

# DNA double-strand break rejoining in radioadapted human lymphocytes: evaluation by neutral comet assay and pulse-field gel electrophoresis

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**Abstract** Adaptive response (AR), an enhanced resistance to a high dose of ionising radiation acquired after pretreatment with a very low dose, was estimated in normal human lymphocytes. The question posed was whether the extent of radioadaptation, assessed by micronucleus test, would be related to the rate of DNA double-strand break (DSB) rejoining. Phytohemagglutinin-stimulated G<sub>1</sub>-lymphocytes from 5 healthy male volunteers were pre-treated (or not) with an adaptive (5 cGy) dose of X-rays, followed by a higher (5 or 10 Gy) challenge dose after 20–22 h. DSB rejoining after the challenge dose was monitored with the use of two methods: neutral comet assay, modified to reduce the contribution of single-strand breaks (SSBs) and thermolabile sites, and pulse-field gel electrophoresis (PFGE), specific for DSBs. At the level of micronuclei, an AR was observed in lymphocytes of 3 of 5 donors. Up to 60 min, comet assay showed no statistically significant differences in DNA break rejoining between adapted and non-adapted lymphocytes, independently of AR appearance. PFGE gave similar results, although in three donors it revealed secondary increases in DSBs levels at 30 min and/or 60 min post-irradiation in the adapted vs. the non-adapted samples. Failure to demonstrate changes in DSBs rejoining rate in the adapted lymphocytes could be due to diversity of AR intensity/timing at the level of DNA repair in not fully homogenous cell populations. Also, “rare” DNA cuts characteristic of early apoptosis/necrosis could overlap the process of DNA break rejoining.

**Key words** human lymphocytes • radioadaptation • DNA double-strand break rejoining • neutral comet assay • pulse-field gel electrophoresis

## Introduction

From 1984 on [19], it is known that eukaryotic cells can exhibit an inducible radioresistance, called adaptive response (AR) or radioadaptation (reviewed in [23, 28, 30, 33, 34, 38]). It occurs when cells, pretreated with a low (‘priming’ or ‘adaptive’) dose of ionising radiation, show an enhanced resistance to a subsequent higher (‘challenge’) dose. There is a relatively narrow range of doses and dose rates which can induce AR [25]. It was shown [26] that low radiation doses stimulate a prolonged translocation of protein kinase C (PKC) alpha from cytosol to plasma membrane, whereas higher doses result in a strong downregulation of PKC activity. PKC is considered as the key enzyme in a signal transduction pathway leading to AR ([8, 22, 36, 37], reviewed in [23, 28]). Radioadaptation is usually identified as a decrease in micronuclei or chromosome aberration frequency. This suggests that the repair of DNA double strand breaks (DSBs), the lesions responsible for chromosomal breaks [2, 15], may be targeted by the adapting signal. It could be expected that AR involves an enhanced rate of DSB rejoining or/and an improved rejoining fidelity.

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The data on the involvement of DNA repair in the AR is conflicting. On the one hand, there are reports that document a faster DNA break rejoining in adapted cells: Zhou *et al.* [40] applied the pulse field gel electrophoresis (PFGE) to measure DNA DSB levels in SR-1 murine carcinoma cells prelabelled with tritiated thymidine. After 30 min of repair, they found a 50% lower level of DSBs in non-adapted, compared to adapted cells. Next, Ikushima *et al.* [7] used the comet assay at neutral pH to measure DNA breaks in Chinese hamster V79 cells and observed higher rejoining rate and less residual damage in the adapted cells. On the other hand, Wójcik *et al.* [32] demonstrated an increased repair capacity in the adapted + challenged, PHA-stimulated human lymphocytes, by using a neutral version of the comet assay, however this did not correlate with a reduction of the aberration frequencies in the cells. Our previous study, with the use of an alkaline version of the comet assay [36], did not show any changes in DNA rejoining kinetics in PHA-stimulated human lymphocytes in donors that displayed an AR, observed at the level of micronuclei.

The aim of the present study was to assess the role of DSB rejoining rate in radioadapted human lymphocytes. We applied two methods for estimation of DSBs, each of them providing somewhat different information: a neutral version of the comet assay [35] allowed us to estimate DNA damage in individual cells and thus, to analyse the damage distribution in the cell population. The damage measured was mainly DSBs, although a slight interference from SSBs and alkali-labile sites still remained [6, 36]. The PFGE specifically estimated DSBs in total DNA from a cell population and gave information about the distribution of DNA fragment sizes [6].

## Materials and methods

### Preparation of lymphocytes and irradiation conditions

Human blood was obtained by venipuncture from 5 healthy male volunteers, non-smokers, aged 20–28 years.

All donors were informed about the aim of the study and the experimental details. X-radiation was generated by an ANDREX defectoscope (Holger Andreasen, Denmark) operating at 200 kV and 5 mA, with 3 mm Al filtration. The dose rate was 0.1 Gy/min for the adaptive dose (5 cGy) and 1 Gy/min for the challenge dose (2, 5 or 10 Gy).

The experimental schedule is presented in Fig. 1. Blood was taken to heparinised plastic syringes (Monovette; Sarstedt). For the micronucleus test, the lymphocytes were cultured in the whole blood: 0.5 ml of blood was added to 4.5 ml of RPMI 1640 medium (Sigma) supplemented with 20% fetal bovine serum (FBS; Boehringer Mannheim) and an antibiotic-antimycotic solution (AA solution; Gibco), in plastic culture tubes and placed into a CO<sub>2</sub> incubator at 37°C. For comet assay and PFGE, lymphocytes were isolated from blood with the use of Histopaque-1077 (Sigma-Aldrich), according to the method provided by the manufacturer. Immediately after isolation, the cells were suspended in RPMI 1640 medium with 20% FBS and AA solution at a density of 10<sup>6</sup> cells/ml. The cell viability was assessed by the nigrosine exclusion test. Phytohemagglutinin M (PHA-M, Gibco, 15 µl/ml) was added to stimulate lymphocytes entering the G<sub>1</sub> phase of the cell cycle. The time course of our experiments was based on the data obtained previously from studies on radioadaptation in PHA-stimulated human lymphocytes [3, 24]. According to them, the adaptive dose delivered as early as 4 h after the stimulation ensured the induction of the adaptive response for three subsequent cell cycles. Here, the adaptive dose of 5 cGy was applied 5 h after PHA stimulation, then, after subsequent 20–22 h, the cells were irradiated with the challenge dose of 2, 5 or 10 Gy (for the micronucleus test, PFGE or the comet assay, respectively). The samples destined for estimation of initial DNA damage were irradiated at 0°C. Those for studying DNA repair were irradiated at 37°C and left at the same temperature until 15, 30 or 60 min from the beginning of the irradiation, then cooled down and processed for the assays.

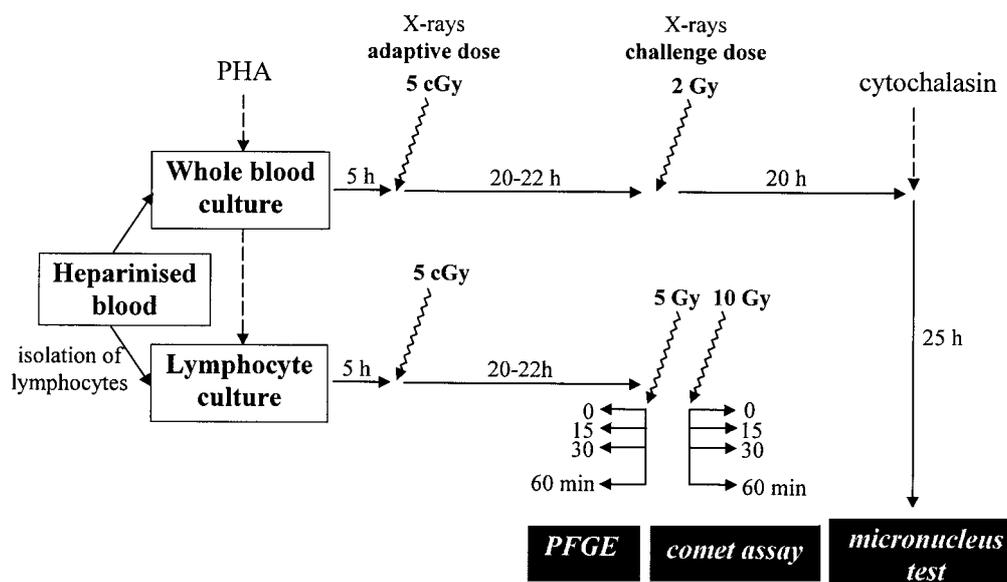


Fig. 1. The schedule of the adaptive response experiments.

Controls were sham-irradiated under conditions identical as those for the test samples.

### Micronucleus test

The micronucleus test was performed as described by Fenech and Morley [4]. Briefly, the lymphocytes at the 47th hour in culture were treated with cytochalasin B (Sigma, 6 µg/ml). After subsequent 25 h (Fig. 1), the cells were hypotonised in 125 mM KCl for 4 min at 37°C and mixed with cold Carnoy's fixative. The cell suspension was spread over chilled glass slides, dried on air and stained with 1% Giemsa. The micronuclei frequency was assessed in the population of binucleated cells as described previously [37]. For each donor, the assay was repeated at least 3 times.

### Neutral comet assay

The method applied here was described in detail in [35]. Lymphocytes, 20–22 h after the adaptive dose, were washed and suspended in cold PBS. The cell suspension ( $4 \times 10^5$  cells/ml) was mixed with low melting point agarose (Sigma-Aldrich) at 37°C, to a final concentration of 0.75% agarose, then layered on the surface of microscopic slides precoated with 0.5% normal agarose, under coverslips, on an ice-cooled aluminium plate. After solidification of the gel, slides were irradiated with 10-Gy challenge dose at 0°C [38] (the initial damage samples), or at 37°C, followed by 15, 30, 60 or 120 min incubation in culture medium at 37°C (the repair samples). All subsequent steps of the procedure were carried out under red light. After the specified repair periods, coverslips were removed and the slides immersed in a lysis buffer containing 2.5 M NaCl, 100 mM EDTA, 1% N-lauroylsarcosine-sodium salt, 0.5% Triton X-100 and 10% DMSO in 10 mM Tris/HCl, pH 9.5, at 4°C, for 1–2 h. After the lysis, slides were washed and equilibrated in an electrophoresis buffer (300 mM sodium acetate, 100 mM Tris/HCl, pH 8.3). Electrophoresis ran at 0.5 V/cm, at 8°C, for 1 h, in the dark. The slides were subsequently washed with a neutralising buffer (400 mM Tris/HCl, pH 7.5) and stained overnight with 1 µM DAPI (4,6-diamino-2-phenylindole). The pictures were captured at 200× magnification with the use of a Labophot-2 epifluorescence microscope (Nikon, Japan) equipped with a UV-1A filter block (an excitation filter of 365/10 nm and a barrier filter of 435 nm) and a video camera. Image analysis of the data was performed by the Comet v. 3.1. software (Kinetic Imaging, Ltd., Liverpool, UK). The measure of DNA damage was tail moment, i.e. the percentage of DNA in the comet tail multiplied by the tail length.

### Pulse-field gel electrophoresis (PFGE)

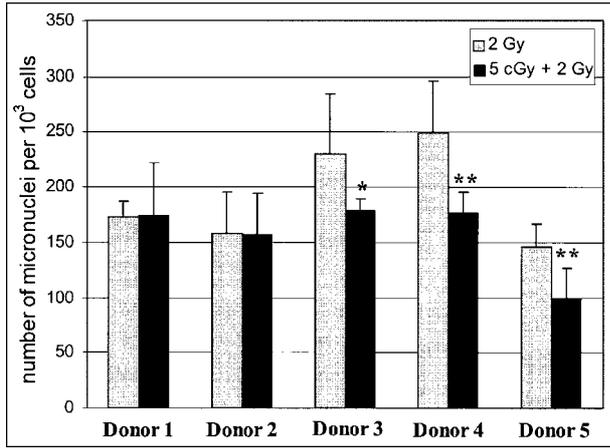
In the PFGE method used [6], prelabelling of cells with tritiated nucleosides was not applied, thus eliminating the possibility of an AR induction by the incorporated radionuclides [14]. The protocol was as follows: directly

after irradiation at 0°C (the initial damage samples) or after 15, 30 or 60 min from the beginning of irradiation at 37°C (the repair samples), the lymphocytes ( $5 \times 10^5$  per experimental point) were spun down, washed with ice-cold PBS and resuspended in 90 µl of a cell suspension buffer (10 mM Tris/HCl, 20 mM NaCl and 50 mM EDTA, pH 7.2). The cell suspension was mixed with 110 µl of 2% clean-cut agarose (BioRad Laboratories, USA) at 50°C and pipetted into plastic moulds (BioRad) on ice. After solidification, the agarose blocks were transferred to 1 ml of a lysing solution, consisting of 1 mg/ml proteinase K (Sigma-Aldrich, nuclease activity not detected), 0.2% sodium deoxycholate, 1% sarcosyl and 100 mM EDTA, pH 8.0. After 1 h preincubation at 0°C, the lysis was being continued for 20 h at 50°C. Subsequently, the plugs were washed four times for 1 h with 2 ml of a wash buffer (20 mM Tris/HCl and 50 mM EDTA, pH 8.0) and stored in the same buffer, in the refrigerator, until use (no longer than 2 days). Before electrophoresis, pairs of equally sized plugs were cut out of the agarose blocks; one plug of each pair was subjected, at 0°C, to 600 Gy of  $\gamma$ -radiation from a  $^{60}\text{Co}$  source, at a dose rate of 55 Gy/min. The pairs of plugs were loaded to the wells in a 0.8% chromosomal grade agarose (BioRad) gel. The electrophoresis was performed in a CHEF-III apparatus (BioRad), in  $0.7 \times$  TBE buffer, pH 8.3, at 1.7 V/cm, at 14°C, with 106° reorientation angle. The run comprised three 31-hour blocks of increasing switch time ramps: 50–200 s, 200–600 s and 2000–2200 s. After the electrophoresis, DNA in the gel was stained with a fluorescent dye, SYBR-Gold (Molecular Probes) and visualised under UV-transillumination. The image was captured with the use of CCD camera (Biometra, Germany) connected to PC. The electrophoretic lanes in the image negative were scanned using GelScan image analysis software (Kucharczyk Electrophoretic Techniques, Poland). In estimation of a DSB level in an individual sample, both DNA fraction released from the plug and an approximate number of DNA fragments in the gel were included [6].

## Results

### Micronucleus test

The criterion of AR appearance in human lymphocytes was the statistically significant decrease in micronuclei frequency observed in cell cultures irradiated with both the adaptive (5 cGy) and the challenge (2 Gy) doses, compared to the value obtained for the challenge dose alone. There was no statistically significant difference in the micronuclei frequencies between the cultures treated with the adaptive dose alone and the non-irradiated controls (not shown). Donors 1 and 2 did not show, while donors 3, 4 and 5 did repeatedly show an AR in at least three independent experiments (Fig. 2). Mean decreases in micronuclei frequencies in the adapted compared with non-adapted samples were: 23%, 29% and 32% for donors 3, 4 and 5, respectively.



**Fig. 2.** Micronucleus test as an indicator of the adaptive response in human lymphocytes from 5 blood donors. The non-adapted cultures (2 Gy) were compared with the adapted ones (5 cGy + 2 Gy). Points are means  $\pm$  SD of at least three independent experiments with 2–3 slides in each and 500 binucleated cells scored per slide. Asterisks mark statistically significant differences between pairs: \*  $p < 0.05$ , \*\*  $p < 0.01$ , with t-test.

**Comet assay**

Figure 3 shows DNA break rejoining after 10 Gy of X-irradiation in adapted and non-adapted lymphocytes from individual donors. In spite of inter-individual differences in tail moment values (not shown), the rejoining courses were fairly similar for all donors when relative values were plotted. We did not find any statistically significant differences in the rejoining rate

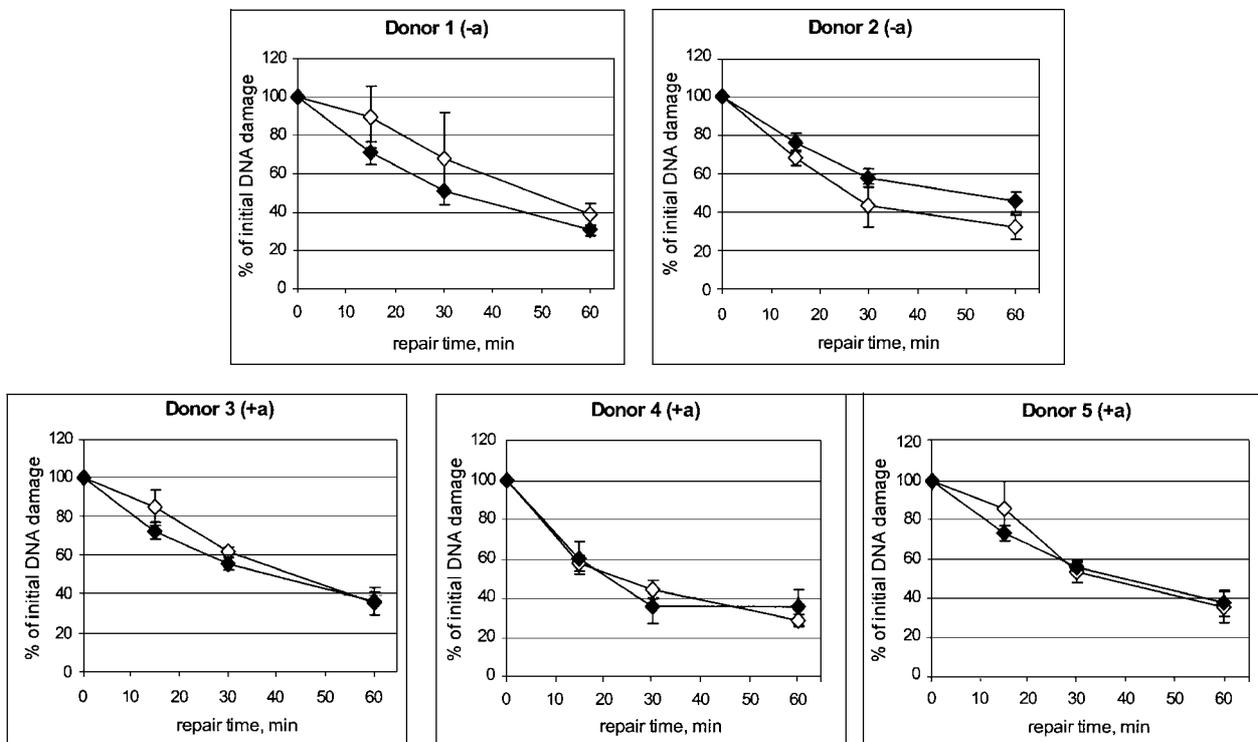
between adapted and non-adapted lymphocytes in either AR-negative or AR-positive group.

**PFGE**

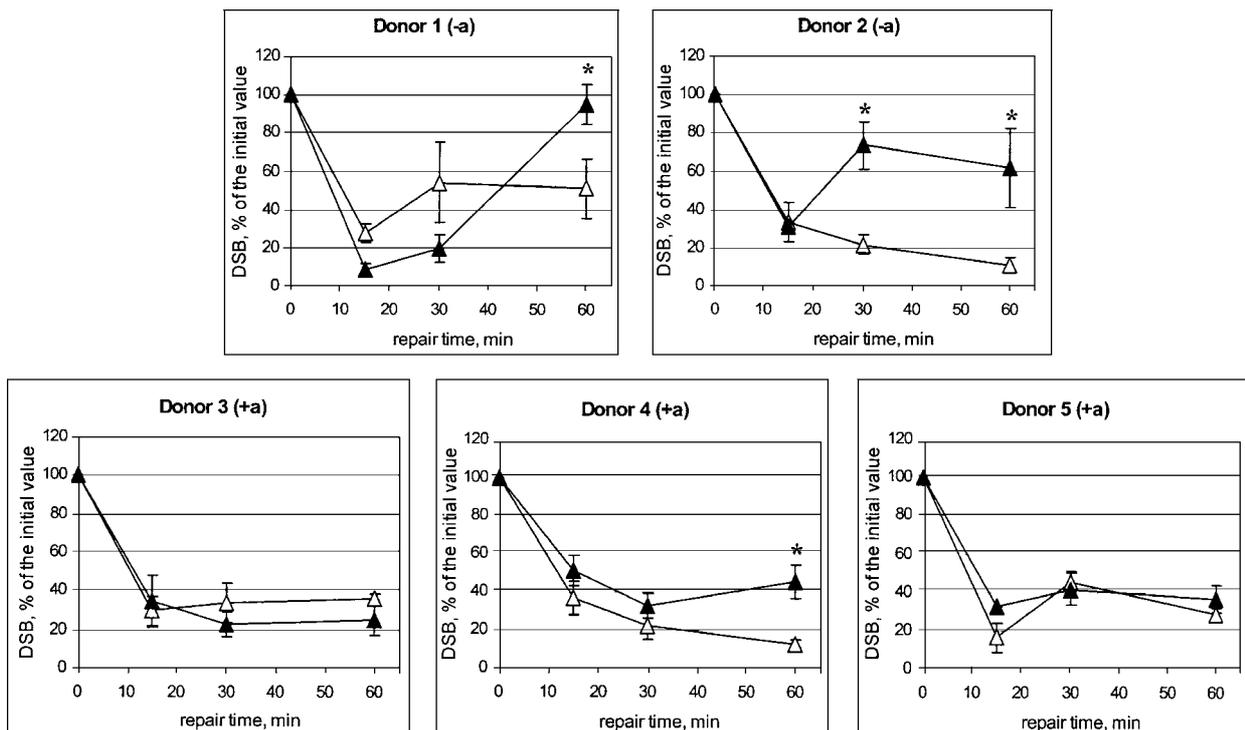
Figure 4 presents DSB rejoining after X-irradiation with 5 Gy. The kinetics of the process revealed by PFGE was different from that showed by comet assay: after 15 min of repair only 20–40% of the initial DNA breaks remained, compared to 60–90% in comet assay (Fig. 3). No significant differences in the initial DSB rejoining rate between non-adapted and adapted lymphocytes from either AR negative (–a) or positive (+a) group of donors were observed, although in donors 1, 2 and 4 a statistically significant secondary increase in DSB level appeared at 30 and/or 60 min of post-irradiation incubation.

**Discussion**

The aim of this work was to answer the question whether AR, seen in human lymphocytes as a decrease in micronuclei frequencies, would be correlated with changes in the rate of DSB rejoining after X-irradiation. The methods applied did not show any differences in the initial DSB rejoining rate either between adapted and non-adapted lymphocytes or between the cells obtained from donors able or not able to develop AR, although PFGE revealed secondary increases in DSB levels at 30 and/or 60 min of repair (Figs. 3 and 4). DSB rejoining measured by the comet assay appeared



**Fig. 3.** Comet assay: time course of DSBs rejoining after 10 Gy of X-irradiation in the adapted (5 cGy + 10 Gy, filled symbols) or non-adapted (10 Gy, open symbols) human lymphocytes. Pairs of curves show the results for individual adaptable (+a) or non-adaptable (–a) donors. Points represent mean tail moments  $\pm$  SD of three independent experiments (100 comets measured per point).



**Fig. 4.** PFGE: time course of DSBs rejoining after 5 Gy of X-irradiation in the adapted (5 cGy + 5 Gy, filled symbols) or non-adapted (5 Gy, opened symbols) human lymphocytes. Pairs of curves show the results for individual adaptable (+a) or non-adaptable (-a) donors. Points are means  $\pm$  SEM of two to five independent experiments. Asterisks indicate statistically significant differences, \*  $p < 0.05$ , with t-test.

to proceed gradually: by 15 min 60–90% of the initial DNA damage still remained. On the contrary, only 20–40% of the damage remained at this time point when DSBs were measured by PFGE. The differences might arise from the fact that in neutral comet assay individual, not fully deproteinised nucleoids are analysed [39]. Thus, alterations in chromatin structure/condensation during DNA repair [37] might affect the results. The increase in the tail moment can be caused both by SSBs that reduce DNA supercoiling, and DSBs that enable DNA strands stretching and migration during electrophoresis [17, 18, 20]. In contrast, PFGE is “insensitive” to chromatin structure: after the lysis less than 0.1% of the total cellular proteins remain associated to DNA [13, 39]. Only DNA fragments that migrate in the gel are considered as the measure of damage. From the distribution of DNA fragments in PFGE, it was possible to estimate the number of DSBs in the nuclear DNA and not only assess the total DNA content in the gel [6]. That was probably the reason why PFGE and not the comet assay revealed secondary increases in DSB levels in the adapted samples from three donors (donors 1 and 2, showing no AR, and donor 4, able to develop AR, see Fig. 4). We observed that the number of DNA fragments within the range of 200–3000 kbp was higher in these donors in the adapted lymphocytes compared to the non-adapted ones (not shown), suggesting the appearance of “rare cuts” in DNA, characteristic of early apoptosis/necrosis [1, 10].

Failure in demonstration of significant changes in the rate of DSB rejoining that correlate with the lowered micronuclei frequencies may be related to the diffe-

rences in the cell composition between the lymphocyte cultures used for the estimation of DNA break rejoining and those for the micronucleus test. In the micronucleus test, almost exclusively PHA-stimulated T-lymphocytes were analysed. For the study of DNA break rejoining, the population of white blood cells was first enriched in T- and B-lymphocytes by centrifugation on Histopaque 1077 (up to ca. 84% of lymphocytes, according to the manufacturer). After subsequent 25–27 h incubation with PHA (Fig. 1), the cell culture consisted in the majority of stimulated T-lymphocytes, contaminated, however, by significant numbers of non-stimulated B-lymphocytes, neutrophils and monocytes [16]. In response to ionising radiation, the cells could differ in propensity and timing of DNA repair and apoptosis [30, 31] and this, in turn, would influence the course of DSBs rejoining.

To the best of our knowledge, for PHA-stimulated human lymphocytes there was no report on consistent increase in DNA break rejoining rate upon adaptation. Wójcik *et al.* [32] showed an increase in DNA repair efficiency in the adapted lymphocytes subjected to X-rays. The same authors [32] noted less initial damage in the adapted lymphocytes and Gajendiran *et al.* [5] demonstrated a similar effect in the adapted lymphocytes treated with a neutron beam. However, these changes did not result in the reduction in the chromosomal aberration frequencies [32]. The analysis of comet distributions by Wójcik *et al.* [32] revealed the presence of a slowly repairing subpopulation of the cells in the non-adapted lymphocytes and lack of such a subpopulation in the adapted ones. They suggest that

the slowly repairing cells were quiescent G<sub>0</sub> lymphocytes which were removed from the adapted cell population, probably by apoptotic-like process. We have also analysed the distributions of various comet parameters in all cell samples and at all repair intervals, but we did not find any lymphocyte subpopulation with slower repair rate (not shown). The only sign of the possible apoptosis/necrosis process observed, was a secondary increase in DSB levels after 30 min and/or 60 min after X-irradiation in the adapted lymphocytes of three donors (see above in the Discussion section). Taken together, the use of highly purified T-lymphocyte cultures is required to ascertain whether the AR is related to the acceleration of DNA break rejoining in these cells.

Enhanced fidelity of DNA rejoining can be another mechanism of AR, possibly complementary to the repair rate acceleration (found in some cell lines) and decreased apoptosis. According to Sasaki *et al.* [23] a feedback signalling pathway involving protein kinase C (PKC), p38 mitogen activated protein kinase (p38 MAPK) and phospholipase C (PLC) would mediate AR by channelling the radiation-induced DSBs into an adaptive legitimate repair pathway and turning off the signals to illegitimate repair and apoptosis. A crucial role in this signalling pathway was ascribed to p53 protein [12, 23]. Increased accuracy of DNA rejoining after priming with low doses of ionising radiation was demonstrated in several human tumour cell lines [11] as well as in human lymphoblasts [27].

One more possibility that can be considered in explanation of the mechanism of AR is chromatin conformation change. It was shown that in more condensed chromatin of centromeres [21] and of G<sub>2</sub>-phase chromatin [9] the repair efficiency and fidelity of radiation-induced DSBs was enhanced. The explanation could be that the condensed chromatin state limits the mobility of break ends, thus increasing the probability for correct rejoining. Also probable is that such alteration in chromatin structure can bring about steric hindrance to the transcription machinery and thus protect against fixation of DNA damage, leaving time for repair [29]. We have indeed observed an increased chromatin condensation in nucleoids obtained from human lymphocytes primed with hydrogen peroxide or X-rays. This property was preserved in nucleoids from the primed cells that received the challenge dose of 1.5 Gy of X-irradiation and showed AR [37].

In conclusion, our results do not support the hypothesis that an accelerated DSB rejoining underlines the cytogenetic AR. We suggest that the mechanism of AR may involve an enhanced fidelity of DNA repair or/and a decreased damage fixation. Still, an enhanced DNA break rejoining rate in the adapted lymphocytes should not be excluded: in the not fully homogenous lymphocyte culture it could be masked by different DNA repair rates of the cell subpopulations, as well as by the interference from apoptosis/necrosis processes.

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