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Validation of the micronucleus-centromere assay for biological dosimetry*

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Abstract

The micronucleus assay is frequently used for purposes of biological dosimetry. Due to high interindividual variability in the spontaneous frequency of micronuclei, its sensitivity in the low dose region is poor. It has been suggested that this problem can be mitigated by selectively analyzing the frequency of those micronuclei which contain only acentric fragments. Using a pan-centromeric FISH probe we have studied the dose dependence of micronuclei with centromeres in peripheral lymphocytes of human donors. In contrast to previous publications, our approach is based on determining the relative frequency of micronuclei with and without centromeric signals. Our results confirm previous observations that in the low dose range of ionizing radiation, the micronucleus-centromere assay is more sensitive than the conventional micronucleus test.

INTRODUCTION

Biological dosimetry is a method by which the level of radiation exposure is estimated on the basis of radiation-induced changes in the human body. The most frequently applied method relies on measuring the frequency of dicentric chromosomes in lymphocytes of the peripheral blood (Bender *et al.*, 1988). A disadvantage of the dicentric assay is the relatively high skill and the time required for a precise analysis. An alternative method is the micronucleus (MN) test (Müller and Streffer, 1994), which is less time consuming. However, its disadvantage is a weak sensitivity at the low dose range of ionizing radiation, resulting predominantly from a high individual variability of the spontaneous micronucleus frequency (Gantenberg *et al*., 1991; Thierens *et al*., 1991).

Norppa *et al*. (1993) were the first to suggest that the sensitivity of the MN assay could be enhanced by discriminating between spontaneous and clastogen-induced micronuclei. The discrimination was based on the observation that spontaneous micronuclei contain predominantly whole chromosomes, whereas clastogen-induced micronuclei contain acentric fragments. Vral *et al*. (1997) used *in situ* hybridization with a pancentromeric DNA probe to investigate if an increased sensitivity for detection of low radiation doses could be achieved by selectively scoring those micronuclei which did not contain centromeres. The results obtained indicated that the sensitivity of the MN assay could indeed be increased by analyzing the frequency of centromere-negative micronuclei.

The aim of the present study was to investigate whether and to what extent an enhanced sensitivity to ionizing radiation could be achieved in our laboratory by combining the micronucleus assay with centromere detection. Although our results were not as clear-cut as those of Vral *et al*. (1997), they confirm that the micronucleus-centromere assay is more sensitive at the low dose range of ionizing radiation than the conventional micronucleus test.

MATERIAL AND METHODS

Lymphocyte culture and irradiation

Samples of heparinized blood were drawn by venipuncture from healthy donors aged between 24 and 55 years, aliquoted into 1-ml Eppendorf tubes and irradiated at room temperature in a 60-Co facility with doses of 0, 0.1, 0.25, 0.5 and 1 Gy, at a dose rate of 0.25 Gy/min. Thereafter, the blood was kept at 37°C for at least 1 h. Subsequently 0.5 ml of each sample was added to 4.5 ml of pre-warmed complete medium consisting of 80% RPMI 1640 (Sigma with HEPES), 20% fetal calf serum, 15 µl/ml phytohemagglutinin (PHA; Gibco, M-form), and 5 µl/ml antibiotic/antimycotic solution (Gibco). All cultures were set up in duplicate. Cytochalasin B (Sigma, final concentration: 5.6 µg/ml) was added after 44 h of culture. After a total culture time of 72 h, the cells were centrifuged and resuspended in 0.14 M KCl. Following a 15-min incubation time at room temperature, the cells were centrifuged again and fixed first in fixative 1 (methanol:0.9% NaCl:acetic acid, 12:13:3) and subsequently in fixative 2 (methanol:acetic acid, 4:1). The cells were washed in fixative 2 until the supernatant was clear, and were dropped onto clean, dry slides.

Slides for the estimation of micronucleus frequency were stained with Giemsa. Slides for centromere analysis were stored at -20°C and subsequently processed as described below.

In situ hybridization

Prior to denaturation, slides were treated with RNase (100 µg/ml 2x SSC at 37°C for 60 min), pepsin (50 µg/ml 0.01 HCl at 37°C for 10 min) and a 1% solution of formaldehyde (in PBS with 50 mM $MgCl_2$, 10 min at room temperature). Following dehydration in an alcohol series, slides were denatured for 30 min at 70°C in 90% formamide 2x SSC and dehydrated in an ice-cold alcohol series. Per slide, 15 µl centromeric DNA probe (all human centromeres, ONCOR, digoxigenin labelled) were denatured for 10 min at 70°C and applied on each slide. Cover slips were sealed with rubber cement and the slides kept at 37°C in a humidified chamber overnight. Next day the slides were washed at 37°C with 50% formamide (3 x 5 min) and 2x SSC (3 x 5 min). The signal detection was performed by incubation with 1) mouse anti-digoxigenin (Sigma), 2) sheep anti-mouse, digoxigenin-labelled (Boehringer), and 3) FITC-labelled sheep anti-digoxigenin (Boehringer). Between the incubations the slides were washed with a 4x SSC/0.05% Tween 20 buffer. Following the final wash, slides were mounted in DABCO (Sigma) antifade containing 1.5 µl/ml propidium iodide counterstain. Slides were analyzed under a fluorescence microscope equipped with filters for FITC and TRITC.

Scoring and statistical analysis

For each treatment group the micronucleus frequency was estimated in at least 1000 Giemsa stained, binucleated cells. Results obtained by different scorers were pooled. The frequency of micronuclei with/without centromeric signals was estimated in 50-100 micronuclei per treatment group. The analysis was restricted to micronuclei located in the vicinity of two main nuclei. The Student two-tailed *t*-test was used for the statistical analysis.

RESULTS AND DISCUSSION

The dose response curve for micronuclei in binucleated cells is shown in Figure 1. Due to the high interindividual variability, a statistically significant increase in the micronucleus frequency is seen only after a dose of 0.5 Gy. The highest frequency of micronuclei was observed in lymphocytes of the oldest donor (top curve of Figure 1), the lowest frequency was seen in lymphocytes of the youngest donor (bottom curve). This result correlates well with the observations of others, that the frequency of micronuclei increases with age (Thierens *et al.*, 1991). A high degree of variability among different donors with respect to the dose response curves has also been reported (Gantenberg *et al.*, 1991; Thierens *et al.*, 1991).

In accordance with the assumption that ionizing radiation-induced micronuclei contain predominantly acentric fragments, the relative frequency of micronuclei containing centromeres decreased with radiation dose (Figure 2). Similar results were observed by others, not only in human lymphocytes (Huber *et al.*, 1996; Darroudi *et al.*, 1996; Vral *et al.*, 1997) but also in other cell lines (Darroudi *et al.*, 1996; Salassidis *et al.*, 1992; Weissenborn and Streffer, 1991). Due to a relatively low degree of interindividual variability, a statistically significant difference in the frequency of MNC+ was observed between 0 and 0.25 Gy. It is thus evident that the micronucleus-centromere assay is more sensitive for the detection of exposure to ionizing radiation than the conventional micronucleus assay.

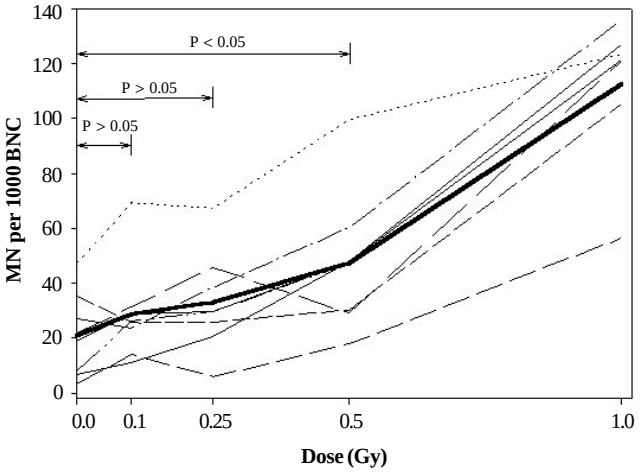

Figure 1 - Individual and average (thick line) dose response curves of micronuclei (MN) in binucleated cells (BNC). Number of donors: 8.

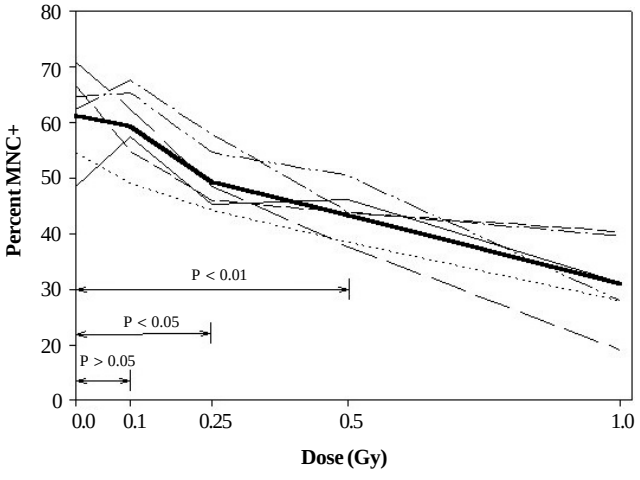

Figure 2 - Individual and average (thick line) dose response curves for the percent of centromere-containing micronuclei (MNC+) in lymphocytes of 5 donors.

The frequency of MNC+ in unexposed micronuclei is a matter of controversy. While Vral *et al.* (1997) observed on average 71.2% of MNC+ in control lymphocytes of seven donors, Darroudi *et al.* (1996) and Huber *et al.* (1996) reported values of between 50 and 60%. The reason for the difference is not clear, since all authors used *in situ* hybridization probes to detect the centromeres. It is obvious that the sensitivity of the micronucleus-centromere assay to ionizing radiation will be greatest with a maximally high percentage of MNC+ in the control cells. We have observed on average 61.2% MNC+.

Vral *et al.* (1997) analyzed the frequency of MNC+ in a given number of binucleated cells. We chose to analyze the percentage of MNC+ in a given number of micronuclei. Our approach arose from the fact that due to the RNAse treatment, the cytoplasm is no longer visible on hybridized slides. Thus, the identification of binucleated cells is difficult, especially if they contain no micronuclei. Therefore, we decided to score a certain number of micronuclei and to express the results as the relative frequency of MNC+. Our results show that this approach is equally well suited for the purpose of biological dosimetry.

In conclusion, our results indicate that, at least under *in vitro* conditions, the micronucleus-centromere assay is more sensitive for the detection of an exposure to ionizing radiation than the conventional scoring of micronuclei in binucleated cells.

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RESUMO

O teste de micronúcleo é freqüentemente usado em dosimetria biológica. Devido à alta variabilidade interindividual na freqüência espontânea de micronúcleos, sua sensibilidade em pequenas doses é baixa. Tem sido sugerido que este problema pode ser atenuado analisando-se seletivamente a freqüência daqueles micronúcleos que contêm apenas fragmentos acêntricos. Usando uma sonda de FISH pan-centromérica, nós estudamos a dependência de micronúcleos com centrômeros em linfócitos periféricos de doadores humanos, com relação à dose. Ao contrário de publicações anteriores, nossa abordagem se baseia na determinação da freqüência relativa de micronúcleos com e sem sinais centroméricos. Nossos resultados confirmam observações anteriores de que, na faixa de baixas doses de radiação ionizante, o teste de micronúcleo-centrômero é mais sensível do que o teste de micronúcleo convencional.

REFERENCES

Bender, M.A., Awa, A.A., Brooks, A.L., Evans, H.J., Groer, P.G., Littlefield, L.G., Pereira, C., Preston, R.J. and **Wachholz, B.W.** (1988). Current status of cytogenetic procedures to detect and quantify previous exposures to radiation. *Mutat. Res. 196*: 103-159.

Darroudi, F., Meijers, M., Hadjidekova, V. and **Natarajan, A.T.** (1996). Detection of aneugenic and clastogenic potential of X-rays, directly and indirectly acting chemicals in human hepatoma (Hep G2) and peripheral blood lymphocytes, using the micronucleus assay and fluorescent *in situ* hybridization with a DNA centromeric probe. *Mutagenesis 11*: 425-433.

Gantenberg, H.-W., Wuttke K., Streffer C. and **Müller, W.-U.** (1991). Micronuclei in human lymphocytes irradiated *in vitro* or *in vivo*. *Radiat. Res. 128*: 276-281.

Huber, R., Salassidis, K., Braselmann, H. and **Bauchinger, M.** (1996). Detection of centromeres in vinblastine- and radiation-induced micronuclei of human lymphocytes using FISH with an alpha satellite pancentromeric DNA probe. *Environ. Mol. Mutagen*. *27*: 105-109.

Müller, W.-U. and **Streffer, C.** (1994). Micronucleus assays. *Adv. Mutagen. Res*. *5*: 1-134.

Norppa, H., Renzi, L. and **Lindholm, C.** (1993). Detection of whole chromosomes in micronuclei of cytokinesis-blocked human lymphocytes by antikinetochore staining and *in situ* hybridization. *Mutagenesis 8*: 519-525.

Salassidis, K., Huber, R., Zitzelsberger, H. and **Bauchinger, M.** (1992). Centromere detection in vinblastine- and radiation-induced micronuclei of cytokinesis-blocked mouse cells by using *in situ* hybridization with a mouse gamma (major) satellite DNA probe. *Environ. Mol. Mutagen*. *19*: 1-6.

Thierens, H., Vral, A. and **Ridder de, L.** (1991). Biological dosimetry using the micronucleus assay for lymphocytes: interindividual differences in dose response. *Health Phys*. *61*: 623-630.

Vral, A., Thierens H. and **Ridder de, L.** (1997). *In vitro* micronucleus-centromere assay to detect radiation-damage induced by low doses in human lymphocytes. *Int. J. Radiat. Biol*. *71*: 61-68.

Weissenborn, U. and **Streffer, C.** (1991). Micronuclei with kinetochores in human melanoma cells and rectal carcinomas. *Int. J. Radiat. Biol*. *59*: 373-383.

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