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Establishment of *in vitro* ¹⁹²Ir γ -ray dose-response relationship for dose assessment by the lymphocyte dicentric assay

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In vitro dose-response relationships are used to describe the relation between dicentric chromosomes and radiation dose for human peripheral blood lymphocytes. The dicentric yield depends on both the dose and the radiation quality. Thus, for reliable dose estimation *in vitro* dose responses must be determined for different radiation qualities. This paper reports the work for setting up the relationship for the dicentric production in the lymphocytes exposed *in vitro* to ¹⁹²Ir γ -rays at Central Laboratory for Radiological Protection (CLOR). In a case of a radiation accident in industrial radiography using ¹⁹²Ir sealed sources, this will be the basis for the indirect evaluation of the γ -ray dose to which an accidental victim was exposed.

Key words: biodosimetry, cytogenetic analysis, radiation exposure

Introduction

In cases of accidental radiation exposures, reliable and accurate dose estimation is crucial in making life saving medical decisions for accident victims, evaluating risks to late health effects or giving reassurance to non-exposed individuals. In accidental radiation exposures, where physical dosimetry measurements are incomplete or absent, biological dosimetry proves to be a valuable method for assessing radiation absorbed doses to individuals. The analysis of dicentric chromosomes in human peripheral blood lymphocytes is recognised to be a valuable method for assessing radiation absorbed dose [6, 7].

Ionising radiation induces structural chromosome aberrations in human peripheral lymphocyte that can be quantifiably measured using cytogenetic analysis. One such aberration is a dicentric chromosome that is caused by misrepair of DNA double strand breaks (DSBs) induced in G₀ or G₁ phase of the cell cycle. The yield of radiation-induced dicentrics depends on both the dose and the radiation quality. Numerous studies have demonstrated that dicentrics induced in peripheral blood lymphocytes after

in vitro exposure to low linear energy transfer (LET) radiation are both qualitatively and quantitatively similar to dicentrics observed after *in vivo* exposure. Dose-response relationships obtained from carefully controlled *in vitro* studies are used to estimate the dose for exposed individuals. Since the cytogenetic analysis uses circulating blood lymphocytes, radiation-induced dicentrics reflect the average total-body dose, independent of specific regions of the body that have been exposed [6, 7].

For producing a dicentric two DNA DSBs in two unduplicated chromosomes are required, and these two DSBs may be induced by one or two ionising particles traversing the cell nucleus. The expected dicentric yield depends on both the dose and the dose rate. A general dose-response model that describes this relationship has been presented by Kellerer and Rossi, using the theory of dual radiation action [9]. Dual radiation action results in the proportionality between the average yield of dicentrics and two components that are proportional to the first and the second power of radiation absorbed dose. The linear term (aD) in the dose response relationship gives the yield of dicentrics produced by one and the same particle track, while the yield of dicentrics produced by two different, independent particle tracks is given by the quadratic term (bD^2). The aD term dominates for very low doses of low LET radiation (e.g. X- and γ -rays), because in the low-dose range the track density is low. The bD^2 component becomes dominant at higher doses, with a/b being the dose at which the contributions to the dicentric formation of these two components are equal. Thus, the dose-response relationship for the dicentric production by low LET radiation is linear-quadratic: $Y=aD+bD^2$. With increasing LET there is an increased probability that two DSBs will be induced by one track. For this reason the aD term dominates over the entire dose-range used. Therefore, the dose-response relationship for the dicentric production by high LET radiation (e.g. neutrons, α -particles) tends to be linear: $Y=aD$.

Dicentrics are the biomarker of choice for biological dosimetry by chromosome aberration analysis for several reasons. They are easily recognised from normal chromosomes (contain two centromeres instead of the one normally present in each chromosome), and are considered relatively specific to radiation (only a few radiomimetic chemicals are known to interfere with the lymphocyte dicentric assay). They have low background frequency (about 1 dicentric in 1000 cells), high sensitivity (it is possible to detect exposure as low as 0,05 Gy), as well as known dose-response relationships for different radiation's qualities and dose-rates. Moreover, dicentrics are characterised by good reproducibility and comparability of *in vitro* and *in vivo* results.

Since its introduction in 1962 by Bender and Gooch [2], the lymphocyte dicentric assay has been applied in nearly every accidental or occupational exposure. Currently this assay is the most documented and validated method of biological dosimetry that is used every time when there is a need to document radiation doses in individuals [6, 7].

The most common radiation accidents are in industrial radiography using γ -rays from multicurie radionuclide sources [6, 7, 12, 13]. There are many reasons for such

accidents. For example: the sealed source may occasionally detach from the cable, which returns it to the housing. At times the cable may fail to return the source to the housing. Or the radiographer may forget that the source is not in its housing at the time he is changing film or positioning the cable.

This paper reports the work for setting up a dose-response relationship for the dicentric assay for γ -rays from ^{192}Ir source at CLOR. ^{192}Ir is commonly used in industrial radiography due to a wide complex energy spectrum of photons emitted from this radionuclide (0.1 - 1.1 MeV, average 0.37 MeV) and its high specific activity (9.1×10^3 curies per gram). In the cases of accidental or suspected radiation exposures in industrial radiography, the *in vitro* ^{192}Ir γ -ray dose-response relationship will be used for converting the observed dicentric frequency in an exposed individual's lymphocytes into the absorbed dose.

Material

Blood samples were obtained from a healthy 65 years old woman and a 29 years old man. Both blood donors were volunteers from CLOR that have been aware of the nature and objectives of the study. The blood samples were taken by vacupuncture using the 7,5 ml S-Monovette enclosed blood collection system (Sarsted) containing calcium-balanced lithium heparin anticoagulant. After the blood collection tubes were inverted several times to dissolve and mix the blood and heparin.

Methods

In vitro γ -irradiation

Whole blood samples from each donor were irradiated in 2 ml polyethylene tubes with seven γ -ray doses in the range 0,175-2,8 Gy.

The irradiation was performed using a Gammamat TSI-3 industry standard γ -ray projector that contained the ^{192}Ir source with an activity 2644 GBq at the time of exposure. The dose rate at the distance of 15 cm between the source and the sample was calculated to be 0,113 Gy/min. The dose rate was measured with a tissue-equivalent ionisation chamber before irradiation. The samples were irradiated at room temperature with doses of 0.175; 0.35; 0.5; 0.7; 1.4; 2.1 and 2.8 Gy. The doses in blood samples were monitored using MCP-N (LiF: Mg, Cu, P) type thermoluminescent dosimeters, which have the shape of pellets with dimension 4,5 mm in diameter and 1 mm in thickness. The TLD were attached to the bottom of the tubes with the blood. The method of TLD using MCP-N material has a very good linear response in a wide range of assessed doses from 0.1mGy up to 10 Gy [3]. Detectors after irradiation were evaluated with a RADOS reader in constant temperature read-out condition, according to the internal procedure of the

laboratory. The standard expanded uncertainty associated with the doses assessed by TLD was lower than 17%. This uncertainty was due to calibration error, inhomogeneity of the detectors, variable position of the detectors during irradiation and variable time for irradiation ending and sample recovering.

Control blood samples were kept at the same room temperature for the same period of time, but received no radiation dose.

Blood culture and slide preparation

After irradiation, the samples were transported back to the laboratory and incubated at 37°C for 2 hours to allow DNA repair processes to take place. From each sample, three whole blood cultures were set up as insurance against poor yields of the first cell cycle metaphases *in vitro*. The cell-culture procedure and chromosome preparations were based on the internationally accepted protocol for lymphocyte chromosome aberration analysis for radiation dose assessment [6, 7].

Lymphocyte cultures were set up using 0.5 ml whole blood and 4,5 ml PB-MAX™ Karyotyping medium (Gibco), containing liquid RPMI 1640 medium, 15% (v/v) foetal bovine serum, gentamicin sulphate at 35 µg/ml, 2 mM L-glutamine, 1% (v/v) phytohaemagglutinin (PHA) and 1% sodium heparin (Sigma-Aldrich). The cultures were incubated in an Assab incubator at 37°C in a 5% CO₂ atmosphere with 95% humidity for 48 h. After 24 h of stimulation with PHA, colcemid (Gibco) was added to a final concentration of 0.05 µg/ml to stop cell cycle progression in the first division metaphases and then incubated for the additional 24 h. Less than 3% of the metaphases were in second division metaphases at this culture time (as was determined by the use of cytochalasin B which allows cells that have completed one nuclear division to be recognised by their binucleated appearance – data not shown).

After hypotonic treatment with 0,075 M KCl for 20 min at 37°C, the lymphocytes were fixed 3 times in methanol-glacial acetic acid (1:3) and dropped onto the centre of clean glass slides. Slides were allowed to dry for at least 24 hours and uniformly stained with 5% Giemsa solution in fresh Gurr buffer for 10 minutes. Slides were then rinsed with distilled water and dried before evaluation.

Scoring criteria

Slides were examined with an optical microscope (Nikon or Zeiss). Dicentrics were scored in well spread metaphases with 46 centromeres and long enough chromosomes to recognise clearly multicentric chromosomes and associated acentric fragments. Chromosomes with three centromeres accompanied by two acentric fragments were recorded as tricentrics and counted as two dicentrics. For the assessment of the

dicentric frequencies only dicentric chromosomes with associated acentric fragments were included.

Statistical methods

Before fitting a linear-quadratic model to the data points given at each dose, a test on the distribution of dicentrics was carried out in order to determine whether the observed frequency at each data point conform to a Poisson distribution. For this purpose the variance/mean ratio (σ^2/Y) and the u-test [5] were used. The u-test of goodness of fit relies on the σ^2/Y value and the overdispersion parameter (u). A σ^2/Y value close to 1 confirms that the data follows the Poisson. Any deviation of σ^2/Y from unity indicates overdispersion compared with the Poisson distribution. A u-value between -1.96 and 1.96 indicates the distribution of dicentrics is Poissonian.

Results

Our *in vitro* dose-response experiment was designed for the simulation of the acute, uniform irradiation scenario. For satisfactory fitting to linear-quadratic dose-response relationship, the blood samples were irradiated with 7 doses in the range 0.175-2.5 Gy. This translates to 5 degrees of freedom equal to the number of data points (eight) minus the number of fitted parameters (three).

Scoring of equally stained metaphases in donors' peripheral blood lymphocytes irradiated *in vitro* with ^{192}Ir γ rays showed that the main types of structural chromosome aberrations observed were dicentrics (Fig.1a), tracentrics (Fig.1b), accompanying and excess acentrics, interstitial deletions (so-called double minutes), and occasionally rings.

Figure 1a. The picture represents an abnormal metaphase containing a dicentric chromosome and its accompanying acentric fragment

Figure 1b. The right picture shows a tracentric and its accompanying acentric fragments. Both pictures are taken from samples irradiated *in vitro* with dose of 2.8 Gy

The dose-response data sets obtained for each donor for the dicentric production in control and irradiated lymphocytes were pooled and presented in Table 1. Progressive increasing in radiation doses resulted in decrease in the number of cells with no dicentrics and increase in the number of cells with multiple dicentrics. The distribution of dicentrics at each dose fits a Poisson statistics as determined by the variance/mean ratio and u-test. These findings are consistent with observations that the distribution of chromosome aberrations for low LET radiation should be Poissonian [5]. Moreover, the Poisson distribution of dicentrics among all scored cells confirms the uniform irradiation of the blood sample.

Table 1. Intercellular distribution of dicentrics in human lymphocytes exposed in vitro to ^{192}Ir γ -rays. A σ^2/Y value close to 1 and u value between -1.96 and 1.96 indicate a Poisson distribution

Dose [Gy]	Analysed cells	Intercellular distribution of dicentrics				Observed dicentrics	Dicentrics per cell	σ^2/Y	u-value
		0	1	2	3				
0	2000	1998	2	0	0	2	0,001	1,00	-0,02
0,175	515	510	5	0	0	5	0,010	0,99	-0,14
0,35	539	531	8	0	0	8	0,015	0,99	-0,23
0,50	287	281	6	0	0	6	0,021	0,98	-0,23
0,7	625	590	33	2	0	37	0,059	1,05	0,91
1,4	660	571	84	3	2	96	0,145	1,04	0,80
2,1	1000	712	251	31	6	331	0,331	0,97	-0,76
2,8	553	341	163	44	5	266	0,481	0,96	-0,59

The points of the dose-response relationship were fitted to the linear-quadratic model:

$$Y = c + aD + bD^2, \quad (1)$$

where Y is the dicentric frequency, D is the dose for lymphocytes, c is the background dicentric frequency at zero dose, and a and b coefficients, theoretically, represent the one track and two track components of the dose-response model. The objective of the fitting was to produce the coefficients that best fit the experimentally obtained data. Calculating the values of c , a , and b coefficients was conducted according to the Papworth method [10], using maximum likelihood and weights of the data points given by multiplying the Poisson derived variance of data points by the ratio of variance/mean. For the fitting procedure a PC-based, freely available program called CABAS was used [4]. The fitted coefficients were $c=0.001$, $a=0.034$ and $b=0.053$. Table 2 summarises the values of c , a and b for orthovoltage X-rays (243 kVp), ^{192}Ir γ -rays and ^{60}Co γ -rays with the mean energy of 0.122MeV, 0.37 MeV and 1.2 MeV, respectively. These three types of radiation were used to produce the coefficients used for purposes of biological dosimetry at CLOR. Values of the coefficients shown in Table 2 were used for producing an illustrative comparison of dose-response calibration curves for these radiation types.

As may be seen in Figure 2, the shape of these curves is consistent with the LET dependence for dicentric yields for low-LET sources. The curves are rather similar, but with γ -rays being less effective at producing dicentrics than X-rays. The value of a maximum relative biological effectiveness for the dicentric production by X-rays or γ -rays was derived from the ratio of the coefficient a of the test radiation to the coefficient a of the reference radiation: ($\text{RBE}_{\text{max}} = a_{\text{test}}/a_{\text{ref}}$). Relative to ^{60}Co γ -rays, the values for the RBE_{max} were found to be 2.9 for 243 kVp X-rays, 2.8 for ^{192}Ir γ -rays and 1 for ^{60}Co γ -rays.

Table 2. A summary of values of c , a , and b and their standard deviations (SD) for the three types of radiation used for setting up *in vitro* dose-response calibration relationships at CLOR

Type of radiation	$c \pm \text{SD}$	$a \pm \text{SD} (\text{Gy}^{-1})$	$b \pm \text{SD} (\text{Gy}^{-2})$
^{60}Co γ -rays	$0,0010 \pm 0,0004$	$0,012 \pm 0,004$	$0,056 \pm 0,002$
^{192}Ir γ -rays	$0,0010 \pm 0,0007$	$0,034 \pm 0,010$	$0,053 \pm 0,006$
243kVp X-rays	$0,0011 \pm 0,0004$	$0,035 \pm 0,003$	$0,064 \pm 0,002$

Figure 2. Graphical presentation of the correlation between dicentrics and radiation dose for human peripheral lymphocytes for three low LET radiations used for setting up *in vitro* dose-response calibration relationships at CLOR

Discussion

Dicentric yield from radiation exposure is dependent not only on the dose, but also on radiation quality (LET) and relative biological effectiveness (RBE). Dicentrics are, thus, a good example of radiation-induced biological effect where it is possible to discriminate differences between radiation qualities that are generally termed low LET (and are ascribed with a weighting factor (w_R) of 1 for use in radiological protection). With dicentrics, differences in biological effectiveness can be seen. They are reflected in the linear yield coefficient (a), which is a measure of intra-track interaction leading to dicentric formation, and are, thus, most evident in low doses. The reason for this stems from differences in lineal energy distributions for radiation of different qualities [8]. The lineal energy, its dose and the dose-weighted mean of the lineal energy and the frequency-weighted mean of the lineal energy are microdosimetric parameters that describe the radiation energy interactions at the microscopic level. Microdosimetric aspects of the dicentric chromosome formation were reviewed by Bauchinger [1].

The aim of biological dosimetry is calculating the absorbed dose on the basis of observed dicentric frequency in an exposed individual's lymphocytes. This process is conducted with the use of the fitted coefficients of the particular *in vitro* dose-response relationship and the statistical-mathematical models described in the IAEA technical manual [7] for a whole-body exposure, partial-body exposure and fractionated or protracted exposure. The assessment of the dose is particularly easy in cases of acute whole-body exposures. Since the dose-response relationship for the dicentric production by low LET radiation is linear-quadratic ($Y=c+aD+bD^2$), an estimate of the unknown dose (D_x) is the positive root of the *in vitro* dose-response equation:

$$D_x = [-a + \sqrt{a^2 + 4b(Y - c)}] / 2b \quad (2)$$

In vitro dose-response relationship is an average for all blood donors of different age, sex and sensitivity to radiation. Therefore, the estimate of dose obtained by reference to this relationship must also be considered as an average estimate for all exposed individuals with the same observed dicentric frequency. An estimate for the exposed individual alone is rather not possible, because it would require a dose response specific to that particular individual.

The difficulty in determining the range of uncertainty in dose arises from the fact that there are two components on this uncertainty. One component originates from the Poisson nature of observed dicentric yield [5], and the other from uncertainties associated with fitting the dose response relationship [11]. The simple method of estimating the 95% confidence limits of dose relies on the use of the Poisson distribution of dicentrics observed in blood samples from irradiated individuals [14]. The method requires determining the yields corresponding to the lower and upper confidence limit on the observed dicentric yield (Y_L and Y_U) and reading off the value of dose for which

Y_L and Y_u crosses the dose-response curve (Figure 3). These doses correspond to the 95% lower and upper confidence limits (D_L and D_u), that define the interval, which enclose the true dose on 95% of the occasions.

Figure 3. Illustration of the method for estimating the 95% confidence limits of the interpolated dose.

CLOR's dose-response relationship for the dicentric assay for ^{192}Ir γ -rays was applied for re-assessment of a dose for an industrial radiographer. The man was irradiated by a 2.63 TBq ^{192}Ir source that had detached from the cable and he was trying to return it back to the housing. Since a personal dosimeter was not worn, a dose to which he had been exposed was estimated by radiation protection officers from the National Atomic Energy Agency. The average total-body dose was about 0.09 Gy (personal communication). This value was based on the reconstruction of the event, dose rate measures and physical calculation by the inverse square law, making no allowance for scattered radiation and assuming that the man was moving.

The case was referred for biological dose assessment in 2011. At that time only *in vitro* dose-response relationships for 243 kVp X-rays and ^{60}Co γ -rays were available at CLOR. The number of dicentrics scored was 7 in 1400 cells. Using the coefficients **c**, **a** and **b** for ^{60}Co γ -rays, the average total-body dose estimate of 0.18 Gy with 95% confidence limits ranging from 0.06 to 0.32 Gy was obtained. Since cobalt-60 is less effective at producing dicentrics than irridium-192, the dose for the exposed radiographer was overestimated. The use of the coefficients for ^{192}Ir γ -rays resulted in an estimate of the average total-body dose of 0.10 Gy with the 95% confidence interval of 0.03 - 0.21 Gy. Then the difference between the biological and physical dose estimates became significantly reduced.

The distribution of dicentrics was Poissonian (the σ^2/Y value of 1 and the u-value of 0.12), therefore no partial body exposure occurred. All calculations were conducted with the use of the chromosome aberrations calculating software (CABAS).

Conclusions

The **a** and **b** coefficients of the *in vitro*-produced dose-response relationship are influenced both by the radiation quality (LET) and the relative biological effectiveness (RBE) of any particular radiation. Therefore, for reliable dose estimates in accidental and suspected radiation exposures, the dose-response relationship to be used should be that of a radiation quality which is the same as, or very similar to, that of the particular type of radiation under study.

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