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Simple Fluorescent Micronucleus Assay-combination of Hg-banding Technique and *In situ* Hybridisation

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Recent development in cytogenetic and molecular techniques, particularly combination of cytochalasin B method and fluorescence *In situ* hybridisation (FISH) now allow sensitive identification of clastogenic versus aneugenic events. To simplify the fluorescence micronucleus assay a technique based on combination of hypotonic banding and *In situ* hybridisation with pancentromeric probe (SO-αAllCen) is described. This work demonstrates more comfortable, easier and faster way to perform fluorescent micronucleus test.

Key words: micronucleus, FISH, hypotonic digestion

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Introduction

The two most common *in vivo* cytogenetic assays, the micronucleus (MN) and metaphase aberration assays are inconsistent among countries worldwide. Since there is a mechanistic link between chromosome breaks and MN, it is generally agreed that these two endpoints detect similar types of chromosomal damages, with the possible exception of numerical chromosome changes that would not be detected in the standard metaphase analysis (Stopper and Muller, 1997). Scoring of MN in cytokinesis-blocked human peripheral lymphocytes frequently used as an alternative method in biological dosimetry (Hall and Wells, 1988; Bauchinger and Braselman 1990). This test is also suitable in studying gentotoxicity of xenobiotcs in mammalian cells systems (Matsuoka *et al.*,1993) and is applied in human biomonitoring (Fenech, 1993). MN are chromatin-containing structures besides the main nuclei and arise from either fragments or whole chromosomes excluded from the daughter nuclei during cell division. Micronuclei arise from chromosomal damage that results from either chromosome breaks or spindle damage and they have been considered to represent both markers of exposure and potential indicators of biological response to genotoxic agents. MN are scored in lymphocytes blocked in their second interphase by addition of Cyochalasin-B. After Giemsa or fluorescent staining these cells are recognized as binucleate cells (B.C.). The frequency of MN in these cells can be quantified microscopically or with flow cytometry (Fenech and Morley, 1985; Nusse and Kramer, 1984). Introduction of the fluorescent *in situ* hybridisation (FISH) technique allows studying the nature of spontaneous and induced MN. All fluorescent MN procedures published in literature up to now use pepsin to permeate the membranes (Surrales *et al.*,1995; Elhajouji *et al.*, 1995; Boei and Natarajan, 1996, Van Hummelen *et al.*, 1995). Such digestion treatment may cause troubleshooting like unstiking or overdigestion of cells.

To simplify the FISH micronucleus procedure for specifying labeling of chromosome regions with repetitive DNA probes a technique based on combination of hypotonic banding and hybridisation with alphoid DNA probe is described. This work demonstrates that the modification of the technique more comfortable, faster and easier to perform.

Materials and Methods

The research work was conducted last year in order to apply fluorescent micronucleus assay in human biomonitoring studies. Peripheral blood used in experiment was obtained from one healthy, non-smoking young male donor (volunteer). Aliquot of heparinized whole blood were put into sterile plastic test-tube, placed in a plexiglas container 15x15 cm^2 and irradiated using ^{60}Co γ-ray source. The radiation dose employed was 2 Gy, the dose-rate 0.45 Gy min^{-1}, the dimensions of radiation field were 20x20 cm^2, and the distance from the source 74 cm. Blood samples were irradiated at room temperature, and as unirradiated samples were set up in cultures one hour after irradiation.

Blood culture: Cultures containing 0.5 ml of the whole blood, 8ml of RPMI-1640 medium, 15% of calf serum, (2mM) L-glutamine and 2.4μg ml^{-1} phytohemaglutinin were cultured at 37°C for 72 hours. To block interphase cell-cytokinesis, Cytochalasin B (6μg ml^{-1}) was added at 44hr. into the culture tubes.

Preparation: Seventy-two hours after culture initiation, cells were washed with 0.9% NaCl; collected by centrifugation and treated 15 min. with hypotonic solution at 37°C. Hypotonic solution was consisted of 0.56 and 0.9% NaCl, respectively (mixed in equal volumes) 0.1 ml of trypsin (final concentration 0.025%) according to method of Novak *et al.*, 1994. Cell suspension was prefixed in methanol/acetic acid 3:1, washed three times with fixative and dropped into clean glass slides. Air-dried slides were hybridised within 2 weeks of preparation. The incidence of spontaneously occurring as well as radiation-induced MN was scored.

Probe: A digogsigenin labelled α-satellite DNA probe (Oncor Inc., USA) hybridising exclusively to the centromeres of all human chromosomes was used.

***In situ* hybridisation**: Subsequently slides were pre-treated with RNAse (100μg ml^{-1} in 2xSSC) for an hour and proteinase K (0.5μg ml^{-1} in 20 mMTRIS, 2mM $CaCl_2$, pH 7.5) for 15 min at 37°C.

The slides were dehydrated in an ethanol series (30, 70, 100%) for 5 min and were denatured in 70% formamide 2xSSC (saline-sodium citrate buffer), pH 7.0 at 70°C for 2 min. After denaturation the slides were dehydrated in ethanol series. Target DNA was denatured at 70°C for 5 min, placed on ice for 10 min and than applied to slides. The slides were incubated for 16 hours in moist chamber at 37°C. After post-hybridisation washes (5 min in 50% formamide, 2xSSC) and two changes in 2xSSC for 2 min each at 37°C, slides were prepared for the detection of the digogsigenin-labelled DNA probe. Subsequently slides were treated with alternating layers of digogsigenin-labelled and avidin-FITC conjugate antibodies (Oncor, Inc.) each followed by three 2 min washes in 2xSSC+0.05 Tween 20. All antibodies were successively incubated for 60 min at room temperature in a dark moist chamber. Slides were stained with antifade solution (Johnson *et al.*, 1981) containing 0.5mg ml^{-1} of propidium iodide.

Slide scoring: Slides were analysed on an Axiophot-2 fluorescence microscope equipped with filters for visualisation of FITC and PI fluorescence. MN identification and signal evaluation were performed using a 40x objective and oil-immersion 100x objective (final magnification 1250), respectively.

Results

The incidence of micronuclei was analysed in control and irradiated sample. Microscopical evaluation was performed by exciting propidium iodide at 580 nm. The incidence of

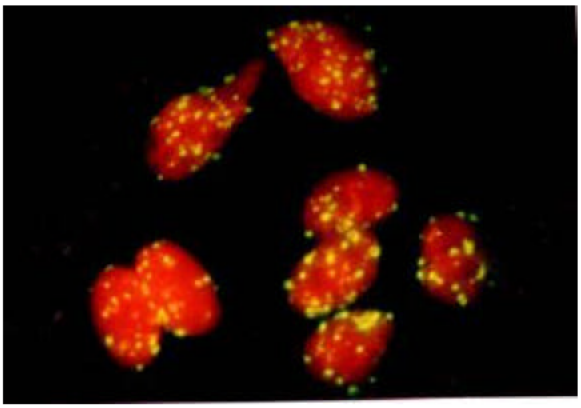

Fig. 1: Two cytokinesis-blocked binucleated lymphocytes and quadrinucleated one after *In situ* hybridisation: upper binucleated cell contains micronucleus; PI excitation

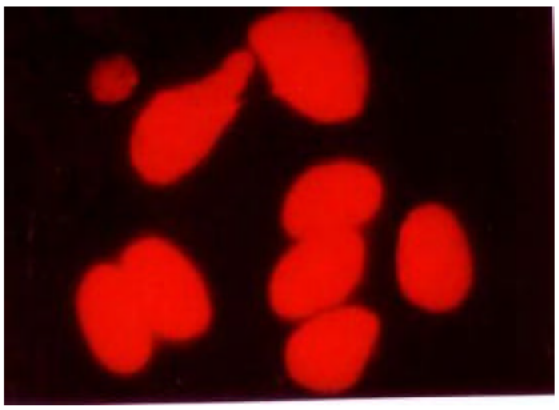

Fig. 2: Cells in Fig 1 visualised with fluorescein excitation showing many alphoid signals in the main nuclei and no signals in micronuclei.

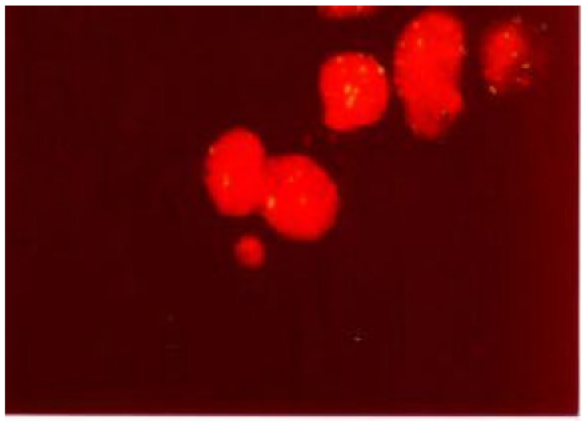

Fig. 3: Cytokinesis-blocked binucleated lymphocyte with micronucleus and one quadrinucleated cell without micronuclei after in situ hybridisation visualised with PI excitation.

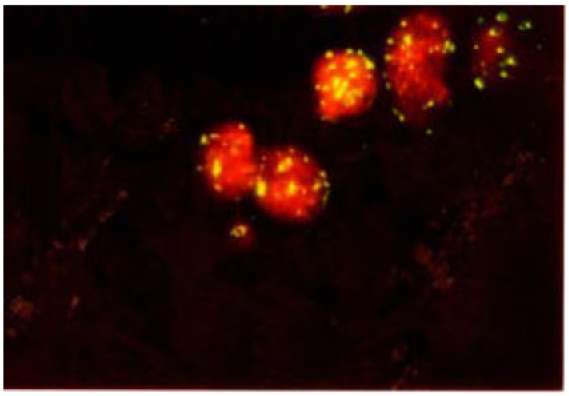

Fig. 4: Cells in Figure 3 viewing by fluorescein excitation showing centromere signals in the micronucleus.

spontaneously occurring of MN was 15 per 1000 BNC. The yield of radiation-induced MN was 224 per 1000 BNC. To evaluate the nature of MN (whether or not they possess a centromere) microscopical analysis was performed by exciting fluorescein at 530 nm. The hybridised segment appeared as yellow spots while the rest of the chromosomal DNA appeared red. MN were analysed for the presence of fluorescent signal by considering a FITC-labelled MN as a centromere-negative MN (MNC-)- Fig. 2, and a centromere-positive MN (MNC+) Fig. 4. In control sample 7 out of 15 MN were MNC+, 16 out of 224 were MNC+ in irradiated sample, respectively. The quality of signals and clarity of cells (irradiated sample) with and without signals are presented in Figs. 1-4.

Discussion

The cytokinesis-block micronuclei assay was developed to screen agents for their potential genotoxic activity. Latter, this assay is adapted for screening aneugenic activity as well. The human lymphocyte MN test is considered a well established system to detect aneuploidy-inducing agents, especially after development of FISH techniques to unequivocally distinguish the origin of the MN (Eastmond *et al.*, 1995; Natarajan *et al.*, 1996). Although there are many biomonitoring studies of populations exposed to the chemical mutagens using conventional methods, there are only a very few studies using FISH on human populations exposed to chemical mutagens. The use of DNA probes directed against repetitive sequences uniquely present in the centromeric region of the chromosome is obviously an accurate method to distinguish clastogenic from aneugenic events (Becker *et al.*, 1990; Miller *et al.*, 1991; Kirsch-Volders *et al.*, 1997). With this approach, both chromosome loss and non-disjunction are simultaneously detected in the same cell type with minimal technical artefacts, thus providing useful information upon the mechanisms of action of the aneugens. The threshold for aneuploidy-inducing chemicals can be successfully detected (Parry *et al.*, 1994). The *In vitro* cytokinesis-block MN test is therefore a very powerful tool for both the screening of mutagens and for understanding their mechanisms of action (Elhajouji *et al.*, 1995). Nevertheless, more effort towards the refinement of protocols is required inorder to improve the accuracy of the method and find out more significance of the interaction of aneugenic chemicals with cellular targets. The

method described in this paper simplifies FISH-micronucleus procedure. Hypotonic digestion of the binucleated cells with trypsin rapidly enhances the permeability of plasma membrane, making unnecessarily all additional digestion with pepsin, as well as additional post-fixations with formaldehyde. The number of washes and dehydrations are reduced; the final results of hybridisation are getting faster. Hypotonic digestion with trypsin enables optimal penetration of DNA probe to target DNA resulting in high efficiency of hybridisation.

Since fluorescent MN assay is a method of choice for genetic monitoring of population exposed to many hazardous agents, which may operate via different mechanisms where it is necessary to detect the sum of genetic damage, it is very important to handle with fast and precise technique.

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