LEUKOCYTE APOPTOSIS AND MICRONUCLEI INDUCTION IN INDIVIDUALS WITH VARYING SENSITIVITY TO IONISING RADIATION

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ABSTRACT
The identification of radiosensitive individuals using test doses of radiation in vitro of blood samples allows one to manage the detriment of exposure to ionising radiation of members of the public and radiation workers. Current methodologies allow one to quantify radiation-induced apoptosis in lymphocytes (Leukocyte Apoptotic Assay – LAA), an endpoint controlled by molecular pathways as well as micronuclei induction, a cytogenetic type of measurement. In this study, blood from 10 donors was used to analyse radiation-induced apoptosis and micronuclei formations in each instance. Micronuclei formations in isolated lymphocytes were numerated in binucleated cells 72 hours after in vitro radiation exposure and leukocyte apoptosis was measured at 48 hours after in vitro radiation exposure. Variation in response for different individuals was noted and compared using both endpoints. However, no significant relationship could be established between the level of apoptosis and micronuclei frequency induced by a test dose of 2Gy of 6MV X-rays. The fundamental differences in these two endpoints, molecular and cytogenetic respectively, may be a possible reason for this. It is concluded that the measure of radiosensitivity of different individuals is dependent on the endpoint in question.

INTRODUCTION
Ionising radiation has the ability to cause chromosomal damage and thereby alter the genetic material of eukaryotic cells[1]. As such, it may be detrimental to individuals exposed to this form of energy. For the most part, radiation protection guidelines assume equal sensitivity of individuals to ionising radiation. This is simply not the case and many studies have demonstrated this, the most recent being that of Slabbert et al[2].

When exposed to DNA-damaging agents such as radiation, cells could undergo apoptosis, which is a physiological response whereby the cell actively participates in its own destruction[3]. Initially cells will shrink due to cytoplasmic condensation. This is followed by chromatin condensation and fragmentation of the nucleus. Eventually the cells separate into a number of small fragments or apoptotic bodies which are then phagocytosed by neighbouring cells[4].

Crompton and Ozsahin[5] developed the leukocyte apoptosis assay (LAA) to predict intrinsic radiosensitivity of normal tissue based on the radiation-induced apoptotic response of CD4+ and CD8+ T-lymphocytes. Of significance is that radiosensitive individuals have an abnormality in their ability to recognise or repair DNA double-strand breaks induced by ionising radiation, leading to enhanced toxicity and a predisposition to cancer[6]. Studies have shown that approximately 5-10% of patients receiving radiotherapy show signs of late toxicity many months after therapy has been completed[7]. If this percentage of patients could be identified prior to radiotherapy, alternative treatment modalities could be used to avoid the late toxicity caused by radiotherapy. Alternatively, patients who are not prone to late toxicity could be treated with higher doses of radiation. The LAA could therefore allow oncologists to individualise each patient’s therapy based on their measured radiosensitivity. The LAA could also be applied to radiation workers. If possible hypersensitivity to radiation of an individual is known, tasks associated with a risk of high radiation exposures should be avoided by such workers who display enhanced toxicity to radiation.

Sensitivity of individuals, as measured by radiation-induced apoptosis, differs from that measured by cytogenetic methods. The normal tissue of an individual with a low apoptotic response is more sensitive to the detriments of ionising radiation following radiotherapy. However when cytogenetic type endpoints are used, radiosensitivity is defined as a higher frequency of chromosomal aberrations to a test dose of radiation.

Chromosomal aberration assays in human peripheral blood lymphocytes have been used for years as the golden standard to detect radiation exposure in individuals[8]. These assays can also be used as biomarkers of cancer risk in a normal population as studies have shown that defects in the processing of radiation-induced DNA damage could lead to cancer predisposition[9].

The cytokinesis-blocked micronucleus assay (CBMN) has been adopted by laboratories worldwide as a reliable method for monitoring chromosomal damage. The assay identifies binucleated cells which express chromosomal damage in the form of micronuclei. Micronuclei are acentric chromosomal fragments that have lagged behind during mitosis. They are smaller than the main nuclei but resemble it in both morphology and staining pattern[10]. Micronuclei show a clear dose response relationship and can therefore be used as an indicator of permanent genotoxic damage[11, 12]. It can also be used as a method for biological dosimetry, allowing the true dose received during accidental or occupational radiation exposure to be estimated[13].

The purpose of this study was to establish if any correlation
exists between apoptosis as noted with the LAA assay and micronuclei frequency as noted with the CBMN assay after the same in vitro radiation exposure.

MATERIALS AND METHODS

Statement of ethics
The study was approved by the Ethics Committee of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology. Written informed consent forms were obtained from the ten volunteer donors.

Cytokinesis-block Micronuclei Assay
Sample preparation: Lithium-heparinised blood was collected from 10 volunteer donors (27-52 years) that consented to participate in this study. Peripheral lymphocytes were isolated with Histopaque-1077 (Sigma Aldrich), washed with phosphate buffered saline (PBS) and diluted in 5ml RPMI 1640, supplemented with 15% heat-inactivated foetal calf serum (FCS) (Delta Bioproducts).

Irradiation: X-ray irradiations were performed using the 6 MV linear accelerator at iThemba LABS, Faure. A dose-rate of 2Gy/min was delivered at a source to surface distance (SSD) of 100cm, a field size of 30x30cm² and a gantry angle of 0°. Tubes were placed side-by-side in the centre of the radiation beam on a 7.4cm thick backscatter Perspex block with a 20mm polyethylene block as build-up material.

Incubation and cell harvest: Immediately after irradiation, lymphocytes were stimulated to divide by adding phytohaemagglutinin (PHA) (Gibco) at a concentration of 3µl/ml and incubated at 37°C in tubes with loose lids in a humidified atmosphere containing 5% CO₂. After 44hrs, 150µl (3µg/ml) of Cytochalasin B (Sigma Aldrich) was added to each tube to block cytokinesis. Cultures were re-incubated for 28hrs resulting in a total of 72hrs incubation time. Tubes were then centrifuged for 5min at 1000rpm. The supernatant was removed and the cell pellet was mixed. Five millilitres of 75mM KCL were added drop-wise while tubes were vortexed at low speed. Tubes were centrifuged for 8 minutes at 800rpm, the supernatant discarded and the pellet resuspended in the remaining fluid. Five millilitres of freshly prepared 0.9% NaCl/Methanol/Acetic acid (ratio of 5:4:1) was added in the same manner as the KCL. Tubes were placed in a refrigerator at 4°C overnight and then centrifuged at 800rpm for 5minutes. Five millilitres of Methanol/Acetic acid (ratio of 4:1) was added in the same manner as the KCL and the tubes were refrigerated at 4°C until slides were prepared for analysis.

Slide preparation and microscopic analysis: Tubes were centrifuged at 800rpm for 5 minutes, the supernatant discarded and

Figure 1 (a-d): LAA scatter plots showing the (a) selection of lymphocytes (R1); (b) selection of CD 4 FITC positive lymphocytes (R2); (c) CD4 apoptotic lymphocytes at 2 Gy (R3) and (d) CD4 apoptotic lymphocytes at 8 Gy (R3).
the cells resuspended in the remaining fluid. Forty microlitres of the suspension was placed onto a slide and left on a flat surface at room temperature to dry for at least 24hrs. Slides were stained with acridine orange at a concentration of 40µg/ml for 2 minutes, washed in distilled water and Gurr buffer pH 6.8 for 1 minute. The slide was covered with a cover slip and sealed with a cement to prevent drying. Slides were analysed immediately using a fluorescence microscope.

Two hundred viable cells were analysed and reported as having 1, 2, 3 or 4 nuclei per cell. The nuclear division index (NDI), an indicator of the PHA's efficiency to stimulate the lymphocytes in G₀ to undergo mitotic division, is calculated as follows:

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NDI = \frac{N₁ \times 1 + N₂ \times 2 + N₃ \times 3 + N₄ \times 4}{\text{Total cells counted}}
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where N₁-4 indicates scorable cells containing 1 to 4 nuclei.

Five hundred binucleated (BN) cells were examined per slide for the presence of micronuclei. The total number of micronuclei was calculated for both dose points. The scoring criteria used in this study were according to the criteria adopted by the HUman MicroNucleus (HUMN) project[12].

The Leukocyte Apoptosis Assay
Lithium heparinised blood from the same 10 volunteer donors was used for this study. Whole blood was diluted in 5ml RPMI 1640, supplemented with 20% foetal calf serum (FCS) (Delta Bioproducts). Following irradiation as described in 2.1 and 48 hour incubation, cells were treated for 20min with 20µl of CD4 and CD8 fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (Becton Dickinson). Erythrocytes were lysed using 4ml FACS Lysing solution (Becton Dickinson). Cells were then washed, centrifuged and resuspended in 400µl of FACS Flow (Becton Dickinson). DNA was stained using 5µl of propidium iodide (PI) for 5min before reading.

Flow cytometry was done using a FACScan (Becton Dickinson). Lymphocytes were identified and gated on a 2D-scatter plot (Figure 1a). A second 2D-scatter plot of antibody fluorescence (FL1-height) vs cellular DNA content (FL3-height) was used to identify the CD4 (Figure 1b) and CD8 positive lymphocytes. A third 2D-scatter plot of cellular DNA content (FL3-height) vs cell size (FSC-height) (Figure 1c & 1d) was then used to determine the proportion of apoptotic cells by identifying the population of cells with reduced DNA content and slightly reduced cell size. Radiation-induced apoptosis was calculated by subtracting the background level (0Gy) of apoptotic cells from the total measured at 2Gy.

Statistical Analysis
The Graphpad Prism Programme (Level 4, 2005) was used to perform the statistical analysis.

RESULTS AND DISCUSSION
The apoptotic response for both CD4 and CD8 lymphocytes after a 2Gy X-ray exposure varied between the 10 donors used in the study (Figure 2). The radiation-induced apoptosis for CD4 lymphocytes ranged from 3.3 to 16.3% (mean: 9.2%) and that for CD8 lymphocytes ranged from 3.2 to 23.4% (mean: 13.4%). The higher levels of apoptosis seen in CD8 lymphocytes are consistent with that noted by Ozsahin et al.[13]

A large variation in micronuclei yield was observed for the different donors (Figure 3). The micronuclei count ranged from 31 to 75 micronuclei per 500 binucleated cells, notwithstanding the fact that the same physical dose (2Gy) of X-rays was given in each instance. This variation was somewhat smaller than the approximate fourfold variation noted by Slabbert et al.[12] It is possible that the more extensive data for each individual analysed by these investigators is responsible for higher variation noted.

The principle reason for this study was to determine if the LAA data can be related to micronuclei frequencies observed. This is shown in Figure 4. It is clear that both endpoints (radiation-induced apoptosis and micronuclei count) vary considerable between different donors. For both CD4 and CD8 lymphocytes there are on average a small increase in the ability of individuals to process the initial radiation damage when they are more sensitive to ionizing radiation as defined by micronuclei frequencies. There is a tendency that both endpoints increase with
radiosensitivity. This gradual increase is however not statistically significant (p = 0.19 CD4 and p = 0.70 CD8). Even so, the findings observed, verify two matters about radiosensitivity as defined by the respective endpoints. Firstly, residual radiation damage as quantified by micronuclei frequency is not related to the apoptotic response of cells subjected to radiation damage. Louagie and co-workers\textsuperscript{14} reported similar findings in their studies. Secondly, if one defines inherent radiosensitivity as micronuclei frequency following a test dose, radiation workers may benefit marginally from their greater apoptotic response.

**CONCLUSION**

The CBMN assay has long been considered as a rigorous endpoint to assess radiosensitivity. The aim of this study was to see if radiosensitivity variations between different donors can be related using the LAA and CBMN assays. South Africa has a large number of people working in applied radiation industries. Each year many radiation workers absorb radiation doses too close to the 20 mSv permitted. Also, accidental over-exposures to ionising radiation are far too frequent in South Africa. These include people both in the health profession as well as industries such as in mining and industrial radiography. It is important therefore to find methods to identify workers who are hypersensitive to ionising radiation in order to allocate jobs of higher risk to individuals better equipped to handle the detriment of ionising radiation. The less labour-intensive LAA assay is in itself very useful to identify workers suitable to work with ionising radiation. Based on the current study, higher apoptotic readings could not be related to the inherent radiosensitivity of individuals.

It would be most valuable to follow micronuclei frequencies over many years in subjects accidentally exposed to high doses of ionising radiation in order to relate residual cytogenetic damage with the apoptotic ability.

**REFERENCES**