate was 10 times lower. Basic parameters taken for the evaluation of the dose rates of fast and thermal neutrons from the Cf-252 source were calculated with the application of MCNP software [12] and are presented in Tables 1a and 1b. To obtain the required doses in the range, from 0.12 to 0.92 Gy, the samples were irradiated for 3.3–13 hours.

To check the influence of the prolonged time of exposure on the level of DNA damage, samples of the isolated unexposed lymphocytes were kept at room temperature, for the same period of time as the irradiated cells.
To evaluate biological efficiency of the source at the molecular and mitotic level, appropriate biological samples were irradiated per each dose:

- for the DNA damage analysis – isolated lymphocytes one sample of untreated and one sample of pre-treated with BSH;
- for chromosomal damage analysis – four whole blood samples untreated or pre-treated with BSH.

As soon as the irradiation procedure was completed, the samples were put into a thermostat less at a temperature of ~37°C and transported to laboratories of the Department of Radiation and Environmental Biology at the H. Niewodniczański Institute of Nuclear Physics where appropriate culturing and molecular procedures were performed immediately.

X-rays irradiation

X-rays irradiation was performed at the Institute of Nuclear Physics using the Department of Radiation and

Environmental Biology Philips X-rays machine with an average dose of 1.72 Gy/h. Isolated lymphocytes resuspended in RPMI 1640 to a final concentration of 100 000 cells per ml, were exposed to the following doses of X-rays: 0, 0.6, 1.2, 1.8 Gy (0, 21, 42, and 63 min, respectively). The irradiation was done at room temperature.

DNA damage analysis by single cell gel electrophoresis assay

Slide preparation

Fully frosted microscope slides (two replicate slides for each dose) were prepared according to the standard procedure described elsewhere [2, 7].

Slides were immersed for 1 h at 4°C in a freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate), with 1% Triton X-100 and 10% DMSO added just before use. The slides were then washed with distilled H_2O (4°C) and placed in a horizontal gel electrophoresis tank filled with fresh electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13) to a level approximately 0.25 cm above the slides. After 20 min of unwinding the DNA, electrophoresis was conducted at 4°C for 30 min at 25 V, 300 mA (0.72 V/cm). All of the steps after lysis were conducted under yellow light to prevent any induction of additional DNA damage. After electrophoresis the slides were gently washed 3 times for 5 min with Tris buffer (0.4 M Tris pH 7.5) and then stained with 60 µl of 17 µg/ml ethidium bromide in distilled water and covered with a coverslip. Before analysis the slides were stored in a light proof box containing moist PBS at 4°C.

Slide analysis

For damage evaluation, the slides were examined at a 200x magnification using an epifluorescence microscope (Olympus BX-50) equipped with a 100 W mercury lamp, an excitation filter of 515–560 nm, a barrier filter of 590 nm and a CCD camera. Automatic evaluation of the comet parameters was performed with a Komet 3.0 software from Kinetic Imaging. One hundred fifty cells were evaluated per each point from the control group and for each irradiation dose (75 cells from each of two replicate slides). The following parameters were used as a measure of the DNA damage:

- tail length (extension of the comet),
- tail DNA (per cent of the DNA in the comet tail),
- tail moment (fraction of the DNA in the comet tail multiplied by the tail length).

To evaluate the DNA damage induced by irradiation itself, the influence of time of prolonged exposure was excluded by subtraction of the damage detected in the cells non-irradiated but only incubated for the period of exposures, from the results of the DNA damage obtained after each dose.

Chromosome damage analysis

Lymphocyte irradiation and cultures

Four samples (2 ml each) of heparinized blood, untreated or pre-treated with BSH, were irradiated per each dose in eppendorfs. The irradiated blood was divided into 5 groups and an appropriate medium was added to start various culturing procedures. Whole blood samples of 1.4 ml were added to 20 ml RPMI 1640 medium with a 20% foetal calf serum and antibiotics. To the cultures prepared for the detection of sister chromatid exchanges and unstable chromosome aberrations, 0.075 µM of 5-bromo-2-deoxyuridine was added to distinguish between the first and subsequent mitosis. Lymphocytes were stimulated with LF-7, the Polish substitute of phytohemaglutinin [1]. Four cultures were incubated at 37°C for 50 h for chromosome aberration induction (one culture with BrdU – and three cultures without it – for fluorescence *in situ* hybridisation). The fifth culture was incubated for 72 h for SCE analysis. Two hours before the end of the culture, colcemid (0.1 µl/ml) was added to each culture to arrest mitosis. The cells were fixed with Carnoy's solution according to standard procedures described elsewhere [1, 5]. The slides were stained using the Hoechst-Giemsa technique to distinguish between the first and subsequent mitosis. Additionally, one slide from the cultures without BrdU was stained with Giemsa solution for comparison of unstable aberration frequency.

Classical cytogenetic analysis

The analysis of chromosome damage was performed in the first mitosis for the presence of chromosome aberrations (CA), each acceptable good spread of the metaphase was analysed for CA. The percentage of aberrant cells (AbC), dicentric and ring frequencies (CAbF), total aberration frequencies including gaps (TAbF), aberration frequencies excluding gaps (AbF) and sister chromatid exchanges (SCE) frequencies per cell were used as a measure of cytogenetic damage. For (SCE) 50 metaphases in the second mitosis were scored. Potential influence on cell cycle kinetics, reported as proliferative rate index, was evaluated according to the following formula:

$$(1) \quad (PRI) = (M_1 + 2 \times M_2 + 3 \times M_3)/(M_1 + M_2 + M_3),$$

where:
M_1, M_2, M_3 are the numbers of cells in the first, second and third mitosis, detected respectively in one hundred of analysed metaphases [14].

Chromosomal damage by fluorescence in situ hybridisation (FISH)

The following chemicals were used for hybridisation procedure; SSC (sodium chloride and sodium citrate solution) and Tween 20 from Sigma-Aldrich, UK, DAPI/PI (4',6-diamidino-2-phenylindole dihydrochloride hydrate) from Cytocell Ltd., a "Chromoprobe-M" kit containing directly labelled whole chromosome painting probes for chromosome 1 and 4 labelled with 1(Cy3)&4(FITc/Cy3) and a pancentromeric probe labelled with fluorescein isothiocyanate (FITC) from Cytocell Ltd. Somerville Court, Oxfordshire, OX 17 3SN, UK.

Preparation of slides and probes

Prior to hybridisation slides were made from the fixed metaphases stored at -20°C. The density of the metaphases and the chromosome spreading were checked under a phase contrast

microscope and the hybridisation area was marked underside of the slide with a diamond pen. All procedures of the hybridisation were performed according to the protocol from the "Chromoprobe-M" kit by Cytocell Ltd. The slides were washed in 2 × SSC for 2 min at room temperature, dehydrated through an ethanol series (2 min. each in 70%, 85% and absolute ethanol) and prewarmed for approx. 5 min at 37°C. Simultaneously, chromoprobe cover slips, covered with probes and 15 µl of hybridisation fluid per slide, were prewarmed and afterwards placed onto the slides. The edges of the cover slips were sealed by the application of rubber solution.

Hybridisation

The probes and target DNA were denatured for 5 min on a hotplate with a surface temperature of 75°C. Hybridisation was performed in a humidified chamber at 37°C overnight. The next day the stringent washes were done for 5 min each by the following solutions (pH 7.0):

- three times in 50% formamide/1 × SSC, at 45°C,
- in 1 × SSC, at 45°C,
- in 1 × ST (4 × SSC and 0.05% Tween 20), at 45°C,
- in 1 × ST at room temperature.

DAPI/PI -Antifade solution was pipetted onto the slides (10 ml per slide) for counter staining.

Slide analysis

For observation and photography, a fluorescent microscope (Olympus BX-50), equipped with a triple wavelength filter (DAPI+FITC+TRITC) was used to detect fluorescence. A Fujicolor 400 film or a CCD camera are used to photograph the translocations detected.

Translocations involving chromosomes 1 and 4 were scored and F_p measured. On the basis of frequencies measured, values of the translocation frequencies per the whole genome (F_G) were evaluated according to Lucas *et al.* [17]:

$$(2) \quad F_G = F_p / 2.05 \times f_p \times (1 - f_p),$$

where: F_p means the observed frequency of translocations measured with FISH and f_p is the fraction of a genome labelled with FISH. For chromosomes 1 and 4 the value of f_p is 0.143.

Statistics

To evaluate a cut-off for HFC distributions of the SCE in control populations were studied with a "Frequency" procedure from SPSS statistical package. The cut-off for HFC, determined as per cent of cells containing a number of exchanges above 95% of the total in control distribution, was 13 ≥ SCE/cell for the normal cells. The least squares best fit for the dose-response curves and correlation coefficients were calculated with the use of Excel 5.0. Student's *t* or ANOVA analysis from SPSS program were applied to determine statistical significance differences between DNA damage due to irradiation or BSH pretreatment.

Results

Results of the DNA damage estimated with the SCGE assay after irradiation with the californium-252 source of human lymphocytes (without and with BSH pre-treatment) are presented in Tables 2a and 2b, respectively. Table 2c presents mean values of the DNA damage evaluated in

Irradiation time (h)	Dose [Gy]	B-10 pre-treatment [ppm]	Tail DNA (%) ± SD	Tail length [µM] ± SD	Tail moment ± SD
3.3	0.23	–	8.04± 1.93	12.78 ± 2.35	1.75 ± 0.71
6.6	0.46	–	12.74 ± 2.08	45.87 ± 5.18	7.37 ± 1.95
13.2	0.92	–	16.56 ± 3.33	49.80 ± 4.99	9.46 ± 1.68
3.3	0.23	18.9	8.23 ± 1.84	14.94 ± 3.28	2.50 ± 1.65
6.6	0.46	18.9	10.52 ± 1.30	46.20 ± 6.64	6.06 ± 1.14
13.2	0.92	18.9	15.33 ± 2.09	53.34 ± 2.87	9.52 ± 1.39

Table 2a. Influence of the irradiation on the DNA damage induced in human lymphocytes untreated or pretreated with BSH.

Tail length (extension of the comet), % tail DNA (per cent of the DNA in the comet tail), and Tail moment (fraction of the DNA in the comet tail multiplied by the tail length).

Incubation time (h)	B-10 treatment [ppm]	Tail DNA (%) ± SD	Tail length [µm] ± SD	Tail moment ± SD
3.3	–	5.46 ± 1.18	11.67 ± 2.12	0.92 ± 0.43
6.6	–	7.21 ± 1.08	32.79 ± 2.77	2.54 ± 0.23
13.2	–	7.43 ± 1.00	35.60 ± 3.23	3.14 ± 0.70
3.3	18.9	6.66 ± 1.89	12.47 ± 3.10	1.19 ± 0.76
6.6	18.9	6.49 ± 0.81	33.10 ± 10.39	2.42 ± 0.62
13.2	18.9	9.39 ± 1.43	36.90 ± 5.50	3.76 ± 0.81

Table 2b. Influence of incubation time on DNA damage in human lymphocytes without and with BSH pretreatment.

Irradiation time (min)	Dose [Gy]	Tail DNA (%) ± SD	Tail length [µM] ± SD	Tail moment ± SD
0	0	3.42 ± 1.46	17.82 ± 5.02	0.88 ± 0.47
21	0.6	5.09 ± 2.04	20.23 ± 6.24	1.19 ± 0.58
43	1.2	6.86 ± 1.97	36.33 ± 5.25	2.84 ± 0.57
63	1.8	9.82 ± 1.95	43.83 ± 4.67	4.65 ± 1.36

Table 2c. The DNA damage evaluated in human lymphocytes irradiated with a low dose rate of X-rays (1.72 Gy/h).

Total dose [Gy]	^{10}B [ppm]	NM	CAbF ± SE	AbF ± SE	TAbF ± SE	AbC ± SE
0.0	–	896	0.002 ± 0.001	0.044 ± 0.007	0.057 ± 0.008	3.68 ± 0.06
0.12	–	179	0.060 ± 0.018	0.117 ± 0.026	0.134 ± 0.027	10.61 ± 0.24
0.24	–	1366	0.102 ± 0.009	0.218 ± 0.013	0.237 ± 0.013	15.96 ± 0.11
0.49	–	647	0.230 ± 0.019	0.423 ± 0.026	0.450 ± 0.026	28.75 ± 0.21
0.0	18.9	449	0.000 ± 0.000	0.036 ± 0.009	0.038 ± 0.009	3.34 ± 0.09
0.12	18.9	760	0.042 ± 0.007	0.120 ± 0.020	0.137 ± 0.013	9.74 ± 0.11
0.24	18.9	357	0.081 ± 0.015	0.224 ± 0.025	0.241 ± 0.026	17.09 ± 0.29
0.49	18.9	586	0.152 ± 0.016	0.326 ± 0.024	0.336 ± 0.024	22.36 ± 0.19

Table 3. Chromosomal damages detected by classical cytogenetics after irradiation with the ^{252}Cf source of untreated or pretreated with BSH human lymphocytes.

NM – number of metaphases; CAbF – dicentrics and ring frequency; AbF – aberration frequency (excluding gaps); AbF – total aberration frequency (including gaps); AbC – per cent of aberrant cells.

human lymphocytes after irradiation with a low dose rate of X-rays (1.72 Gy/h).

Figs. 1a and 1b show dose-response curves for biological effects measured as Tail DNA and Tail moment after subtracting the values of damage detected in the control samples performed respectively for each dose of radiation. The open markers present the results obtained in the normal lymphocytes, and the filled markers present the results obtained in the pretreated with BSH cells. There is a close to linear dose-dependent response throughout the dose range under study. Dose response relationship shows a linear shape at a lower dose range with the tendency to saturation at higher doses. Finally, a better approximation of the dose response relationship is given by a polynomial fit. No significant difference between boron pre-treated and normal cells was observed for the levels of the DNA damage detected by the Comet assay. Although, after irradi-ation of the normal cells, the length of the comet tail is slightly lower compared to its value in BSH pretreated lymphocytes.

Results from the measurements of unstable chromosomal aberrations induced by the californium-252 source without and with BSH pretreatment are presented in Table 3. The analysis of chromosomal damage, present in the first mitosis, revealed a dose-dependent increase of unstable aberration frequencies reported as dicentrics and rings frequency (CAbF), aberration frequency excluding gaps (AbF) and including gaps (TAbF), and per cent of aberrant cells (AbC). Dose-dependent increase was observed after irradiation both without and with BSH pretreatment of the cells.

Figs. 2a and 2b show the chromosomal damage reported as CAbF and AbF, respectively after Cf-252 irradiation of the lymphocytes without or with BSH pretreatment. There is a close to linear dose-dependence observed after irradiation of untreated cells, and a non-linear one in cases of irradiation of BSH pre-treated lymphocytes. Finally, a slightly better approximation of the dose response relationship is given by a polynomial fit. No significant differences were observed in the whole dose range under study between the frequencies of chromosomal aberrations detected after irradiation of not treated and pretreated with BSH cells, ($p < 0.17$ for the CAbF and $p < 0.4$ for AbF, respectively).

Studies of the californium-252 source efficiency in the induction of the stable chromosomal aberrations (translocations) were done by the fluorescence *in situ* hybridisation method (FISH) with the application of the whole painting probes for chromosomes 1 and 4. Results of the screening for the presence of translocations are shown in Table 4 for both types of cells without and with BSH pretreatment. There is an increase in the frequencies of translocations with the dose for both types of lymphocytes; pretreated or not pretreated with BSH. Fig. 3 present dose-response curves conducted for the frequencies of translocations detected and evaluated for the whole genome in presence and absence of BSH pretreatment. There is close to linear dependence of translocation induction on dose and no difference is observed for lymphocytes pretreated or non treated with BSH.

In Figs. 4a and 4b are presented the dose response curves for the frequencies of translocations assessed for the whole

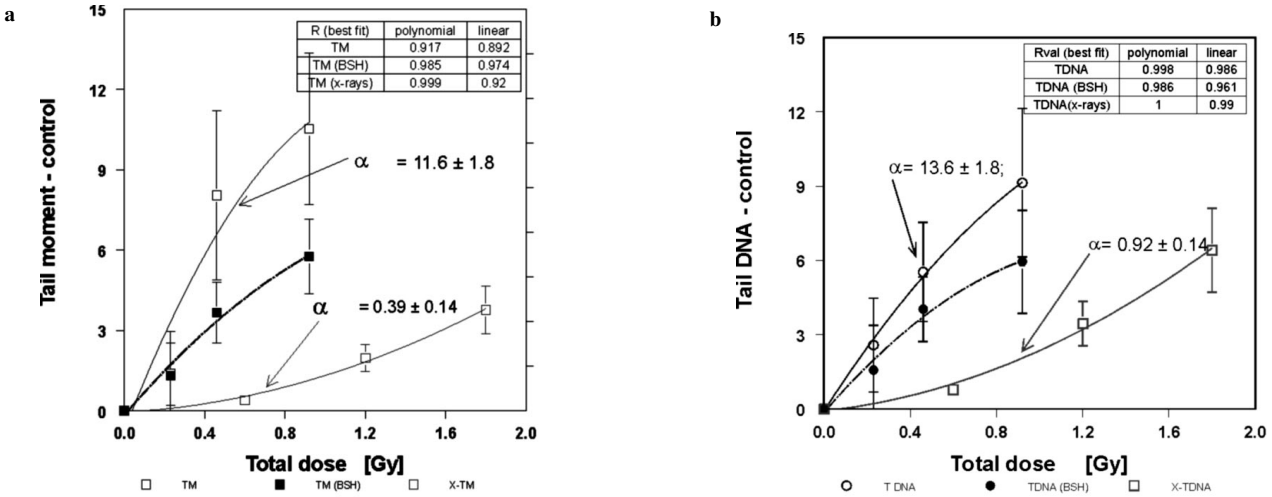

Fig. 1. Tail moment (a) and Tail DNA (b) detected after irradiation with X-rays (squares) and with Cf-252 source (circles) of human lymphocytes untreated (open markers) or pretreated with BSH (filled markers).

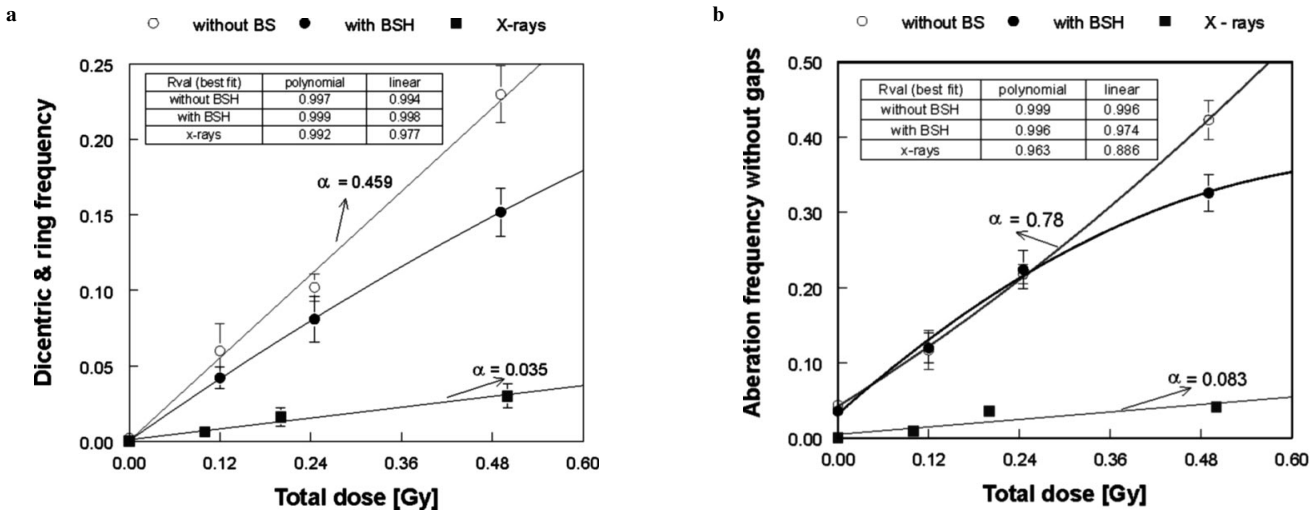

Fig. 2. Frequencies of unstable chromosome aberration (dicentric and ring). a – and all aberration without gaps (AbF); b – after lymphocytes irradiation with the Cf-252 source at presence and absence of BSH.

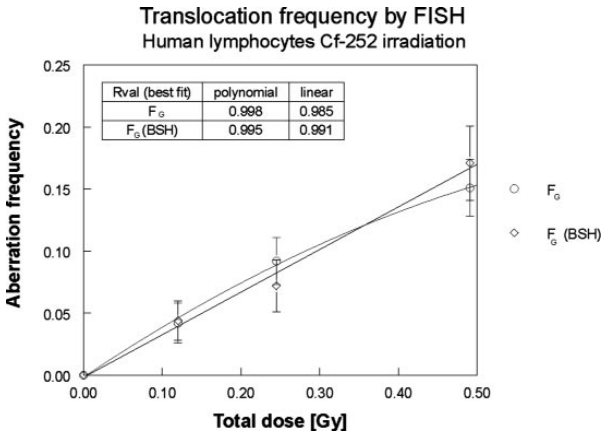

Fig. 3. Translocations frequencies evaluated with FISH technique for the whole genome (from the frequencies of translocation detected in the chromosome 1 and 4) after a Cf-252 source irradiation of lymphocytes without or with BSH pretreatment.

genome, and the dose response curves conducted for dicentric frequencies in the case of untreated lymphocytes (Fig. 4a) and pretreated with BSH (Fig. 4b). Comparison of the frequencies of the dicentrics measured with the classical method, and the translocations measured with FISH, revealed only a slightly higher frequency rate of the detected unstable chromosomal aberrations. Due to the slightly lower efficiency of the induction of unstable aberration in BSH pretreated cells (CabF, in Table 3) a similar efficiency in the induction of stable and unstable chromosomal damage is observed in the whole dose range in case of irradiation of cells after BSH pretreatment (Fig. 4b).

Analysis of the changes observed in the second cell division showed a very small and insignificant influence of the irradiation on the SCE frequencies induced in the normal and pretreated cells (Tables 4 and 5, Fig. 5) and none on high frequency cells (HFC). None of the proliferating rate indexes showed any evidence of the irradiation influence on the cell cycle kinetics (Table 5). Again, on the average, there is no difference observed in the response of cells untreated or pretreated with BSH.

Table 6 presents the values of a coefficients from the dose response curves for the DNA damage measures and chromosomal aberrations evaluated after irradiation of the lymphocytes with the Cf-252 and X-ray sources in our previous studies [11].

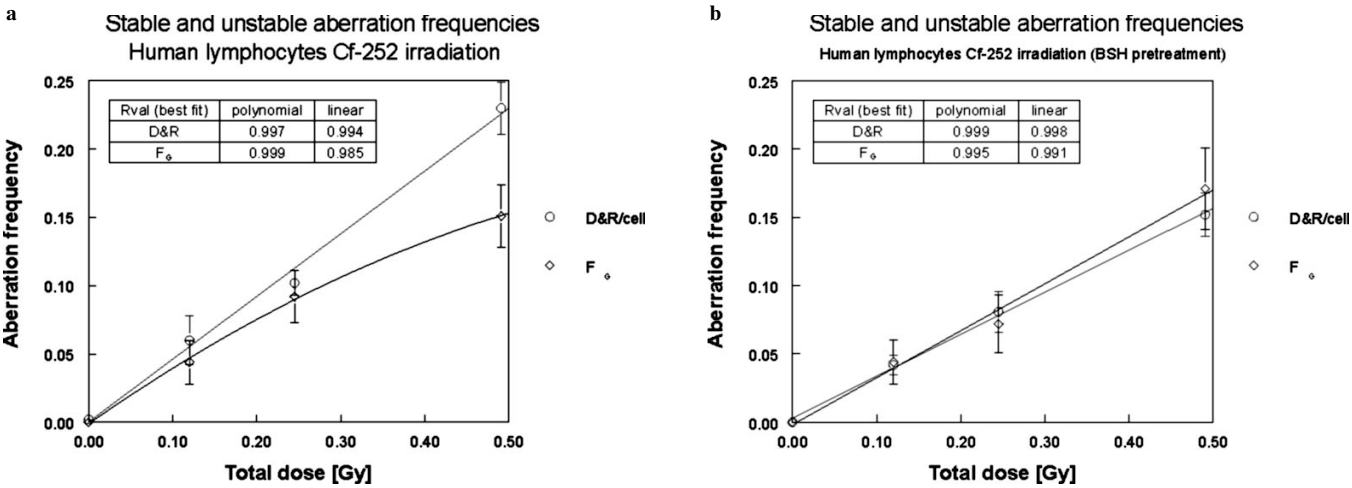

Fig. 4. Comparison of stable and unstable chromosomal aberration frequencies after a Cf-252 source irradiation of lymphocytes without (Fig. 4a) and with (Fig. 4b) BSH pretreatment.

Table 4. Translocation frequencies by irradiation with the ^{252}Cf source of untreated or pretreated with BSH human lymphocytes.

Total dose [Gy]	^{10}B [ppm]	NM	MT	T1, T4	$F_p \pm SE$	$F_G \pm SE$
0.0	–	119	0	0	0.000 ± 0.000	0.000 ± 0.000
0.12	–	168	2	2	0.011 ± 0.008	0.044 ± 0.016
0.24	–	260	6	6	0.023 ± 0.009	0.092 ± 0.019
0.49	–	264	9	10	0.038 ± 0.012	0.151 ± 0.023
0.0	18.9	215	0	0	0.000 ± 0.000	0.000 ± 0.000
0.12	18.9	180	2	2	0.011 ± 0.008	0.044 ± 0.016
0.24	18.9	164	3	3	0.018 ± 0.010	0.072 ± 0.021
0.49	18.9	185	7	8	0.043 ± 0.015	0.171 ± 0.030

NM – number of metaphases; MT – metaphases with translocations; T1,T4 – number of translocations involving chromosome 1 or 4; F_G – frequency of translocations per the whole genome acc. [17]: $F_G = F_p/2.05f_p(1-f_p)$, where F_p – observed frequency of translocations, f_p – fraction of genome covered with FISH probes (for chromosomes 1 and 4 $f_p = 0.143$).

Discussion

The objective of this study was to evaluate the effectiveness of the californium-252 isotopic source in the induction of the DNA and chromosomal damage in human lymphocytes. Californium-252 neutrons are taken for this study as this source has a spectrum of neutron energy similar to that of fission neutrons, and besides it, also a useful tool in cancer therapy. There are relatively few data on the biological efficiency of ^{252}Cf. The reported results obtained after irradiation with ^{252}Cf showed in somatic mutations in Trad-SH assay the efficiency of beam lower than expected from the RBE energy dependence curve estimated for mono-energetic beams [11, 12].

In this studies, irradiation of human lymphocytes was done with ^{252}Cf at very low dose rates, i.e. 37.25–70.9 mGy/h, in the dose range 0–0.92 Gy. A special attention was paid to check whether there is any enhancement effect due to the presence of the boron-10 ions inside the cells. Three methods were applied to investigate the problem: SCGE assay at the molecular level and classical cytogenetics and fluorescence *in situ* hybridisation with the whole painting probes for chromosomes 1 and 4 at the mitotic level. Irradiation with the ^{252}Cf source showed very close to the linear dose response relationship for almost all measured endpoints detected in the lymphocytes with or without chemical pretreatment, either for the DNA damage or for stable and unstable (translocations) aberrations frequencies. As it is seen in the presented Figures, most of the results gave slightly higher correlation coefficients for polynomial fits, but the differences between the coefficients were insignificant. The relationships observed are in good agreement with our expectations, because for the high LET radiation a close to linear dependence on the dose should be anticipated from the theory of radiation action [20].

Table 6. The α coefficients values and maximal RBE evaluated from chromosomal aberrations.

	Radiation	α ± SE	RBE_{max}
CAbF	X-rays	0.035 ± 0.010	–
CAbF	^{252}Cf	0.46 ± 0.05	13.1
Tail moment	X-rays	0.39 ± 0.14	–
Tail moment	^{252}Cf	11.6 ± 1.8	29.7
Tail DNA	X-rays	13.6	–
Tail DNA	^{252}Cf	0.92	14.8

Table 5. SCE, HFC frequencies and proliferating index (PRI) measured after a 252-Cf source irradiation of the cells without and with BSH pretreatment.

Dose [Gy]	without BSH			with BSH		
	SCE ± SE	HFC (%)	PRI	SCE ± SE	HFC (%)	PRI
0.0	7.96 ± 0.49	4	2.45	7.35 ± 0.51	4	2.71
0.12	7.76 ± 0.40	4	2.56	7.52 ± 0.39	2	2.62
0.24	8.94 ± 0.39	4	2.33	7.92 ± 0.37	4	2.55
0.49	10.26 ± 0.39	7.3	2.45	8.36 ± 0.41	6	2.63

SCE – sister chromatid exchanges per cell, scored in 50 cells; HFC – high frequency cells (per cent of cells displaying number of exchanges per cell higher than 95% of the control population distribution); PRI – proliferating rate index = $(M_1+2M_2+3M_3)/(M_1+M_2+M_3)$.

The values of α coefficients, describing the dose response curves slopes, and the value of maximal relative biological efficiency (RBE_{max}) evaluated from the α coefficients are presented in Table 6. The RBE_{max} value for the DNA damage estimated on the basis of the Tail moment parameter was almost 30. This value seems to be too high for the mixed beam described in Table 1, particularly that the value of the RBE_{max} obtained for 5.6 MeV fast neutrons from the previous study was about 4.6 [7]. The value of maximal relative biological efficiency (RBE_{max}) evaluated from the a coefficients obtained from the dose response relationships for the DNA in the comet tail is 14.8. The latter value is close to the (RBE_{max}) observed in cytogenetic measurements. This might suggest that percentage of the DNA in the comet tail is a much better estimator for the damage induced by ionising radiation at low doses. The maximal value of the relative biological effectiveness (RBE_{max}) for cytogenetic endpoints in untreated lymphocytes was calculated as the ratio of the fitted linear coefficients of the dose response curves for dicentrics and rings induced with the californium source ($\alpha = 0.287 \pm 0.014$), and for X-rays ($\alpha = 0.035 \pm 0.010$) from our previous studies [11]. The RBE_{max} value, estimated for chromosomal aberrations, was 13.1, whereas at of the RBE_{max}, calculated for fast neutrons (5.6 MeV) and fission neutrons from the previous studies, were about 10 and 20, respectively [11]. The RBE value obtained by Tanaka *et al.*

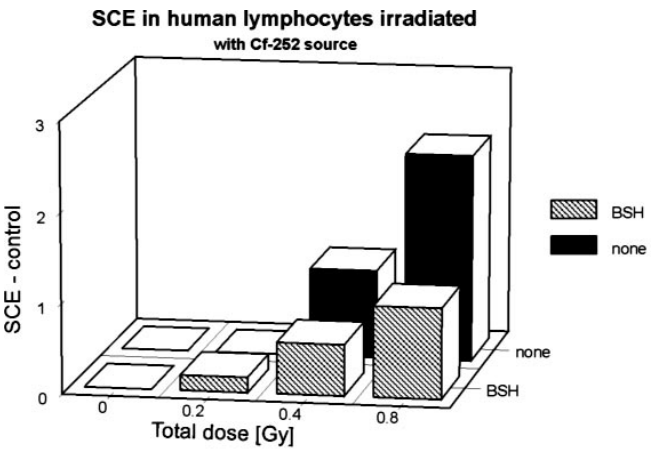

Fig. 5. SCEs frequency observed after irradiations with the Cf-252 source of lymphocytes with and without BSH pretreatment.

for the filtered Cf-252 neutrons for chromosomal aberrations was 12.3 [21]. Only a slightly higher RBE value evaluated for chromosomal aberrations in our study (13.1 *vs.* 12.3), compared to the RBE value obtained by Tanaka *et al.*, could be a result of different conditions of cells irradiation (in our studies lymphocytes were irradiated in the infinite block and in a polyethylene chamber filled with distilled water, while those by Tanaka *et al.* [21] were irradiated in air). In our exposure conditions a gamma ray impact was about 40%, and in the conditions of Tanaka experiments it was neglected. Nevertheless, our results are not conflicting with his findings because irradiation in the infinite block not only resulted in the higher impact from gamma radiation, but also in lowering the energy of neutrons, which, according to the dependence of biological efficiency on the neutrons energy increases the RBE value [20].

Fluorescence *in situ* hybridisation with the whole chromosome painting probes appears to be especially useful for the analysis of stable cytogenetic damage (translocations) as a very rapid and sensitive test that is not dependent on the time between irradiation and sampling, although, quite expensive. Comparison of the dose-response curves for dicentrics, analysed after conventional staining and translocations visualized by chromosome painting, revealed similar frequencies of radiation-induced translocations and dicentrics. Other authors have also reported similar frequencies of dicentrics and translocations [15, 16, 18]. To investigate an association between molecular and mitotic types of damage detected after irradiation with the californium source, we determined the Pearson Product Moment correlation coefficients (r^2). Results from the analysis of percentage of the aberrant cells and tail DNA gave $r^2 = 0.99$. The percentage of the aberrant cells and Tail moment is expressed by a value of $r^2 = 0.95$. The dicentrics and ring frequency and tail DNA gave $r^2 = 0.98$, and for the dicentrics and ring frequency and the tail moment $r^2 = 0.98$. In our previous [9] studies a comparison of the DNA damage in the Comet assay and cytogenetic damage, induced by radiation or chemical treatments, revealed also a very high correlation for the two types of damage.

Results of statistical analysis of our data have shown that pretreatment with the BSH for 1 h before irradiation with ^{252}Cf with a dose of 0–0.49 Gy, did not significantly alter neither the DNA strand breakage, reported as Tail moment in SCGE assay, nor the various types of cytogenetic damage detected in the first and second cell divisions. The effectiveness of boron neutron capture depends on the number of ^{10}B atoms delivered to the cells, the subcellular distribution of ^{10}B and the thermal neutron flux. According to Pöller *et al.* [19] the presence of 600 ppm of ^{10}B (boric acid) in the medium during irradiation of cells with fast d(14)+Be neutrons (mean energy 5.8 MeV) in a phantom enhances the DNA damage measured with SCGE assay by 20% compared with neutron irradiation alone. This is remarkable, as probability of the BNC reaction decreases dramatically (by two or three orders) with increasing energy of neutrons. In studies reported by Cebulska-Wasilewska *et al.* [10] of the lethal and colourless mutation frequencies induced in somatic cells of Trad-SH assay by californium neutrons (the mean energy probably at least 5 times lower than at used by Pöller *et al.*), values of the RBE were much higher for plants pretreated with borax. The evaluated RBE values from the comparison of α parameters, estimated from dose response curves, were almost five times higher in the presence of 40 ppm of B-10 from borax and being dependent on the biological end-point. Statistically significant alterations in RBE values were observed in the case of lethal mutations (from 6.2 to 34.3) and in the colourless mutations (from 1.6 to 5.6).

The lack of enhancement effect due to boron-10 pretreatment in our study should not be due to too low concentration of ^{10}B in the lymphocytes (~20 ppm). According to designs for therapy [3, 13] an optimum for the epithermal neutrons is said to be ~30 ppm. In our other studies [8] with the Brookhaven Medical Research Reactor beam (specially modified for the purpose of BNCT) we have used boric acid to introduce the boron ion into the cells. The latter were incubated at 37°C for two hours. According to Capała *et al.* [6], this procedure was resulting in the final concentration of boron atoms in cells of 10 ppm for GS-L9 rat gliosarcoma cells and 10 or 20 ppm for human lymphocytes. The total dose was estimated taking into account the high LET radiation from the BNC reaction per each ppm of boron-10. The RBE_{max} value for the neutrons part in this beam was evaluated as being 28.3 and this was much higher than expected for neutrons of such low energy [4]. That was an additional proof of the high efficiency of the BNC reaction products. Therefore, the evident lack of any enhancement effect due to BSH pretreatment in the presented results can be caused by two reasons; too low impact from the thermal or epithermal neutrons in the beam under study, or a different and much slower kinetics of diffusion into the cells of the boron enriched compound. It is also possible that BSH (that is one of the chemicals applied for therapeutic introduction of boron-10) is penetrating lymphocytes more slowly than boric acid, which in the case of BNCT is a positive sign for healthy cells. Further studies are needed to explain which factor is responsible for the lack of boron enhancement effects in our results.

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