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ORIGINAL PAPER

DNA damage in subpopulations of human lymphocytes irradiated with doses in the range of 0–1 Gy of X-radiation

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Abstract. We compared three methods usually applied in biological dosimetry for estimation of radiation-induced DNA damage in human T and B lymphocytes: alkaline comet assay, micronucleus (MN) test and formation of histone γ-H2AX foci. Human peripheral blood lymphocytes were fractionated using T cells and B cells isolation kits. Cells were irradiated with doses in the range of 0–1 Gy of X-rays. Induction of DNA damage was assessed by the standard alkaline comet assay, MN test and histone γH2AX foci immunofluorescence assay. Notwithstanding different end-points measured by the applied methods, all tests revealed a similar induction of DNA damage in B lymphocytes as compared with T lymphocytes. The results indicated that all three tests detect DNA damage with similar sensitivity, the lowest dose being approximately 0.3 Gy. The difference between irradiated and control cells was expressed as the ratio of the value obtained for irradiated cells (1 Gy) to that for control cells. The highest ratio was obtained for formation of γH2AX foci and was 6.2 for T and 13.8 for B lymphocytes, whereas those for comet assay and micronucleus test were 3.5; 3.6 and 5.6; 4.8, respectively.

Key words: alkaline comet assay • biodosimetry • micronucleus test • histone γH2AX foci immunofluorescence assay • T lymphocytes • B lymphocytes • ionizing radiation • DNA damage

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Introduction

Deleterious lesions directly or indirectly induced by ionizing radiation are generated in proportion to the absorbed radiation dose. Hence, biological dosimetry can be based on measuring specific DNA lesions or the ensuing cytogenetic damage (see [10] for review). Depending on the aim, early or late time range of examination may be advantageous. Usually, however, time between irradiation and damage estimation may take several hours in the case of radiation accidents. Therefore, rapidly repaired DNA single strand breaks (SSB) usually are not suitable for dosimetric purposes. In contrast, SSB may be a measure of damage in the case of chronic exposure. DNA double strand breaks (DSB) are repaired with longer half-times and may last as long as 24 h. Far better end-points are chromosomal aberrations, especially dicentrics which are specific for ionizing radiation, or micronuclei, which, however, can also be formed by other DNA-damaging agents. Nevertheless, determination of chromosomal aberration frequency is too time-consuming and not cost-effective enough to be applied for dose estimation in numerous subjects.

A recently discovered basis for direct DSB estimation in single cells is generation of discrete foci containing phosphorylated histone H2AX (γ-H2AX) in

irradiated cells (review in [15]). Furthermore, there is an approximately 1:1 relation of foci to the calculated number of DSB for a given radiation dose [11, 14]. A confounding factor here is that such foci may occasionally form during DNA replication due to collapsed replication forks [15]. The "repair foci" are now an acknowledged marker of DSB, however, the kinetics of their disappearance is different from that of DSB rejoining. On the other hand, they are an excellent and sensitive marker of residual DNA damage.

Peripheral blood lymphocytes are the most popular subject of dosimetric measurements, thus constituting everybody's personal dosimeter always ready to be used. One practical question is to what extent the differential radiosensitivity of lymphocyte subfractions and the individual variability of their proportions affect dose estimation with the use of standard tests. In this report we checked how T and B lymphocytes differ in radiation response estimated on the basis of various end-points. In parallel, we aimed at attaining a maximal sensitivity of each test. We used the alkaline comet assay as a reference test to characterize radiosensitivity differences between B and T lymphocytes and micronuclei frequency and γ-H2AX foci as potentially suitable for screening human populations chronically exposed to low radiation doses.

Materials and methods

Lymphocyte isolation and irradiation

Human peripheral blood (450 ml) was collected from a healthy donor by venipuncture and used for lymphocyte isolation by density gradient centrifugation. Fractionation was carried out with the use of Pan T Cell Isolation Kit II and B Cell Isolation Kit II (Miltenyi Biotec), according to the manufacturer's recommendations. 2–3 × 10^6 cells/ml were placed in the RPMI 1640 medium (Gibco) supplemented with 20% foetal calf serum (FCS). Homogeneity of isolated cell subpopulations was 97–99%, as estimated by flow cytometry.

Cells were irradiated with doses in the range of of X-rays (0–1 Gy) using an ANDREX defectoscope (Holger Andreasen, Denmark) operating at 200 kV and 5 mA, with 3 mm Al filtration, at a dose rate of 1.48 Gy/min.

Alkaline comet assay

The comet assay (single cell gel electrophoresis) was performed as described in [16]. Briefly, an aliquot of cell suspension was mixed with an equal volume of 2% low melting point agarose (Type VII, Sigma), put on a microscope slide pre-coated with 0.5% regular agarose (Type I-A, Sigma) and left on ice. After agarose solidification slides were irradiated on ice with a dose of 2 Gy of 200 keV X-rays, the slides were then immersed in a cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris and 1% Triton X-100, pH 10) for 1 h. After lysis, the slides were placed on a horizontal gel electrophoresis unit filled with fresh electrophoretic buffer (1 mM Na_2EDTA and 300 mM NaOH) and allowed to stay in this buffer for 40 min for DNA unwinding. Next, electrophoresis was performed (1.2 V/cm^{-1}, 30 min, 10°C), the slides were washed with 0.4 M Tris, pH 7.5 (3 × 5 min) and stained with DAPI, 50 μl, (1 μg/ml^{-1}). Pictures of 75 randomly selected comets per slide were captured at 200× magnification using a fluorescent microscope (Labophot-2, Nikon). Image analysis of data was performed by the Comet v.3.0 software (Kinetic Imaging Ltd., Liverpool, UK). Tail moment was taken as a measure of DNA damage.

The same test was applied for the measurement of base damage. Incubation of irradiated cells with the formamidopyrimidine glycosylase (FPG, BioLabs) was carried out as described in [7]. Briefly, after lysis, the slides were washed 3 × 5 min with a buffer (40 mM Hepes, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8) at 4°C. Further, 50 μl of FPG solution (4.8 × 10^{-2} U) in the buffer was placed on each slide, covered with a cover glass and incubated for 30 min in a tight box at 37°C.

Micronucleus test

The micronucleus test was performed according to the method of Fenech and Morley [4] with slight modifications.

Following irradiation, 0.5 ml of whole blood were re-suspended in 5 ml of the culture medium and phytohaemaglutinin A (PHA) or pokeweed mitogen (PWM) were added to the final concentration 3 μg/ml and 2.5 μg/ml, respectively, to stimulate B or T lymphocytes [5, 8]. After 48 h (lymphocytes T, PHA) or 72 h (lymphocytes B, PWM) cytochalasin B (Sigma) was added to a final concentration of 5.6 μg/ml. After 24 h incubation, the cells were centrifuged and carefully resuspended in a hypotonic solution, 0.125 M KCl, added dropwise at room temperature. The cells were kept for 5 min at room temperature, centrifuged again and the pellet was fixed in a cold fixing solution, 0.9% NaCl/methanol/acetic acid, 12:13:3, for 10 min. The cells were then centrifuged and fixed again in a cold acetic acid/methanol 1:4 solution. After fixing, the cells were dropped on microscopic slides, air-dried and stained with 10% Giemsa (Sigma). Coded slides were scored at 200× magnification. The frequency of micronuclei in binucleated cells with well-preserved cytoplasm was determined, as proposed by Almassy *et al*. [1]. Only rounded bodies approximately one-fifth to one-tenth of the size of the main nucleus were scored as micronuclei. One thousand of binuclear cells were analysed per sample.

Histone H2AX immunofluorescence

Formation of γH2AX foci was assessed by immunofluorescence according to [3]. In brief, lymphocytes were irradiated and the cells incubated for an appropriate period of time and settled on polylysine-treated (0.01%, Sigma) microscopic slides. After 15 min incubation, the slides were washed with cold PBS and fixed with cold methanol (–20°C) for 5 min in a freezer. After fixation, the cells were re-hydrated by incubating in PBS for

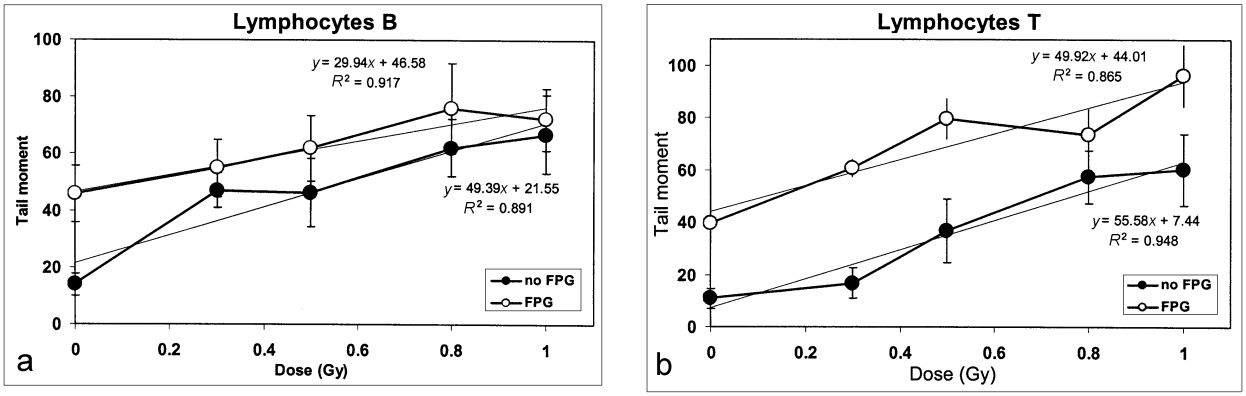

Fig. 1. Dose-effect relationship for SSB (no FPG) and base damage (FPG) recognized by the repair glycosylase, FPG, measured in lymphocyte subfractions: a – lymphocytes B; b – lymphocytes T. Mean ± SD of 2–3 experiments. R^2 – correlation factor.

30 min with three changes of PBS. Non-specific binding was blocked by incubation for 1 h at room temperature in a blocking buffer (2% BSA, 10% skimmed milk powder in KCMT buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.1% Triton X-100, pH 8.0) and a primary antibody was added (anti-γH2AX rabbit polyclonal, Upstate, UK) for 2 h at room temperature. The slides were then washed in the KCMT buffer using three washes of 15 min each on a platform shaker. Secondary fluorescent antibody (AlexaFluor 488 conjugated goat anti-rabbit IgG, Molecular Probes) in the blocking buffer was then added and slides were incubated for 1 h at room temperature in the dark. After washing, slides were mounted in a Vectashield mounting medium with DAPI, VectorLabs) and sealed with clear nail varnish. Images of at least 25 nuclei per slide were captured on the next day and the number of γH2AX foci was counted for each nucleus.

Results and discussion

Alkaline comet assay

The alkaline comet assay detects DSB, SSB and alkali-labile sites. Furthermore, incubation of irradiated cells with FPG allows to assess oxidative DNA base damage because it detects 8-OH guanine and other oxidatively damaged purines. Oxidative base damage is generated in irradiated cells in less than 50% of the number than the sum of DSB and SSB [9]. FPG sensitive sites are generated by γ-rays with a yield of 0.044 per 10^6 base pairs per Gy. For the SSB (including DSB and alkali-labile sites), the yield is 0.123 per 10^6 base pairs per Gy [9]. As shown below, the use of FPG in the alkaline comet assay for biodosimetric purposes does not give any advantage in sensitivity of the comet assay, as indicated by the dose-effect relationships determined for both types of lesions.

Figures 1a and 1b shows the results obtained by the comet assay for quiescent T and B lymphocytes. The dose-effect relationship is described by the equation $y = ax + b$, where y is the tail moment, x is the dose of X-rays in Gy and b is the tail moment determined in unirradiated cells. For lymphocytes, T the relationship of the sum of DSB and SSB vs. dose is $y = 55.58x + 7.44$, whereas that for base damage, $y = 49.92x + 44.01$. The respective equations for lymphocytes B are $y = 49.39x + 21.55$; $y = 29.94x + 46.58$. When measured by SSB induction expressed as tail moment, the radiosensitivity of lymphocytes B is slightly higher than that of lymphocytes T (difference not statistically significant, Student's t-test, p = 0.09).

The ratio of the value obtained in the alkaline comet assay for irradiated (1 Gy) lymphocytes to that for control cells was 5.6 for lymphocytes T and 4.8 for lymphocytes B.

Micronuclei frequency

MN test detects chromosomal damage generated due to the presence of chromosome fragments resulting from incomplete DSB repair or misrepair. The results obtained for lymphocyte subfractions are presented in Figs. 2 and 3. When MN frequency is the end-point, the difference in radiation sensitivity between lymphocytes B T is much less evident than in the case of other criteria of the cellular response, like proliferation. The difference degree depends on the dose, as noted by Wuttke *et al.* [17] who also observed that proliferation of lymphocytes B and T is differentially affected by irradiation and of considerable importance for micronuclei expression.

The sensitivity of the test was satisfactory, the dose of 0.3 Gy gave a marked increase in micronuclei frequency

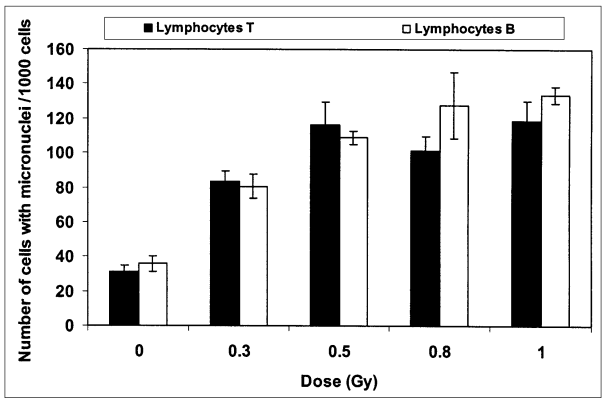

Fig. 2. Number of cells with micronuclei per 1000 cells in lymphocyte subfractions irradiated with doses in the range of 0–1 Gy of X-radiation. Mean ± SD of 2–3 experiments.

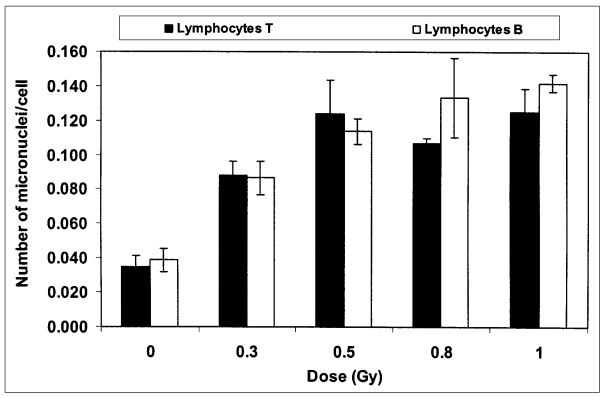

Fig. 3. Micronuclei frequency per cell in lymphocyte subfractions irradiated with doses in the range of 0–1 Gy of X-radiation. Mean ± SD of 2–3 experiments.

over the control value (Student's t-test, $p < 0.002$ and $p < 0.021$ for number of cells with micronuclei/1000 cells for lymphocytes T and $p < 0.001$ and $p < 0.035$ for number of micronuclei/cell for lymphocytes B).

The ratio of the value obtained for irradiated (1 Gy) lymphocytes to that for control cells was 3.5 for lymphocytes B and 3.6 for lymphocytes T.

Histone γH2AX foci

Generation of foci containing phosphorylated histone H2AX (γ-H2AX) in X or γ-irradiated cells is the basis of a sensitive test to asses DSB presence in the nuclear DNA. It was applied to lymphocyte subfractions and the results are shown in Figs. 4 and 5. The data indicate that DSB induction by X-irradiation with graded doses is similar in lymphocytes B and T and can be described by the equations $y = 11.04x + 0.79$ ($R^2 = 0.999$) and $y = 8.87x + 2.29$ ($R^2 = 0.979$) where y is foci frequency and x is the dose of X-rays in Gy. Differences between the foci frequencies in lymphocytes B and T are not statistically significant. The ratio of the value obtained for irradiated (1 Gy) lymphocytes B or T to that for control cells is 13.8 for lymphocytes B and 6.20 for lymphocytes T. The rate of foci disappearance differs in lymphocytes B and T; the differences are statistically significant after 1 and 3 h incubation (Fig. 6). Nevertheless, the end level (5 h repair time) is identical. The rate of foci disappearance is slower than that of DSB rejoining [2] and this may be an advantage in biodosimetic tests.

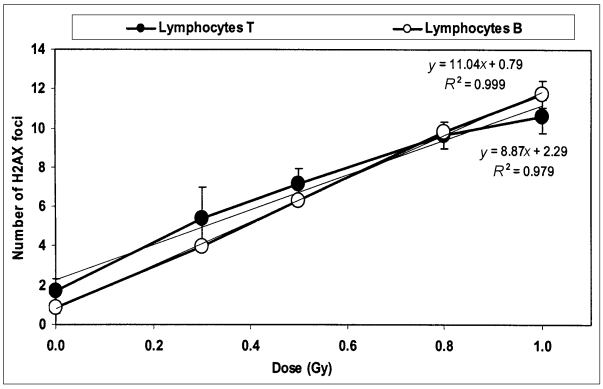

Fig. 4. The dose-effect relationship for lymphocytes T and B with initial DSB as end-point, estimated from the mean number of γH2AX foci per cell 15 min after X-irradiation. Mean ± SD of 2–3 experiments.

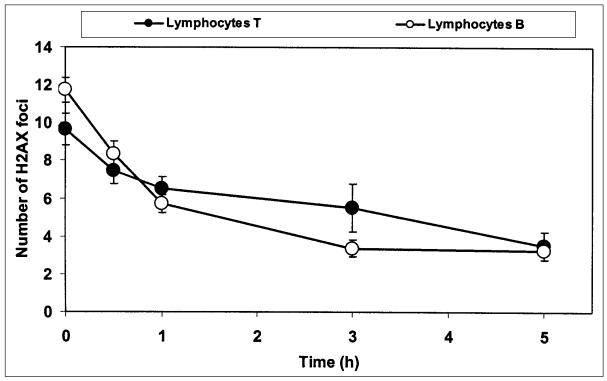

Fig. 5. The repair of DSB in lymphocytes T and B irradiated with 1 Gy of X-rays, as estimated from the mean number of γH2AX foci per cell. Mean ± SD of 2–3 experiments.

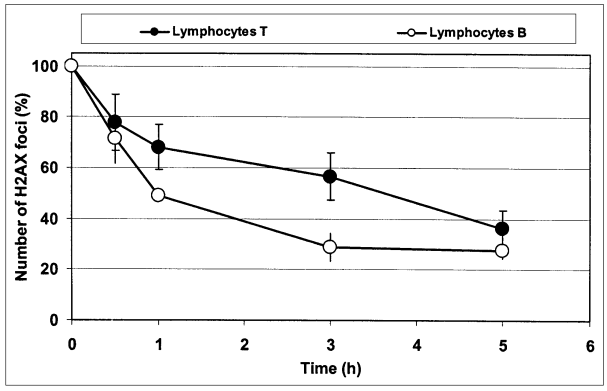

Fig. 6. The repair of DSB in T and B lymphocytes irradiated with 1 Gy of X-rays, expressed as percent of the initial number of γH2AX foci. Mean ± SD of 2–3 experiments.

In summary, our report indicates that the γH2AX foci assay is an advantageous alternative for MN test and comet assay for radiation dose estimation in ionizing radiation-exposed individuals. Furthermore, in spite of considerable individual variation in the proportion of various lymphocyte subpopulations and their differential radiosensitivity reported by other authors [6, 12, 13], application of the standard biodosimetry tests to quiescent lymphocytes does not reveal any marked differences between B and T lymphocytes. So, the varying individual proportion of lymphocyte subpopulations does not weaken the reliability of biodosimetric assessment of the dose for total lymphocytes from peripheral blood as applied for screening human populations chronically exposed to low doses of ionizing radiation.

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