Characterizing of γ H2AX response of human lymphocytes to ionizing radiation

by

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Abstract

This work used flow cytometry to examine the feasibility of γ H2AX as a marker for biological dosimetry and investigated the behavior of the H2AX histone for estimating the relative biological effectiveness of neutron irradiation. The work studied the dose rate effect for high and low dose rates. The behavior of different populations of human lymphocytes was investigated to determine whether one population has a more appropriate response for biological dosimetry.

The data showed that although the γ H2AX biomarker has a linear dose response relationship, the usefulness of the biomarker is hampered with its short lifetime. Also, for the low dose rate irradiation, the plateau of the dose response curve makes it difficult to estimate dose. It was also shown that for human lymphocytes there is a clear dose rate effect for the γ H2AX endpoint, with more damage measured when cells were irradiated at high dose rate than irradiated at low dose rate. The measurement of RBE using the γ H2AX response gives unity; supporting a notion that the number of double strand breaks endpoint is mostly independent from linear energy transfer.

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LIST OF ABBREVIATIONS

ADC	Analogue to digital conversion
APC	Allophycocyanin (emission maximum – 660 nm, excitation maximum – 650 nm)
CD	Cluster of differentiation
DNA	Deoxyribonucleic acid
DRE	Dose rate effect
DSB	Double strand break
ESR	Electron spin resonance
FBS	Fetal bovine serum
FISH	Fluorescent <i>In Situ</i> Hybridization
FITC	Fluorescein isothiocyanate (emission maximum – 520 nm, excitation maximum – 495 nm)
FL1, FL2 etc.	Fluorescence parameter 1,2, etc., on the flow cytometer
FSC	Forward scatter
GPA	Glycophorin A
Gy	Gray, SI unit for absorbed dose
HDR	High dose rate
LDR	Low dose rate
LET	Linear energy transfer
mM	Millimolar

NaCl	Sodium chloride
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PC7	Phycoerythrin-Cyanine7 (emission maximum – 780 nm, excitation maximum – 565 nm)
PCC	Premature chromosome condensation
PE	Phycoerythrin (emission maximum – 575 nm, excitation maximum – 565 nm)
RBE	Relative biological effectiveness
RPMI	Roswell Park Memorial Institute medium
SF2	Surviving fraction after 2 Gy
SSB	Single strand break
SSC	Side scatter
T _{1/2}	Repair half time
TBS	Tris buffered saline
TLD	Thermoluminescent dosimeter
TST	Tris-HCl-buffered saline
UV	Ultra violet light

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1. Introduction

The interaction of ionizing radiation with living things leads to changes in several biological parameters. The purpose of the biological dosimetry is to reconstruct the level of the exposure to radiation based on these changes. The estimation of the dose level can be obtained by comparison to a dose-effect calibration curve derived from samples irradiated in a controlled environment.

A need for biological dosimetry arises when physical dosimetry is lacking or in dispute. In cases of the industrial accidents, professional exposures, and military conflicts information obtained from biological dosimetry can assist in medical treatment of patients.

Biological indicators of radiation exposure have been developed and applied to populations exposed to acute and relatively large doses, such as atom bomb survivors and Chernobyl liquidators but they are mostly labor intensive and time consuming¹. Also there is increasing interest in developing biomarkers for lower doses and low rate exposures². Biological assays specific for more densely ionizing radiation, e.g. high LET radiation, could be applied in high radon areas, to airline personal, or for extended space flights.

Any biological indicator considered to be apt for biological dosimetry has to have a number of useful characteristics^{1,3}:

- 1) The time required to perform assay should be short, especially in an accident or a military conflict where a large number of individuals may have been exposed. In these circumstances, medical care should be applied as soon as possible, particularly for those

who exceeded medical dose limits. In the case of military conflicts such as Hiroshima and Nagasaki, or accidents such as Chernobyl, large populations would require monitoring; therefore an automated assay is highly preferable.

2) The longevity of the biological indicator is critical. Some biological markers live for years and some disappear within a few hours. In order to avoid underestimating the dose, longevity of the marker must be taken into account.

3) The inter- and intra-individual variations should be small to allow the same dose-response curve to be applicable to all exposed individuals,

4) The specificity of the biological marker for ionizing radiation is another characteristic of the good biological marker. A biological marker should not be affected by the other factors such as smoking or age.

5) And finally a clear dose-response relationship is necessary.

Consideration of all these factors makes it clear that it is very hard to establish one biological indicator for all applications.

1.1 Existing methods of biological dosimetry

The chromosomal unstable aberration assay, also known as dicentric assay, is currently considered as a “gold” standard⁴ and has been in use since the 1960’s⁵. Having 1-2 dicentric aberrations (Figure 1.1) per 1000 cells for background⁶, the assay is very radiation specific. Also it has small variability between individuals with a clear dose-response relationship (linear for high LET radiation and linear quadratic for low LET radiation^{5, 7}(Figure 1.2)). But the assay requires a few days to complete and requires

highly skilled personnel for the analysis. The dicentric assay is also not very useful for retrospective dosimetry since T-lymphocytes, which are used in this assay, gradually

Figure 1.1 Two dicentric chromosomes and one centric ring with their associated fragments

Figure 1.2 Typical linear and linear quadratic dose response curves
(Figures 1.1 and 1.2 adopted from Cytogenetic analysis for radiation dose assessment. A Manual. Technical reports series No. 405, IAEA Vienna, 2001.)

disappear from the blood stream. These lymphocytes have life span ~ 3 years but their replacement may be affected by widespread apoptosis² after radiation exposure.

Blood count assays don't require the technical expertise of the dicentric assay, and have low variability. For example, the total lymphocyte count falls rapidly in the first week after irradiation^{1,8} (Figure 1.3). But several counts have to be conducted daily for a several days immediately following radiation exposure and all counts return to normal after a few weeks or months rendering this assay impossible to use for retrospective biodosimetry¹. Also the assay can be confounded with physical stress, injuries, or toxic exposures¹.

Figure 1.3 Lymphocyte count after single acute gamma irradiation
(Figure 1.3 adopted from Guskova A.K, ed. Acute Radiation Effects in Victims of the Chernobyl Nuclear Power Plant Accident. United Nations, UNSCEAR 613-647 (1988).)

Electron spin resonance (ESR) assay overcomes the time restraints, since at 25° C a lifetime of 10^7 years was determined for radiation-induced radicals⁹. But the assay is

very invasive since tooth enamel is working material. Also it uses large and expensive equipment, and is very inconclusive about doses to parts of body other than the head. This last problem could be solved using cloth and fingernails of victims, although this work is still very preliminary. ESR dose reconstruction procedure is also considered to be time and labor consuming. It is also limited by being minimally sensitive to neutrons¹⁰, and could be applied to measure only accumulated dose. Other disadvantages of this assay include UV exposure affecting the total dose assessment, and inability to distinguish between different types of radiation. Also in the case of bone-seeking radionuclides (e.g. ⁹⁰Sr) the reconstruction of the individual dose is complicated, and in the certain cases impossible^{9, 10}.

The glycophorin A somatic mutation assay^{3, 10} (GPA assay) has a few practical advantages. Only 1ml of blood per subject is required. Blood collected from study subjects can be stored at temperature (4 °C) up to 1 week prior to analysis, making it practical in studies with limited resources available in the field conditions. The GPA assay can be performed on a commercially available flow cytometer, reducing the amount of labor and time, which makes it attractive for large population studies. Glycophorin A bears the M or N blood group. Individuals who inherit two N alleles have blood group N. Individuals who are homozygous for the M allele have blood group M. Heterozygous individuals produce both proteins and have blood group MN. The M allele encodes serine at position 1 (Ser-1) and glycine at position 5 (Gly-5) of glycophorin A. The N allele encodes leucine at position 1 (Leu-1) and glutamic acid at position 5 (Glu-5). Glycophorin A from individuals with the MN blood type contains equimolar mixtures of each species.

Only the individuals having the MN form and constituting about 50% of the population are eligible for the GPA assay. Inter-individual variability, especially at doses above 1 Gy, makes the GPA assay less useful as an individual biodosimeter, but the assay can be used to determine average doses in population groups. Also the assay has relatively high sensitivity threshold of 1 Gy and can be used only for estimating cumulative dose.

Radiation damage can also be detected in interphase cells by the premature chromosome condensation (PCC) assay^{1, 5}. For potential high-dose irradiation above the median lethal dose, such as in a radiation disaster, it is expected that delay in cell cycle progression into mitosis will interfere with dose estimation. In addition, high-dose radiation accident victims will also suffer from lymphopenia; therefore, few cells will be available for cytogenetic studies. In order to overcome this limitation, quantitative analysis of radiation-induced damage may be performed using resting lymphocytes *in lieu* of metaphase spreads. Use of interphase cytological assays, such as PCC assay, eliminates these inherent problems associated with the use of metaphase-spread cytogenetic assays. The PCC assay is useful to determine exposure to low doses as well as to life-threatening acute high doses. This method uses fusion of the test cells with mitotic cells of another type, which transmit a signal for dissolution of the nuclear membrane and condensation of the interphase chromosomes as if in preparation for mitosis. Pieces in excess of the expected 46 chromosomes occur when breaks are present within the interphase chromosomes. Excess PCC fragments have been shown to increase with radiation exposure¹¹. The PCC assay could be used with PCC rings being scored in

the event of a very high overdose⁵. Dose estimates for PCC assay are obtainable within 48 hours of receipt of blood in a laboratory.

Recently developed assays such as a micronucleus and stable chromosomal (Fluorescent *In Situ* Hybridization) aberration assays are very promising, but they are still developing¹², without a clear generally accepted procedure and with relatively high cost².

There are many more methods than described here, but all of them have certain shortcomings^{1, 2}. At this time, there is no ideal bio-dosimetry indicator that meets all our needs.

1.2 Organization of DNA

DNA consists of two strands that form a double helix. Each strand is composed of a series of nucleotides, the sequence of which contains the genetic code. Sugar moieties and phosphate groups form the backbone of the double helix. The bases on opposite strands are complementary; adenine pairs with thymine, and guanine with cytosine¹³⁻¹⁵.

DNA is the principal target for the biological effects of ionizing radiation. Therefore, it is important to understand the organization of DNA in chromosomes.

The basic structural unit of the chromosome is a complex called the nucleosome. Nucleosomes consist of the class of the basic proteins called histones. There are five major types of histones: H1, H2A, H2B, H3, H4^{13, 14, 16} (Figure 1.4).

Figure 1.4 Nucleosome structure

Each nucleosome is made up of an octamer of histones wrapped around with 146 nucleotide pairs of DNA. The octamer contains each of two histones H2A, H2B, H3, and H4 (Figure 1.4). The formation of nucleosomes produces the 10-nm fiber (Figure 1.4), resembling “beads on a string” by electron microscopy, and provides a level of compaction of about six fold¹³.

The next level of packaging involves the linking together of the nucleosome particles to form the 30-nm^{13, 14} solenoid (Figure 1.5). The association of the nucleosomes with each other is mediated by histone H1 and extends the degree of compaction to about 42-fold. Histone H1 is not a part of the nucleosomal structure but associated with the spacer DNA between nucleosomes. H1 is thought to bring adjacent nucleosomes into close juxtaposition, firming a chromatin fiber and also called the linker histone.

Under the right conditions, further compaction of the DNA is provided by higher order folding of the 30-nm solenoid. The solenoid structure coils to form a hollow tube about 200 nm in the diameter (Figure 1.5). The solenoid coiling into the 200 nm tubes condenses the length of the DNA by additional factor of 18, making the overall packing

ratio from linear DNA molecule to the hollow tube of interphase chromosome about 750. In the cells that are not dividing, this is the extent of chromosome condensation. For metaphase chromosomes, the packing ratio is still higher (Figure 1.5) because of the further condensation that takes place as the nucleus enters division. For example, a typical human chromosome contains about 75 mm of DNA and has length of about 4 or 5 μm at metaphase. The overall packing ratio must be somewhere in the range of 15,000 to 20,000.

Figure 1.5 Packaging of Nucleosomes into Chromosomes. a) Nucleosomes about 10 nm each. b) Chromatin fiber forms a helical coil called a solenoid, with thickness of about 30 nm c) The solenoid structures coil to form 200 nm tubes. d) In mitosis, a higher degree of coiling generates the 600 nm. chromatids.

(Figures 1.4 and 1.5 adopted from Wayne M. Becker, *The World of the Cell*, The Benjamin/Cummings Publishing Company, Menlo Park, CA, 1986.)

1.3 Double-strand breaks and repair

The effect of ionizing radiation on DNA has been studied for many years. Such studies are justified because of the central role DNA plays as the major cell target^{15, 17}. When cells are irradiated, many lesions are induced. Those are single strand breaks (SSBs), double strand breaks (DSBs), base damage, and DNA protein cross links⁵ (Figure 1.6). But in intact DNA base damage, SSB, and cross-links are of little biological consequence because they are readily repaired^{15, 17, 18}. By contrast, if the breaks in two

Figure 1.6 Radiation induced DNA lesions.
(Figure 1.6 adopted from Cytogenetic analysis for radiation dose assessment. A Manual. Technical reports series No. 405, IAEA Vienna, 2001.)

strands are opposite one another, or separated by only a few base pairs, this may lead to a DSB. DSBs are believed to be the most important lesion produced by radiation and responsible for most visible chromosome aberrations such as dicentrics, centric rings, acentrics, reciprocal translocations, etc⁵. The interaction of DSBs may result in cell killing, mutation, or carcinogenesis^{15, 18, 19}.

1.4 Linear Energy Transfer effects

Amount and quality of DNA damage depends heavily on the quality of radiation such as linear energy transfer (LET), and dose rate.

When ionizing radiation passes through matter, it ejects electrons from the atoms through which it travels leaving positively charged ions, in the case of neutron irradiation interaction with nuclei takes place. The distribution of primary events, ionizations and excitations along the path of an ionizing particle will vary according to the type of radiation. The average separation of these events decreases with increasing charge and mass of particles^{5, 15}.

LET of charged particle in medium is defined by the International Commission on Radiation Units and Measurements as quotient $d\varepsilon/dl$, where $d\varepsilon$ is the average energy locally imparted to the medium by a charged particle of specific energy in traversing a distance of dl ^{5, 15}. LET may be obtained by weighting each interval according to the energy deposited (or dose) or according to the length of the track traveled. These give, respectively, dose (or energy) average and track average LET. Track average appears to be the better quantity to describe the RBE variations for chromosomal damage^{5, 15}. The

track average LET for 250 kVp (kilovolts peak) X rays is about 2 keV/mm, as compared with heavy charged particles that have track average LET values of 100–2000 keV/mm or greater^{5, 15}. The essential point to consider is that different types of radiation can vary considerably in the quantity of energy imparted per micrometer of track, and this can clearly change the biological efficiency of different types of radiation. As the LET of the radiation increases, there is a greater probability that two lesions within the target will be induced by two ionizing events along the same track, resulting in DSBs.

It is known that SSBs induced in the DNA that can be converted into DSBs can be repaired, taking from a few minutes up to several hours, depending on the particular lesion^{5, 19}. If the two SSBs needed for inducing a DSB are produced by separate tracks, and the dose rate is low, there is a probability that the lesion produced by the first track will be repaired before the DNA is traversed by a second track, forming the second lesion. Although two lesions have been produced within the target, they cannot form a DSB. The probability of the two lesions being able to interact will decrease with decreasing dose rate — the lower the dose rate, the lower the frequency of ionization tracks per unit time and therefore the longer the time available for repair of the first lesion before the second can be induced. As a result the situation for low LET radiation is the following: lowering the dose rate decreases the DSB frequency per unit dose. The same argument holds true for fractionated or split doses. If two or more doses are received, lesions from the first can interact with lesions produced by the second, or subsequent, dose, provided that the time interval between the first dose and the subsequent dose fraction is not longer than the time it takes to repair the lesions induced by the first or previous dose. Thus, if doses are separated by times longer than the repair time, the

frequency of DSBs created by the total dose will be less than that from the total dose delivered at one time. The situation is different with regard to high LET radiation, since both lesions involved in the induction of DSBs are usually produced by a single track. Therefore, lowering the dose rate does not alter the frequency of DSBs, because repair of the lesions during longer exposures will not be an influencing factor. The same argument applies to fractionated exposures — the repair of lesions between the fractions does not have much influence since both are produced coincidentally by a single track.

1.5 Relative biological effectiveness

The effectiveness of different types of radiation for inducing a certain biological effect is commonly represented by the term ‘relative biological effectiveness’ (RBE). The RBE is defined as the ratio of the dose of the reference radiation (usually X rays) to the dose of the particular radiation being studied that produces the same biological effect. That is ^{5, 15},

$$\text{RBE} = \frac{\text{dose of 200–250 kVp X rays producing effect Z}}{\text{dose of test radiation producing effect Z}}$$

For example, Figure 1.2 shows the typically shaped linear and linear quadratic dose response curves obtained with, respectively, high and low LET radiations using the dicentric assay. The RBE at a high dicentric yield that would be related to high doses is shown by the upper horizontal dashed line, which intercepts the two curves at 1.0 and 3.5 Gy. The RBE is the ratio of the two doses that is $3.5/1.0 = 3.5$. The lower horizontal dashed line intercepts at 0.1 and 1.0 Gy, resulting in a higher RBE: $1.0/0.1 = 10$.

From the earlier discussion it is apparent that dose, dose rate or dose fractionation can affect RBE. In addition RBE depends on radiation quality (LET), and biological system or endpoint¹⁵ making it a complex measurement.

1.6 γ H2AX and DNA double strand breaks

The introduction of double strand breaks into DNA triggers a complex set of responses in eukaryotic cells, including cell cycle arrest, relocalization of DNA repair factors, and some cases apoptosis¹⁹. Failure to arrest cellular functions can lead to a high level of genomic instability and oncogenic transformations. Several factors known to be involved in DNA repair or in signaling the presence of damage have been shown to accumulate in large nuclear domains (foci) after double strand breakage of DNA^{16, 21, 22, 23}.

One of the first cellular responses^{16, 24} to the introduction of the double strand breaks is phosphorylation of H2AX, one of three types of histone H2A molecules in eukaryotic cells. As far as has been examined, phosphorylated H2AX (or γ H2AX) forms at sites of all types of DSBs, regardless of their sources²⁴. In mammals, the H2AX variant constitutes from 2 to 25 % of total H2A^{16, 21, 24, 25}. Serine 139 in unique C-terminal tail of H2AX is phosphorylated within few minutes after damage, producing a γ H2AX species^{16, 24}. The C-terminal end of histone H2AX is intimately involved in the efficient recognition and repair of chromatin regions containing DSBs^{16, 24}. It was shown that although mice lacking H2AX are viable, they are smaller and sensitive to ionizing radiation. H2AX is necessary for G2/M cell cycle checkpoint competency at

physiological doses of ionizing radiation. Also, H2AX negative cells display substantially increased numbers of genomic defects in mitotic spreads²⁶.

Using two-dimensional gel analysis, it has been demonstrated that γ H2AX is detectable within 3 minutes²⁴ of exposure to radiation. The phosphorylated fraction of H2AX rapidly increases until a plateau is reached 10-30 min.^{24, 25, 27} (for a Chinese hamster ovary cell line) with the level of the plateau being proportional to dose. Also it was found that the number of γ H2AX molecules increases linearly with severity of the damage²⁴. An antibody specific to γ H2AX was produced recently and used to show that γ H2AX is localized in large chromatin domains (foci) at the sites of DNA double strand breaks²⁴ (Figure 1.7). Also it was shown that hundreds to thousands of H2AX molecules become phosphorylated at DSBs^{24, 27}.

Figure 1.7 γ H2AX focus pattern. Cells were irradiated with 5 Gy and allowed to recover for 6 h before fixation and antibody staining. The bright dots represent γ H2AX foci. (Figure 1.7 adopted from K. Camphausen, W. Burgan, M. Cerra, K. A. Oswald, J. B. Trepel, M. Lee, and P. J. Tofilon. Enhanced Radiation-Induced Cell Killing and Prolongation of H2AX Foci Expression by the Histone Deacetylase Inhibitor MS-275. *Cancer Research* 64, 316–321, 2004)

It has been generally established that γ H2AX foci attract repair factors, assembling higher concentration of repair proteins, and proteins involved in cell cycle checkpoint activation, near a DSB site. The more persistent the break, the larger the γ H2AX foci is and presumably the higher concentration of repair and checkpoint proteins²⁴.

1.7 White blood cells

Suspended in blood plasma are two classes of cells: red blood cells which transport oxygen, and white blood cells whose function is defense^{13, 14}. A third cellular element, platelets^{13, 14}, is pieces of cells that are involved in clotting. Red blood cells, or erythrocytes are not of interest, since erythrocytes in mammals have no DNA. The same can be said about platelets.

The only blood cells having nuclei (and therefore DNA) are white blood cells or leukocytes. The density of these leukocytes in the blood is 5000-10000/ mm³¹⁴ and there are three different types: granulocytes, monocytes, and lymphocytes¹⁴.

All leukocytes develop from a common source: a single population of cells called pluripotent stem cells in the red marrow of bones¹⁴. Pluripotent stem cells first differentiate into myeloid and lymphoid stem cells. Later on myeloid stem cells mature into granulocytes and monocytes, when lymphoid stem cells become lymphocytes.

Lymphocytes are quite common in the blood (~ 30%)¹⁴ and are generally smaller than the other leukocytes. The nucleus is round and large in comparison to the cell, occupying most of it. Human peripheral lymphocytes as well as granulocytes and monocytes represent a cell population, which is predominantly in a DNA presynthetic

stage of the cell cycle (i.e. the G_0 phase). The total number of lymphocytes in a healthy young adult has been estimated to be approximately 500×10^9 cells⁵.

The lymphocyte population is made up of several different subpopulations, mainly B, and T cells. Both these types originate from lymphoid stem cells in the bone marrow or liver of a developing fetus. Early lymphocytes are all alike, but they later develop into T cells or B cells, depending on where they continue their maturation. Lymphocytes that migrate to the thymus develop into T cells (T stands for thymus). Lymphocytes that remain in the bone marrow and continue their maturation become B cells (we can equate B with bone). On the basis of their surface markers, T lymphocytes could be further subdivided into CD4 and CD8 lymphocytes, which are the most common cell type used for biological dosimetry⁵.

CD8 cells are responsible for lysing foreign cells. CD4 lymphocytes are involved in suppressing immune response. Also CD4 helper lymphocytes induce CD8 lymphocytes and CD4 suppressor and cytotoxic (killer) lymphocytes to proliferate and differentiate.

B lymphocytes (or CD19 lymphocytes, due to specific surface markers) develop into antibody producing plasma cells after stimulation by CD4 cells.

1.8 Flow cytometry

Flow cytometry is a technique for making rapid measurements on particles or cells as they flow in a fluid stream one by one through a sensing point^{28, 29}. Measurements are made separately on each particle within the suspension in turn.

The ability of flow cytometers to measure multiple cellular parameters, based on light scatter and fluorescence, and to physically purify subpopulations of cells has led to the increasingly widespread use of this instrumentation in biology and medicine.

The applications of flow cytometry are numerous. A wide range of fluorescent stains are available for directly estimating cellular parameters such as nucleic acid content, nuclear antigens, cell surface antigens, cell viability, etc. Conjugation of fluorescent dyes to ligands and polyclonal and monoclonal antibodies has enabled the density and distribution of cell surface and cytoplasmic determinants and receptors to be studied, as well as allowing functional subpopulations of cells to be identified. Many of these fluorescent dyes and reagents can be used in combination to produce multiple correlated measurements. It is commonplace to measure two light-scatter and three fluorescence parameters on the each cell.

Modern flow cytometers can analyze several thousands particles every second in real time and can actively separate particles having specified properties.

An apparatus has five main components:

1. Flow cell – liquid stream carries and aligns the particles so that they pass in single file through the light beam for sensing;
2. Light source – arc lamps (e.g. mercury, xenon), lasers (e.g. argon, krypton, red-Ne, green-HeNe), and diode lasers;
3. Detection and analogue to digital conversion (ADC) system – generating FSC (forward scatter) and SSC (side scatter) as well as fluorescent signals;
4. Amplification system – linear or logarithmic;
5. Computer for analysis of the signals.

A beam of the light (usually laser light) of the single frequency is directed onto a hydrodynamically focused stream of fluid (Figure 1.8). A number of detectors are aimed at point where the stream passes through the light beam; one in line with the light

Figure 1.8 Diagram of the FACSCalibur flow cytometer. The numbers with FL1, FL2, etc. represent the wavelength and bandwidth collected by this channel (Figure 1.8 adopted from Introduction to Flow Cytometry: A Learning Guide. Manual Part Number: 11-11032-01 April, 2000, BD Biosciences).

beam (FSC) and several at right angle to it (SSC and fluorescent detectors). Each suspended particle passing through the focal point scatters the incident laser beam, producing forward and side scatter. Fluorescent probes in the particle may be excited into emitting light of lower frequencies than the incident light. The detectors pick up emitted and scattered light. From fluctuations of brightness at each detector it is possible to deduce various facts about physical and chemical structure of each individual particle. For example FSC correlates with the particle volume, and SSC with inner complexity of

the particle. The use of the different cell surface antigens (CD markers) stained with fluorescent dyes allows singling out various cell populations.

1.9 Hypothesis

The number of double strand breaks of DNA reflects dose received by the cell ²²⁻²⁷, and this quantitative information on DSBs could be obtained by use of anti- γ H2AX fluorescently stained antibodies and the methods of flow cytometry.

The hypothesis of this thesis is that the γ H2AX bioindicator is a useful tool for applications of biological dosimetry.

Specific aims are:

- To verify usefulness of γ H2AX histones as biomarkers, using different subpopulations of human lymphocytes, assuming that different types of lymphocytes may have different radiosensitivity³⁰⁻³².
- Use of γ H2AX biomarker for study of dose rate effect.
- Measurements of RBE (relative biological effectiveness) by means of γ H2AX biomarker.

2. Materials and methods

2.1 The cell lines

Human peripheral venous whole blood was used in all experiments. Healthy volunteers of both genders were in range from 19 to 40 years old.

2.2 Isolated lymphocyte culture for high dose rate (HDR) irradiation

Peripheral blood mononuclear cells (PBMCs) from volunteers were isolated from 5-7 ml of venous whole blood collected in a sodium heparinized vacutainer tube (Becton Dickinson, Oakville, Ontario, Canada). The whole blood was layered over 10 ml Histopaque 1077 (Sigma, St. Louis, MO), separated by density centrifugation (at room temperature, 400 g, for 30 min) and washed with 1x phosphate-buffered saline (PBS: 58 mM sodium phosphate dibasic-anhydrous, 17 mM sodium phosphate monobasic-monohydrate, 68 mM NaCl; all from Fisher Scientific, Hampton, NH), pH 7.4. Cells were diluted to 5×10^5 cells/ml with complete media (RPMI 1640 Invitrogen Canada, Burlington, ON) supplemented with 15% fetal bovine serum (FBS, Sigma), 1% L-Glutamine (Invitrogen Canada) and 1% penicillin-streptomycin (Invitrogen Canada). The cells were divided into 1 ml samples for irradiation with 0, 0.1, 0.2, 0.5, 1, 2, 4, and 10 Gy and 30 min of incubation time or 0, 0.25, 0.5, 0.67, 0.83, 1, 1.5, 2, 4, 24, 48 h of incubation time after 2 Gy of irradiation. Before irradiation cells were stained with anti-human fluorescently labeled CD markers: 0.625 μ l CD4 PC7, 5 μ l CD8 APC, 5 μ l CD19 PE in each sample and incubated for 1 h in a humidified chamber at 37°C in 5% CO₂ in the dark.

Irradiation was conducted on ice ($\sim 4^{\circ}\text{C}$) at a dose rate of 0.74 Gy/min using a ^{137}Cs Gammacell-40 (MDS Nordion, Kanata, ON). After irradiation the samples with an incubation time less than 1.5 h were placed in water bath at 37°C . The samples with an incubation time of 1.5 h or more were placed in the humidified chamber.

At the desired time point, the samples were refrigerated for 5 min to stop cellular activity and centrifuged (8 min , $300 \times g$, at 0°C). After the supernatant was aspirated and discarded, pellets were re-suspended in $500\text{ }\mu\text{l}$ cold (4°C) PBS and fixed by adding $500\text{ }\mu\text{l}$ 0.5% formaldehyde (Fisher Scientific, Hampton, NH) in PBS dropwise while vortexing. The samples were incubated for 15 min on ice and centrifuged (8 min , $300 \times g$, at 0°C). The supernatant was aspirated and cells were re-suspended and fixed with 1 ml of very cold (-40°C) 70% methanol (Fisher Scientific, Hampton, NH). The samples were kept in a freezer for the next $3\text{-}4$ days before staining. The samples were stable for two weeks in the freezer.

The cells were washed (5 min , $400 \times g$, at 0°C) with 1 ml tris buffered saline (TBS: 20 mM Trizma HCl (Sigma-Aldrich), 0.5 M NaCl (Fisher Scientific)), pH 7.4 then incubated for 10 min on ice with 1 ml cold (4°C) TST (TBS containing 4% FBS and 0.1% Triton X-100 (T-9284, Sigma-Aldrich)). Then the cells were centrifuged and resuspended in $200\text{ }\mu\text{l}$ mouse antihuman anti-phosphohistone H2AX FITC conjugated antibody (Upstate Biotechnology, Waltham, MA, USA), which was diluted $1/4000$ in TST. The tubes were placed on ice for a 2 h incubation. After rinsing and centrifuging (5 min , $400 \times g$, at 0°C) with 1 ml cold (4°C) TBS containing 2% FBS (TBS+ 2% FBS) cells were resuspended in $500\text{ }\mu\text{l}$ TBS+ 2% FBS.

Flow cytometry and statistical analysis: The data acquisition gates were set to count 70 000 lymphocytes on the forward scatter (FSC) vs. side scatter (SSC) plot in the region R1 as shown in Figure 2.1. The region R1 was determined visually. The acquisition gates were set so that monocytes, granulocytes, and debris were excluded, leaving only lymphocytes for analysis.

Figure 2.1. Forward scatter vs. side scatter plot of human lymphocytes. The gate is set around the peripheral blood lymphocytes (R1), irradiated with 0.5 Gy after 30 min of incubation.

Figure 2.2 (a), (b) shows dot plots of the CD4 PC7 vs. CD8 APC T cells and CD4 PC7 vs. CD19 PE cells. Regions CD4+ and CD8+ are to identify the CD4 and CD8 T cells. Similarly we have the CD19+ region to identify B lymphocytes. Information on the DSBs was obtained using histograms (Figure 2.3 (a), (b)) for the FITC channel for the regions R1, R1 and CD4+, R1 and CD8+, and R1 and CD19+. The geometric means of the histograms were considered as a response (since the geometric mean is better suited

for use with data collected with a logarithmic amplifier), with error bars representing the standard error of the mean.

a)

b)

Figure 2.2. a),b) Dot plots of CD8 APC vs. CD4 PC7 and CD19 PE vs. CD PC7 as identified in Figure 2.1 (R1 region).

a)

b)

Figure 2.3 anti γ H2AX FITC vs. count histogram for region R1, 0Gy (a), and 10Gy (b), 30 min incubation

2.3 Whole blood culture for HDR

Peripheral blood from volunteers (4 ml) was drawn into a sodium heparinized vacutainer tube (Becton Dickinson, Oakville, Ontario, Canada). 100 μ l of blood was transferred into each 5 ml flow cytometry tubes and diluted 1/10 with 15% complete media for irradiation. Again as in the case of lymphocyte assay, the diluted blood was divided into 1 ml samples for irradiation with 0, 0.1, 0.2, 0.5, 1, 2, 4, and 10 Gy and 30 min of incubation time or 0, 0.25, 0.5, 0.67, 0.83, 1, 1.5, 2, 4, 24, 48 h of incubation time after 2 Gy of irradiation. Irradiation was conducted on ice ($\sim 4^{\circ}\text{C}$) at a dose rate of 0.74 Gy/min using a ^{137}Cs Gammacell-40. After the irradiation the tubes were incubated for appropriate times and blood was separated from the media by centrifugation (5 min, 250 x g, at 0°C). 65 μ l of 10% formaldehyde was added and gently mixed in each tube. The tubes were left to incubate for 10 min at room temperature, followed by addition of 1 ml Triton X-100 (0.12% diluted in PBS). After another 30 min of incubation at room temperature, 1 ml of cold wash buffer (PBS with 4% of FBS) was added. The samples were centrifuged (8 min, 300 x g, at 0°C), resuspended in 1 ml cold 50% methanol in PBS and incubated on ice for 10 min. The cells were washed twice with 2 ml of wash buffer. CD markers and anti- $\gamma\text{H2AX-FITC}$ antibody were added and incubated for 30 min at room temperature. After incubation the cells were washed with 2 ml of wash buffer and resuspended in 300 μ l of wash buffer.

Flow cytometry and statistical analysis: The data acquisition gates were set to count 70 000 lymphocytes on FSC vs. side SSC plot in the region R1 as shown in Figure 2.4. Again, the region R1 was determined visually. The acquisition gate (region R1) was set so that only lymphocytes were included for analysis. Dot plots for different

lymphocyte populations were similar to Figure 2.2 of the lymphocyte assay, i.e. CD4 PC7 vs. CD8 APC, and CD19 PE vs. CD4 PC7 lymphocytes. Regions CD4+ and CD8+ identified the CD4 and CD8 T cells and the CD19+ region identified B lymphocytes.

Again, the information on the DSBs was obtained using histograms (Figure 2.3) for the FITC channel for the regions R1, R1 and CD4+, R1 and CD8+, and R1 and CD19+. The geometric means of the histograms were considered as a response with error bars representing the standard error of the mean.

Figure 2.4 Forward scatter vs. side scatter plot of whole blood. The gate is set around the peripheral blood lymphocytes (R1).

2.4 Low dose rate (LDR) and neutron irradiation

For low dose rate (LDR) irradiation, blood diluted 1/10 with 15% complete media was irradiated in 24 well cell culture plates (BD Biosciences, USA), by an array of ^{137}Cs sources in a temperature controlled incubator. The irradiations were carried out at $37\pm 1^\circ\text{C}$ to allow cells to repair during the irradiation. Temperature was continuously checked by thermocouples. The dose rate was determined by using TLD dosimetry.

To determine the dose rate, a 24 well cell culture plate, with each well having 3 TLDs, was positioned on the center of the irradiation plane and irradiated for 20 hours. The average dose rate was calculated by averaging dose for all TLDs and dividing the average dose by 20 hours. The average dose rate was 0.27 ± 0.03 Gy/h. The irradiation times required to achieve the desired doses were calculated by using this average dose rate.

For the neutron and low dose rate irradiations, the whole blood assay was used except that for the low dose rate irradiation there was no 30 min incubation time after irradiation. After obtaining the desired doses the cells were processed according to the assay described in the whole blood culture section.

Flow cytometry and statistical analysis for LDR irradiations were conducted according to flow cytometry and statistical analysis described for whole blood culture section.

Irradiation with a neutron source was conducted at McMaster Accelerator Laboratory of McMaster University with an accelerator-based $^7\text{Li}(p,n)$ neutron source.

For the neutron irradiation, blood diluted 1/10 with 15% complete media was irradiated in 5 ml flow cytometry tubes. Irradiation was conducted on ice in order to

minimize cell repair. Dose rate distribution and the tube positioning were based on Monte Carlo modeling^{34, 35}.

A continuous energy spectrum of neutrons with mean energy 340 keV was produced by means of the ${}^7\text{Li}(p,n){}^7\text{Be}$ reaction. The impinging proton beam energy and current were 2.3 MeV and 300 μA . The total dose rate in the experiment consisted of two components produced by neutrons and γ rays, which were 23.7 mGy/min. and 8.19 mGy/min respectively.

After obtaining the desired doses, the tubes with diluted blood were transferred to a water bath at 37°C for a 30 min incubation and processed according to the whole blood culture assay.

Flow cytometry and statistical analysis for neutron irradiations were conducted according to flow cytometry and statistical analysis described for the whole blood culture section.

3. Results

Flow cytometry was used to quantify anti- γ H2AX antibody binding in human lymphocytes after exposure to ionizing radiation. The γ H2AX response is a measure of the change in the fluorescence measured by the flow cytometer channel number. Data for the dose response curves were normalized for differences in background staining intensity so that the response at 0 Gy was equal to 0.

3.1 Time and dose response after HDR irradiation

The purpose of these experiments was to explore the effectiveness of measuring the phosphorylation of the H2AX histone as a biological indicator for biological dosimetry in lymphocytes and different lymphocyte populations.

3.1.1 Isolated Lymphocyte Cultures for the HDR irradiation

Figure 3.1 shows response of all lymphocytes and different lymphocyte subpopulations to different doses in the range from 0-10 (Gy). Irradiations for isolated lymphocyte cultures were conducted on ice (4° C) in order to minimize cell repair. After irradiation the cells were placed in the water bath (at 37° C) for 30 min of recuperation.

Each experiment was conducted 7 times using 3 different donors. The error bars represent the standard error of the mean of the distribution.

Figure 3.1. Dose response curves of different lymphocyte populations for isolated lymphocyte culture after HDR irradiation.

Figure 3.2 shows response of all lymphocytes and different lymphocyte populations to different doses in the range from 0-10 (Gy) for donor #21.

There were 4 experiments conducted with this particular donor. Again, the error bars represent the standard error of the mean of the distribution.

Figure 3.2. Dose response curves of different lymphocyte populations for isolated lymphocyte culture after HDR irradiation for donor #21.

For the both Figures 3.1 and 3.2 all populations of lymphocytes show similar dynamics and are almost linear.

Figure 3.3 shows the response of different lymphocyte fractions relative to the whole lymphocyte response for all donors' data. The relative response was calculated by the ratio of the response of one subpopulation of lymphocytes over the response of the whole lymphocyte population. Error bars for the Figure 3.3 were calculated using the error propagation formula.

Figure 3.3 Relative dose response curves of different lymphocyte populations for isolated lymphocyte culture after HDR irradiation.

Figure 3.4 Relative dose response curves of different lymphocyte populations for isolated lymphocyte culture after HDR irradiation for donor #21.

Figure 3.4 shows the response of different lymphocyte fractions relative to lymphocyte response for donor #21.

Although response of CD19 lymphocytes varies from responses CD4 and CD 8 lymphocytes at low doses, there are no significant differences in the response of different lymphocyte subpopulations throughout the whole dose range. Student's two-sided t-test for the γ H2AX response values when comparing different populations of lymphocytes was applied (a p-value smaller than 0.05 was considered to be statistically significant). P values between different cell types for all doses were considerably greater than 0.05 for all donors and for donor #21 data.

Figure 3.5 represents the time response of all lymphocytes and different fractions of lymphocytes after irradiation on ice with 2 Gy, for different incubation times. Samples were incubated at 37°C for different times after irradiation. Each experiment was conducted 5 times using 4 different donors. Error bars represent the standard error of the mean.

It is seen that again that all lymphocyte populations show a similar behaviour. The damage increases quickly during first 40 min of incubation, reaching a "plateau" and staying on this level for approximately 1 h. CD4 and CD8 fractions showed a slightly stronger response to irradiation than the CD 19 fraction. After 24 hours all fractions returned to levels close to background and stayed at this level for the next 24 hours.

Again there is no significant difference in response for different lymphocyte populations for the time response curve.

Figure 3.5. Time response curves of different lymphocyte populations for isolated lymphocyte culture after HDR irradiation for different incubation times. The error bars are absent for clarity. The insert shows the time response curve for incubation time up to 4 hour.

3.1.2 Whole Blood Culture for HDR irradiation

Figure 3.6 shows the response of lymphocytes and lymphocyte subpopulations to different doses in the range from 0-10 (Gy). Again, irradiations were conducted on ice (4° C) in order to minimize cell repair and after irradiation the samples were placed in the water bath (at 37° C) for 30 min of recuperation.

Each data point was produced by 9 experiments for 6 different donors. The error bars represent the standard error of the mean of the distribution.

Figure 3.6. Dose response curves of different lymphocyte populations for whole blood culture after HDR irradiation.

Figure 3.7 represents data for donor #21. There were 3 experiments conducted for this donor. Again, irradiations were conducted on ice (4°C). After irradiation the samples were placed in the water bath (at 37° C) for 30 min. of recuperation.

The error bars represent the standard error of the mean of the distribution.

Figure 3.7. Dose response curves of different lymphocyte populations for whole blood culture after HDR irradiation for donor #21.

As for the case of lymphocyte assay, different fractions of lymphocytes (and all lymphocytes as well) show similar patterns (linear dependence), but in the case of whole blood assay, the difference in response for the various fractions is more pronounced. CD19 lymphocytes show the lowest response to irradiation and CD4 lymphocytes demonstrate the highest response.

Figures 3.8 and 3.9 show responses of different lymphocyte fractions relative to all lymphocytes for all donors' and donor's #21 data. Error bars for the Figures 3.8 and 3.9 were calculated using the error propagation formula.

Figure 3.8 Relative dose response curves of different lymphocyte populations for whole blood culture after HDR irradiation.

Figure 3.9 Relative dose response curves of different lymphocyte populations for whole blood culture after HDR irradiation for donor#21.

From Figures 3.8 and 3.9, it is seen that CD4 lymphocytes have the highest response to irradiation compared with the other fractions of lymphocytes; CD19 lymphocytes have the lowest response to irradiation compared with the other fractions of lymphocytes.

P values of two-sided t-test for all donors' data are given the Table 3.1:

Dose (Gy)	CD4vsCD19	CD8vsCD19
0.1	0.53	0.63
0.2	0.28	0.33
0.5	0.24	0.33
1	0.2	0.28
2	0.12	0.22
4	0.1	0.19
10	0.06	0.13

Table 3.1 Results of two-sided t-test for different lymphocyte populations after HDR irradiation for all donors' data, the dose response curve.

Again we see that since there are no p values less than 0.05, there is no significant difference in response for different lymphocyte populations but there is a larger difference than in the lymphocyte culture.

Figure 3.10 represents time dependence curves for all lymphocytes, and different lymphocyte populations. The samples were irradiated with 2 Gy on ice, and kept in the water bath for different times after irradiation. Error bars represent the standard error of the mean of the distribution. There were 9 experiments conducted for 7 different donors. From Figure 3.10, it is seen again that the damage response increases quickly for all subpopulations during first 40 minutes of incubations, reaching a "plateau" at approximately 1 hour after irradiation and staying on this level for approximately 1 hour. After 24 hours all fractions returned to levels somewhat higher than background level and slowly decreased for the next 24 hours. All lymphocyte populations show a similar

dynamic, with CD4 lymphocytes demonstrating the highest response and CD19 the lowest. Although CD4 lymphocytes showed the highest response, they showed the highest speed of loss of the γ H2AX foci.

Figure 3.10. Time response curves of different lymphocyte populations for isolated lymphocyte culture after HDR irradiation for different incubation times. The error bars are absent for clarity. The insert shows the time response curve for incubation time up to 4 hour.

Naturally that time response curves (Figure 3.11) for donor #21 show dynamics similar to the ones of Figure 3.10. There were 3 experiments conducted for donor #21.

Figure 3.11. Time response curves of different lymphocyte populations for whole blood culture after HDR irradiation for different incubation times for donor #21. The error bars are absent for clarity. The insert shows the time response curve for incubation time up to 4 hour.

Table 3.2 shows p values for all donors' data:

Time (hour)	CD4vsCD19	CD8vsCD19	CD4vsCD8
0	0.26	0.32	0.83
0.25	0.01	0.04	0.34
0.5	0.01	0.02	0.41
0.67	0.01	0.049	0.36
0.83	0.01	0.04	0.34
1.5	0.01	0.056	0.32
2	0.01	0.03	0.34
4	0.06	0.11	0.69
24	0.97	0.94	0.91
48	0.79	0.72	0.92

Table 3.2 Results of two-sided t-test for different lymphocyte populations after HDR irradiation for all donors' data, the time response curve.

It is clearly seen that there are significant differences in response between CD4 and CD19 populations for times up to 4 hours (except for 0 hour point); also there are significant differences again in responses between CD8 and CD19 lymphocytes, again up to 4-hours time (except for 0 and 1.5 hour point).

For the single donor (donor #21) we have the similar response (P values for the donor #21 are shown in the Table 3.3.):

Time (hour)	CD4vsCD19	CD8vsCD19	CD4vsCD8
0	0.047	0.06	0.96
0.25	0.03	0.07	0.54
0.5	0.02	0.01	0.71
0.67	0.03	0.048	0.58
0.83	0.06	0.09	0.67
1	0.02	0.04	0.47
1.5	0.04	0.09	0.55
2	0.02	0.03	0.5
4	0.04	0.05	0.82
24	0.81	0.68	0.87
48	0.50	0.48	0.88

Table 3.3 Results of two-sided t-test for different lymphocyte populations after HDR irradiation for donor #21 data, the time response curve.

Again it is seen that there is a significant difference in response between CD4 and CD19 lymphocyte populations up to 4 hours time. Also we can observe significant difference in responses for CD8 and CD19 populations for some data points up to 4 hour point.

3.1.3 Comparison between different lymphocyte populations for whole blood and isolated lymphocyte cultures for HDR

Figure 3.12 Relative dose response curves of different lymphocyte populations for whole blood culture vs. isolated lymphocyte culture after HDR irradiation.

Figure 3.12 shows relative responses in whole blood and isolated lymphocyte cultures for different lymphocytes fractions. For Figure 3.12, W.B. stands for whole blood and Lymphs stands for isolated lymphocyte culture. Error bars for the Figure 3.12 were calculated using the error propagation formula. Although it is seen that for all dose

ranges (except dose 0.1Gy) the response for whole blood culture is higher than for isolated lymphocyte culture, significant differences are rarely observed. Table 3.4 with p values for different lymphocyte populations for all donors' data is shown below.

Dose (Gy)	Lym vs Lym	CD4vsCD4	CD8vsCD8
0.1	0.93	0.86	0.73
0.2	0.5	0.38	0.42
0.5	0.58	0.28	0.35
1	0.41	0.21	0.26
2	0.44	0.19	0.27
4	0.24	0.11	0.13
10	0.12	0.04	0.046

Table 3.4 Results of two-sided t-test for different lymphocyte populations after HDR irradiation for all donors' data for isolated lymphocyte and whole blood cultures.

3.2 Dose response for LDR irradiations

The purpose of these experiments was to explore the effectiveness of measuring the phosphorylation of the H2AX histone as a biological indicator for biological dosimetry and investigate dose rate effect in lymphocytes and different lymphocyte populations. Figure 3.13 shows the response of all lymphocytes and different lymphocyte subpopulations to low dose rate irradiation in the range from 0-4.5 (Gy). Irradiations were conducted at 37°C, allowing repair to occur in order to observe the dose rate effect and to keep the cells alive during a long exposure. The dose rate was ~ 0.27 Gy/h, and the experiments were conducted 4 times for 4 donors. The error bars represent the standard error of the mean. From Figure 3.13 it is seen again that all lymphocytes populations show similar dynamic, with CD4 lymphocytes demonstrating the highest response and CD19 the lowest. It is seen that at ~ 1 Gy (or 4 hours) the curves reach their maximum and decrease after that, even though the dose continues to increase.

Figure 3.13. Dose response curves of different lymphocyte populations for whole blood culture after LDR irradiations.

Again, although it is seen that CD4 and CD8 lymphocytes have the highest response to irradiation compared with CD19 lymphocytes, which have the lowest response to irradiation compared with the other fractions of lymphocytes, significant differences are observed only once between CD4 and CD19 populations for D=1.89 Gy, Table 3.5:

Dose(Gy)	CD4vsCD19	CD8vsCD19
0.13	0.56	0.54
0.27	0.25	0.23
0.55	0.23	0.26
1.1	0.05	0.07
1.89	0.046	0.08
4.52	0.89	0.72

Table 3.5 Results of two-sided t-test for different lymphocyte populations after LDR irradiation for all donors' data.

Figure 3.14 represents the response of different lymphocyte fractions relative to all lymphocyte response. Error bars for the Figure 3.14 were calculated using the error propagation formula.

Figure 3.14 Relative dose response curves of different lymphocyte populations for whole blood culture after LDR irradiation.

3.2.1 Comparison LDR with HDR and dose rate effect

Figures 3.15-3.18 show the response for different lymphocyte fractions for both high dose rate (HDR) and low dose rate (LDR).

Figure 3.15. Dose response curves for all lymphocytes for whole blood culture after HDR and LDR irradiations.

Figure 3.16. Dose response curves for CD4 lymphocyte populations for whole blood culture after HDR and LDR irradiations.

Figure 3.17. Dose response curves for CD8 lymphocyte populations for whole blood culture after HDR and LDR irradiations.

Figure 3.18. Dose response curves for CD19 lymphocyte populations for whole blood culture after HDR and LDR irradiations.

It is seen that the initial slopes of curves for LDR follow closely slopes of the curves for HDR.

3.3 Whole blood culture for neutron irradiation

The purpose of these experiments was to investigate the relative biological effectiveness (RBE) of high LET neutron irradiation compared with low LET ^{137}Cs irradiation for γH2AX endpoint.

Neutron irradiations were conducted over ice in order to minimize cell repair. After irradiations the samples were placed in the water bath at 37°C for 30 min of recuperation. Each data point was produced by 6 experiments for 6 different donors. The error bars represent the standard error of the mean of the distribution.

Figure 3.19 represents dose response curves for different lymphocyte fractions after neutron irradiation.

Figure 3.19. Dose response curves of different lymphocyte populations for whole blood culture after neutron irradiation.

The data for all doses except for first non-zero point ($D=0.319\text{ Gy}$) is not very reliable, due to cell repair so a linear approximation based on first non-zero point was

made. Figure 3.20 represents original data superimposed with the linear approximation of original data.

Figure 3.20. Dose response curves of different lymphocyte populations with their linear approximations based on the first non-zero point for whole blood culture after neutron irradiations.

Again it is seen that CD4 population of lymphocytes has the highest response and CD19 population has the lowest response, like in all previous cases.

3.4 Relative biological effectiveness

In order to find the relative biological effectiveness, the linear approximation of data for whole blood culture for HDR is used with **linest** excel function, which calculates the statistics for a line by using the "least squares" method to find a straight line that best fits our data.

Data for whole blood culture for HDR, linear approximation gives the next functions for 95% confidence interval:

$$\text{Lymphocyte response} = (9.4 \pm 1.4) * D$$

$$\text{CD4 lymphocyte response} = (12.2 \pm 1.6) * D$$

$$\text{CD8 lymphocyte response} = (10.5 \pm 1.3) * D$$

$$\text{CD19 lymphocyte response} = (6.8 \pm 0.9) * D.$$

Where D stands for received dose in Gy.

Figures 3.21-3.24 show responses for whole blood cultures for HDR, linear approximation for HDR, and response for neutron irradiation (the first non zero point) for the different lymphocyte populations.

The error bars represent the standard error of the mean of the distributions.

Figure 3.21. Linear approximation for dose response curve of all lymphocyte populations for whole blood culture after HDR irradiation and one based on the first non-zero points after neutron irradiations.

Figure 3.22. Linear approximation for dose response curve of CD4 lymphocyte populations for whole blood culture after HDR irradiation and one based on the first non-zero points after neutron irradiations.

Figure 3.23. Linear approximation for dose response curve of CD8 populations for whole blood culture after HDR irradiation and one based on the first non-zero points after neutron irradiations.

Figure 3.24. Linear approximation for dose response curve of CD19 populations for whole blood culture after HDR irradiation and one based on the first non-zero points after neutron irradiations.

Finally we can calculate RBE if the delivered dose for neutron irradiation is 0.32 Gy and summarize our data in Table 3.6 for different populations of lymphocytes for HDR and neutron irradiation:

	Dose (HDR)	Dose(neutrons)*	RBE
Lymphocytes	0.31 ± 0.05	0.32 ± 0.05	0.97 ± 0.21
CD4	0.27 ± 0.04	0.32 ± 0.05	0.84 ± 0.17
CD8	0.28 ± 0.04	0.32 ± 0.05	0.88 ± 0.17
CD19	0.36 ± 0.05	0.32 ± 0.05	1.13 ± 0.22

Table 3.6 RBE of neutron irradiation for lymphocytes and different lymphocyte populations.

*The uncertainty of dose measurements for neutron irradiation was 15% at the 95% confidence level.

It is seen that for the neutron irradiation RBE stays relatively close to unity as compared to HDR (^{137}Cs) irradiation.

4. Discussion

Analysis of γ H2AX using flow cytometry provides a sensitive and objective way to quantify the amount of phosphorylated H2AX protein. Although the integral signal measured doesn't provide the sensitivity of counting the number and/or size of the γ H2AX foci (these could be obtained using other techniques), flow cytometry proves to be a very useful tool in measuring of DNA damage relevant to DSBs.

The analysis of human peripheral blood lymphocytes has the advantage that access is easy and fast and doesn't require massive medical intervention.

And although whole blood and lymphocyte cultures are widely applied techniques to determine the cellular response to ionizing radiation, both of the assays involve procedures with uncertainties. These are variations in the different media and solutions employed and time and temperature differences. Potential inaccuracy in the irradiation or the cell concentration counting could contribute to possible errors of these experiments. Also variability is an intrinsic part of all living organisms. All the above mentioned may have contributed to the lack of precision of these experiments.

4.1 Dose and time response curves for HDR irradiation for isolated lymphocyte cultures

From Figures 3.1 and 3.2, obtained from data from all donors and donor #21, it is seen that the dose response curves were almost linear, bending slightly at about 2 Gy. Both curves showed a relatively high variability (the standard error of the mean reaches up to 20% of the mean value), indicating fairly high inter and intra variability of the γ H2AX biomarker. Results similar to these have been previously described for the dose range 0-40 Gy^{24, 25, 27, 36-40}. A linear relationship between the amount of γ H2AX histone and the dose was often found with a gradual saturation above this dose range. Most of these studies, however, were conducted using cell cultures that can differ in response from human lymphocytes and counting the number of γ H2AX foci per a cell as an endpoint, which is more laborious compared with use of flow cytometry. Also, in this study, there was no observed significant difference in the response to irradiation (response is considered significantly different if $p < 0.05$) between different populations of lymphocytes in the delivered dose range.

Our data largely support the notion of a linear relationship between HDR dose and amount of γ H2AX histone. This linear relationship, along with the speed of this assay, could make γ H2AX histone a very suitable biomarker for biodosimetry, although variability makes it hard to calculate the received dose very accurately.

Figure 3.5 shows the time response curves for the times up to 48 hours. It is seen that, up to 40 min, the γ H2AX response increases rapidly, with the maximum response plateau somewhere between 1 and 2 hours of incubation. After 2 hours of incubation the

response decreases gradually, reaching levels close to background after 24 hours and decreasing very slightly for next 24 hours. The samples were incubated up to 72 hours, but for 72 hours of incubations apoptosis was excessive and data not very reliable. Also 48 hours incubation the sample could be considered background level⁴¹, since the 0 hour sample showed the higher response, probably indicating that some phosphorylation had taken place at low temperatures (4 °C) within very short time. One study, however, showed residual phosphorylation, above background could be observed for 2 weeks after irradiation³⁷. Again this study was conducted counting the number of foci/cell.³⁷

Similar results have been reported in other work^{25, 27, 37, 40, 42, 43}, with phosphorylation being detected as soon as 1-3 min post irradiation^{25, 27, 43} and the maximum response at 9-30 min. Again these studies were performed with cells different from human lymphocytes and the number of γ H2AX foci per cell was counted. Although work was conducted for HFFF2 human fibroblast for both high and low LET irradiation and a small dose range 0.5-2 Gy, approximately the same maximum response time was shown⁴². On the other hand work, with human lymphocytes, showed a 30 min maximum response time, for the dose range applied for computed tomography examination⁴⁰. All of these previous studies support the idea that the exact form of time response varies with received doses, the type of cells and even changes between individuals³⁷⁻⁴⁰.

Again, statistical analysis showed no difference between populations of lymphocytes for all time ranges, although CD4 and CD8 populations demonstrated slightly stronger response compared to CD19 population.

Although γ H2AX biomarker shows very clear the dose response linear relationship, very short de-phosphorylation time most likely makes it impractical to use in biodosimetry.

4.2 Dose and time response curves for HDR irradiation of whole blood cultures

Figures 3.6 and 3.7 show dose response curves for data from all donors and donor #21 alone. A linear dose response curve is clearly seen, supporting previously cited references. Although the CD4 population shows the strongest response compared to CD19 throughout dose range 0.5-10 Gy (also Figures 3.8 and 3.9), there is no significant difference between these two populations.

Yet again, the time dependence curves for whole blood cultures (Figures 3.10, 3.11) for different lymphocyte populations demonstrated curves resembling ones reported for isolated lymphocytes cultures and previously referred works, with a maximum response between 1 and 2 hours of incubation and with a plateau duration of approximately 1 hour. Again it is seen that the responses of all populations abated quickly after reaching a maximum value (with CD4 fastest and CD19 slowest) and approached background levels after 24 or 48 hours of incubation. The responses of CD4 and CD8 populations were higher than for the CD19 population. P values were less than 0.05 for most points between 0.25 and 2 hours.

Also, besides the fact that CD4 and CD8 populations have the strongest response, they clearly de-phosphorylate the γ H2AX histone pool faster than CD19 lymphocytes, perhaps manifesting more radioresistance compared to CD19 cells (Figures 3.10, 3.11).

It is known that radioresistant cultures dephosphorylate faster than radiosensitive ones^{37-40, 42, 44}. In addition, previous studies observed a good correlation between the half time of loss of γ H2AX foci and the relative radiation resistance (surviving fraction after 2 Gy).^{38,39} The lower the half time of loss of γ H2AX, the more radioresistant cells.

Work based on post radiation apoptosis and survival curves using the clonogenic assay found that CD19 lymphocytes are more radiosensitive than CD4 and CD8 lymphocytes³⁰. Relative radiosensitivity of CD4 and CD8 lymphocytes is more contentious. Some authors find CD8 more radiosensitive than CD4 lymphocytes³¹ while others find no differences between these two populations³².

Figures 3.10 and 3.11 could be used to estimate half time ($T_{1/2}$) of DSB repair of human peripheral blood lymphocytes. This parameter could be roughly estimated as 2-2.5 hours and is consistent with estimate from another source⁴⁵, $T_{1/2} > 1$ hour.

As in the case of whole lymphocyte cultures (but even more pronounced) a linear dose response relationship is observed for the dose response curves of whole blood cultures, a similar time response curve, which unfortunately shows a short life (close to 24 hours) for the γ H2AX biomarker. It also appears that the processes of DSB repair and de-phosphorylation of the γ H2AX histone follow each other very closely.

4.3 Comparison of whole blood and isolated lymphocyte cultures

Figure 3.12 shows the relative responses of different lymphocyte populations for whole blood and isolated lymphocytes cultures. Although responses of populations in whole blood are higher than those in isolated lymphocytes culture, a significant difference for CD4 and CD8 populations was only observed for the highest dose of 10 Gy.

This is a somewhat unexpected result; since the opposite result has been shown before⁴⁶, suggesting that a decrease in radiation induced strand breaks in whole blood could be the result of the presence of radioprotective substances in blood. This discrepancy could be a result in part of slightly different treatments of lymphocytes in whole blood and isolated lymphocytes assays. There has been some evidence that lysis of whole blood in vitro can introduce DNA strand breaks⁴⁷. Although this has only been measured for single strand breaks and alkali-labile sites it could also cause DSBs and, therefore, increase γ H2AX.

4.4 Dose response curves for LDR irradiation

Figure 3.13 represents the dose response curves for different lymphocyte populations, for dose rate 0.27 Gy/h at 37°C.

The curves have three distinctive regions. Up to 0.5 Gy there is a nearly linear dose response, past 0.5 Gy the curves bend and reach a maximum in the vicinity of 1 Gy, then the response decreases, reaching a certain threshold. Statistically significant difference between CD4 and CD19 lymphocytes were observed only for 1.9 Gy.

A mathematical model for LDR dose response curves for the number of DSBs⁴⁸ exists that suggests that DSB induction and repair are similar in cells irradiated at 4°C and allowed to repair at 37°C and in cells irradiated at 37°C at different dose-rates. The results of irradiations at 37°C at different dose-rates were predicted from the dose response curves for the induction of DSBs and repair kinetic data from cells irradiated at 4°C and allowed to repair at 37°C. Qualitative analyses of the curves obtained in this thesis are in agreement with this work.

According to this model, the initial slope of the curve for DSB induction after LDR irradiation at 37°C has to be the same as the initial slope of the curve after HDR irradiation at 4°C due to non-zero repair half time. Although from Figures 3.15-3.18 it seems not to be the case, the curves representing the dose response for HDR irradiation were obtained after a 30 min incubation, whereas the number of DSBs would have been better represented by curves with an incubation time of 1-1.5 hours (maximum response for time response curves⁴³). Since the dose response curve for 1-1.5 hours, representing initial damage due to phosphorylation delay are steeper than curves for 30 min of incubation, 1.5 hours dose response curve may have been a better fit for the initial LDR region of Figure 3.13. The next region of the dose response curve for LDR represents the region where the repair mechanism is initiated (a repair halftime of 2-2.5 hours as was estimated) and takes over. The last flat region indicates DSB repair and induction balancing each other. The exact form of the dose response curves for LDR irradiation mostly depends on half repair time and dose rate.

The curves in Figure 3.13 reflect a competition between induction and repair of DNA DSBs. This struggle starts soon after the beginning of the irradiation and causes a bending of a dose response curve as observed in cells acutely irradiated at 4°C.

In Figures 3.15-3.18, it is observed that the amount of damage measured after LDR irradiation was considerably lower than in cells irradiated with HDR at 4°C. There was a clear dose rate effect^{48, 49, 50}, with more damage measured for a given dose when cells were irradiated at high dose rate than when cells were irradiated at a lower dose rate.

Interest in LDR irradiation also comes from evidence that a correlation between remaining DSBs and radiosensitivity may be found after exposure of human cells to radiation at 37°C at different dose rates^{48, 49}. But there is no difference in response for different types of lymphocytes for the maximum dose.

Regarding applications of the γ H2AX biomarker for biodosimetry, it is seen that dose calculations after LDR irradiations could be hindered if the dose lies in flat region of dose response curve and also if there is a long time delay before receiving the sample after exposure due to the short life-span of γ H2AX.

4.5 Dose response curves for neutron irradiation and RBE calculations

Figure 3.19 shows a curve based on original data for neutron irradiation. It was concluded that the curvature of the lines was probably due to cell repair, since it was difficult to keep the temperature at 4°C throughout the exposures, which lasted up to 1 hour. There is some resemblance to the dose response curves for LDR irradiation supporting the conclusion that repair was occurring. As a result, a linear approximation⁵¹,

⁵² based on the first non zero point was adapted (Figure 3.20) as an estimate of the dose response to neutrons with no repair occurring during the exposure.

As in all previous experiments it is seen that CD4 lymphocytes have the strongest response and CD19 the weakest.

From the Figures 3.21-3.24 it could be seen that response for HDR and neutron irradiation follow each other closely.

It is known that RBE values vary greatly according to cell lines and/or endpoints studied^{15, 53}. With respect to cytogenetic damage⁵⁴ (e.g. micronuclei, chromosome aberrations) it is well established that high LET or densely ionizing radiation such as neutrons are more effective than low LET or sparsely ionizing radiation. On the other hand, no differences in RBE values for neutrons versus X rays were found in thymocytes and peripheral blood lymphocytes⁵⁴.

In the present study, it was demonstrated that the RBE value for 340 keV neutrons relative to ¹³⁷Cs for this γ H2AX endpoint was very close to unity. RBE values from 1 to 1.5 have previously been obtained for fast neutrons^{51, 52, 55} using the endpoint of the number of DSBs, which relate to γ H2AX.

Other endpoints (e.g. apoptosis) show independence from LET. RBE values equal to unity were shown in a number of works^{53, 54}. One of the results⁵³ was obtained from neutron energy spectrum very close to the spectrum of this work. The RBE value obtained by this flow cytometry method compared well with the other results. Corresponding RBE values for neutrons with respect to DNA DSB formation and apoptosis may suggest that the latter is correlated with yield of DNA DSBs and not repair process⁵⁴.

5. Conclusions

The purpose of this study was to investigate the response of the γ H2AX histone in human lymphocytes and lymphocyte subpopulations to ionizing radiation, its application for biological dosimetry and for RBE calculations with regards to neutron irradiation.

Our experiments showed high sensitivity of human lymphocytes to ionizing radiation. For peripheral human blood lymphocytes, whole blood and isolated lymphocyte culture assays were applied for HDR irradiation. For isolated lymphocyte culture there were no differences observed between the lymphocyte populations for the dose and time response curves over the range studied. On the contrary, the whole blood culture assay showed the highest response for CD4 and CD8 lymphocytes compared to CD19 population, although there is no statistically significant difference for the dose response curves. A longer incubation time may have been required to observe statistically significant differences, because significant differences were observed for the time response curves particularly for incubation times from half hour and up to 4 hours.

Both culture conditions showed linear relationships between dose and γ H2AX response in the dose range from 0 to 10 Gy. It was shown that in the time response curves, γ H2AX increased quickly in the first 30 min, reached a maximum value between 1 and 2 hours and decreased to background levels within the next 24 hours. This indicated that a time between 1 and 2 hours would have been more appropriate for dose response experiments where there was need to detect the maximum response. Even though the isolated lymphocyte culture assay didn't show a different response for different types of lymphocytes, the whole lymphocyte culture showed that, for incubation times up to 2 hours, the CD4 cells had the highest response and CD19 the lowest. From

the time response curves, the approximate repair half time for DSB repair of human lymphocytes was estimated as 2-2.5 hours.

Both assays showed high intra and inter individual variability in γ H2AX response, and demonstrated that even though there is clear linear dose response relationship for phosphorylation of H2AX histones, applicability of this biomarker for biodosimetry is problematic because of the short post irradiation lifetime of γ H2AX histone, effectively not more than 24 hours.

The isolated lymphocyte culture assay showed no real advantages versus the whole blood culture assay. The isolated lymphocyte culture requires more time and blood than whole blood culture assay per experiment. Also the responses of isolated lymphocyte cultures were not different for different types of lymphocytes and were lower than expected. Differences in cell processing may have had something to do with it.

For LDR irradiations, the whole blood assay was applied. Difference in the response to irradiation between CD4 and CD19 lymphocytes was observed, but only at 1.9 Gy. The shape of dose response curve was in the agreement with a previously published theoretical model. For the LDR irradiation (0.27 Gy/h) and low dose range flow cytometry demonstrated very high sensitivity compared with the other methods. A dose rate effect was clearly seen as there was a lower response for the LDR in comparison with the HDR irradiation.

The calculation of RBE of high LET (neutrons) versus low LET (^{137}Cs) irradiation gives value close to unity, which is in agreement with results obtained for this endpoint from the other methods.

Overall, the γ H2AX marker meets a few requirements of a good biological indicator. These traits are a clear linear relationship between delivered dose and γ H2AX response, automation through the means of flow cytometry, short time to perform the assay (2 hours is a feasible time) and specificity of double strand breaks to ionizing radiation. However, the high inter and intra variability and a very short longevity of phosphorylated H2AX histones make this assay hard to apply in practical situations.

5.1 Future research

In all experiments, except for isolated lymphocyte culture, CD4 and CD8 lymphocytes showed the highest response whereas CD19 demonstrated the lowest response, although the difference was not always statistically significant.

Levels of γ H2AX histones for different types of lymphocytes (or any other cells) for the incubations times 20-24 hours, indicating residual non-repaired DSBs could be relevant to different radiosensitivity of the cells and could be used as an indicator of the cellular sensitivity⁴⁹, but more research is needed.

The repair halftime for the different types of cells also would be interesting to measure more accurately and compare to sensitivity, since evidence exists about reciprocal relationship between repair halftime and cellular radiosensitivity^{38, 39}.

Also the development of new fluorescent probes for flow cytometry opens new applications in the biological dosimetry. Applications of flow cytometry in micronuclei, PCC, or FISH are very promising.

References

- 1) Sally A Amundson, Michael Bittner, Paul Meltzer, Jeffrey Trent and Albert J Fornace Jr. Biological indicators for the identification of ionizing radiation exposure in humans. *Expert Rev. Mol. Diagn.* Jul; 1(2), 211-9 (2001).
- 2) A. Léonard, J. Rueff, G. B. Gerber and E. D. Léonard. Usefulness and limits of biological dosimetry based on cytogenetic methods. *Radiation Protection Dosimetry*, 115 (1-4), 448-454 (2005).
- 3) Philippe Voisin, Laurence Roy and Marc Benderitter. Why can't we find a better biological indicator of dose? *Radiation Protection Dosimetry*, 112(4): 465-469 (2004).
- 4) A. Montoro, P. Rodriguez, M. Almonacid, J. I. Villaescusa, G. Verdu', M. R. Caballin, L. Barrios and J. F. Barquinero. Biological dosimetry in a group of radiologists by the analysis of dicentrics and translocations. *Radiation research*, 164, 612–617 (2005).
- 5) International atomic energy agency, Vienna, 2001. Cytogenetic analysis for radiation dose assessment. A Manual. Technical reports series No. 405.
- 6) Bauchinger M. Quantification of low-level radiation exposure by conventional chromosome aberration analysis. *Mutat Res.* 339(3), 177–189 (1995).
- 7) A. T. Natarajan, and P. C. Kesavan. Cytogenetics for dosimetry in cases of radiation accidents and assessing the safety of irradiated food material. *Current Science*, vol. 89, N. 2, 360-365 (2005).
- 8) Guskova A.K, ed. Acute Radiation Effects in Victims of the Chernobyl Nuclear Power Plant Accident. United Nations, UNSCEAR 613-647 (1988).

- 9) IAEA, Use of electron paramagnetic resonance dosimetry with tooth enamel for retrospective dose assessment. Report of a coordinated research project. IAEA-TECDOC-1331. International atomic energy agency, Vienna, December 2002.
- 10) R. A. Kleinerman, A. A. Romanyukha, D. A. Schauer and J. D. Tucker. Retrospective assessment of radiation exposure using biological dosimetry: chromosome painting, electron paramagnetic resonance and the glycophorin A mutation assay. *Radiation research*, 166, 287–302 (2006).
- 11) Prasanna P.G., Kolanko C.J., Gerstenberg H.M., Blakely W.F. Premature chromosome condensation assay for biodosimetry: studies with fission-neutrons. *Health Phys.* 72, 594–600 (1997).
- 12) P. Voisin, N. Paillole, M. Benderitter, M. Claraz, V. Chambrette, M. Delbos, V. Durand, A. Leroy and I. Sorokine-Durm. Improvement Of Biological Dosimetry By Cytogenetics For Operational Purposes: Dicentrics Or Micronuclei? Institut de Protection et Sûreté Nucléaire, Département de Protection de la santé de l'Homme et de Dosimétrie. IPSN, BP6, F-92265 Fontenay-aux-Roses Cedex, France.
http://net-science.irsn.org/net-science/liblocal/docs/docs_DPHD/IRPA10-P3A-217.pdf
- 13) Wayne M. Becker, *The World of the Cell*, The Benjamin/Cummings Publishing Company, Menlo Park, CA, 1986.
- 14) Neil A. Campbell, Jane B. Reece, *Biology*, 6th Edition. Benjamin Cummings, 2002.
- 15) Eric J. Hall, *Radiobiology for the Radiobiologist*, 4th Edition. J.B. Lippincott Company, 1994.
- 16) R. Pusarla, P. Bhargava, Histones in functional diversification. Core histone variants. *FEBS Journal*, 272, 5149-5168 (2005).

- 17) J.F. Ward. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. *Prog. Nucleic Acid Res. and Mol. Biol.*, 35, 95-125 (1988).
- 18) R.D. Stewart. The Nature of a Fatal DNA Lesion. PNNL-SA-30810, June 25, (2001).
http://www.pnl.gov/berc/bg/fatal_lesion.html
- 19) Smith J., Smith K., Mezard C. Tying up Loose Ends: Generation and Repair of DNA Double-Strand Breaks. (2001).
<http://www.infobiogen.fr/services/chromcancer/Deep/DoubleStrandBreaksID20008.html>
- 20) Lankinen M. H., Vilpo L. M., Vilpo J. A., UV- and gamma-irradiation-induced DNA single-strand breaks and their repair in human blood granulocytes and lymphocytes. *Mutat. Res.*, 352(1-2), 31-8, (1996).
- 21) Thiriet C., Hayes J.J. Chromatin in need of a fix: phosphorylation of H2AX connects chromatin to DNA repair. *Mol Cell.*, 18(6):617-22, (2005).
- 22) Paull T.T, Rogakou E.P, Yamazaki V, Kirchgessner C.U, Gellert M, Bonner W.M. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr. Biol.* 10(15): 886-95 (2000).
- 23) E. R. Foster and J. A. Downs. Histone H2A phosphorylation in DNA double-strand break repair. *FEBS Journal*, 272, 3231-3240 (2005).
- 24) D. R. Pilch, O. A. Sedelnikova, C. Redon, A. Celeste, A. Nussenzweig, and W. M. Bonner, Characteristics of γ -H2AX foci at DNA double-strand breaks sites. *Biochem. Cell Biol.* 81(3): 123–129 (2003).
- 25) E. P. Rogakou, D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner, DNA Double-stranded Breaks Induce Histone H2AX Phosphorylation on Serine 139.

J. Biol. Chem. 273 (10), 5858-68 (1998).

26) A. Celeste , S. Petersen , P. J. Romanienko , O. Fernandez-Capetillo , H. T. Chen, O. A. Sedelnikova , B. Reina-San-Martin , V. Coppola , E. Meffre , M. J. Difilippantonio , C. Redon , D. R. Pilch , A. Olaru , M. Eckhaus , R. D. Camerini-Otero , L. Tessarollo , F. Livak , K. Manova , W. M. Bonner , M. C. Nussenzweig , A. Nussenzweig, Genomic Instability in Mice Lacking Histone H2AX, *Science*, 296 (5569), 922 – 927 (2002).

27) E. P. Rogakou, C. Boon, C. Redon, and W. M. Bonner, Megabase Chromatin Domains Involved in DNA Double-Strand Breaks In Vivo. *J. Cell Biol.*, 146 (5), 905-916 (1999).

28) H. M. Shapiro, *Practical Flow Cytometry*, Fourth Edition, John Wiley & Sons, Inc. (2003).

29) *Flow Cytometry: A Practical Approach*, Third Edition, Edited by M. Ormerod, Oxford University Press. (2000).

30) S.K. Durum , N. Gengozian, The Comparative Radiosensitivity of T and B Lymphocytes, *Int. J. Rad. Biol.*, 34 (1), 1-15 (1978).

31) R. C. Wilkins , B. C. Kutzner , M. Truong , J. R. N. McLean, The effect of the ratio of CD4⁺ to CD8⁺ T-cells on radiation-induced apoptosis in human lymphocyte subpopulations, *Int. J. Rad. Biol.*, 78 (8), 681 - 688 (2002).

32) Nakamura N., Kusunoki Y., Akiyama M. Radiosensitivity of CD4 or CD8 positive human T-lymphocytes by an in vitro colony formation assay, *Radiat Res.* 123(2): 224-7 (1990).

33) Chow S., Hedley D., Grom P., Magari R., Jacobberger J.W., Shankey T.V. Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry

of intracellular phosphorylated epitopes in leukocyte subpopulations. *Cytometry A*. 67(1), 4-17 (2005).

34) D. A. Allen and T. D. Beynon, Design for an accelerator-based orthogonal epithermal neutron beam for boron neutron capture therapy, *Phys. Med. Biol.* 40, 807-821 (1995).

35) Irradiation/Shielding Cavity for the Accelerator Neutron Beam at McMaster.

36) W. M. Bonner, Low-dose radiation: Thresholds, bystander effects, and adaptive responses, *PNAS* 100(9), 4973-4975 (2003).

37) K. Rothkamm and M. Löbrich, Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses, *PNAS* 100(9), 5057-5062 (2003).

38) MacPhail S.H., Banath J.P., Yu T.Y., Chu E.H., Lambur H., Olive P.L., Expression of phosphorylated histone H2AX in cultured cell lines following exposure to X-rays, *Int. J. Rad. Biol.* 79(5), 351-359 (2003).

39) J. P. Banáth, S. H. MacPhail and P. L. Olive, Radiation Sensitivity, H2AX Phosphorylation, and Kinetics of Repair of DNA Strand Breaks in Irradiated Cervical Cancer Cell Lines, *Cancer Research*, 64, 7144-7149 (2004).

40) Lobrich M., Rief N., Kuhne M., Heckmann M., Fleckenstein J., Rube C., Uder M. In vivo formation and repair of DNA double-strand breaks after computed tomography examinations. *PNAS* 102(25), 8984-9 (2005).

41) M. Lobrich, B. Rydberg, P.K. Cooper, Repair of X-Ray-Induced DNA Double-Strand Breaks in Specific Not I Restriction Fragments in Human Fibroblasts: Joining of Correct and Incorrect Ends. *PNAS* 92,12050-12054. (1995).

42) A. Antoccia, A. Sgura, Cavinato, M., R. Cherubini, S. Gerardi and C. Tanzarella, Histone H2AX phosphorylation in human primary fibroblasts after X-ray and low-energy

proton irradiation. University of Rome and INFN-Sezione “Roma Tre”, INFN, Laboratori Nazionali di Legnaro, Italy,

http://www.inl.infn.it/~annrep/read_an/2003/contrib_2003/pdfs/B041STT.pdf

- 43) F. Antonelli, M. Belli, G. Cuttone, V. Dini, G. Esposito, G. Simone, E. Sorrentino, M. A. Tabocchini, Induction and Repair of DNA Double-Strand Breaks in Human Cells: Dephosphorylation of Histone H2AX and its Inhibition by Calyculin A, *Radiat Res.* 164(4), 514-7 (2005).
- 44) N. Taneja, M. Davis, J. S. Choy, M. A. Beckett, R. Singh, S. J. Kron, and R. R. Weichselbaum, Histone H2AX phosphorylation as a predictor of radiosensitivity and target for radiotherapy, *J. Biol. Chem.* 279(3), 2273-2280 (2004).
- 45) Tobin S.E., Itzhaki R.F., Three classes of DNA strand breaks induced by X-irradiation and internal B-rays. *Int. J. Radiat. Biol.* 63(5), 617-622 (1993).
- 46) Banath, J.P, M. Fushiki, and P.L Olive, Rejoining of DNA single- and double-strand breaks in human white blood cells exposed to ionizing radiation, *Int. J. Radiat. Biol.*, 73(6), 649–660 (1998).
- 47) S. Narayanan, M.R. O'Donovan, S.J. Duthie, Lysis of whole blood *in vitro* causes DNA strand breaks in human lymphocytes, *Mutagenesis*, 16(6), 455-459 (2001).
- 48) Foray N.; Fertil B.; Alsbeih M. G. A.; Badie C.; Cvavaudra N.; Iliakis G.; Malaise E. P, Dose-rate effect on radiation-induced DNA double-strand breaks in the human fibroblast HF19 cell line, *Int. J. Radiat. Biol.*, 69(2), 241–249 (1996).
- 49) Dikomey E, Brammer, Relationship between cellular radiosensitivity and non-repaired double-strand breaks studied for different growth states, dose rates and plating conditions in a normal human fibroblast line, *Int. J. Radiat. Biol.*, 76(6), 773-781 (2000).

- 50) A. Vral, H. Thierens, L. DE Ridder, Study of dose-rate and split-dose effects on the In vitro micronucleus yield in human lymphocytes exposed to X-rays, *Int. J. Radiat. Boil.*, 61(6), 777-784 (1992).
- 51) I. Furuno, T. Yada, H. Matsudaira, T. Maruyama, Induction and Repair of DNA Strand Breaks in Cultured Mammalian Cells Following Fast Neutron Irradiation, *Int. J. Radiat. Biol.*, 36(6), 639 – 48 (1979).
- 52) M. Spothem-Maurizot; M. Charlier; R. Sabattier, DNA Radiolysis by Fast Neutrons, *Int. J. Radiat. Biol.*, 57(2), 301-313 (1990).
- 53) L.A. Ryan, R.C. Wilkins, N.M. McFarlane, M.M. Sung, J.P. McNamee, and D.R. Boreham, Relative Biological Effectiveness of 280 keV Neutrons for Apoptosis in Human Lymphocytes, *Health Physics*, 91(1), 68-75 (2006).
- 54) Vral, A., Cornelssen, M., Thierens, H., Louagie, H., Philippe, J., Strijckmans, K., and De Ridder, L., Apoptosis induced by fast neutrons versus ⁶⁰Co gamma-rays in human peripheral blood lymphocytes, *Int. J. Radiat. Biol.*, 73(3), 289-295 (1998).
- 55) R. A. Britten, L. J. Peters, D. Murray, Biological Factors Influencing the RBE of Neutrons: Implications for Their Past, Present and Future Use in Radiotherapy, *Radiation Research*, 156(2), 125–135 (2001).