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PCC-ring Induction in Human Lymphocytes Exposed to Gamma and Neutron Irradiation

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PCC-rings/Dose-effect curves/High doses radiation/Calyculin A/Human lymphocytes.

In case of an accidental overexposure to ionizing radiation where the dose received by the victim is over 5 Gy, the conventional biological indicator of dose, the dicentric assay, does not provide an accurate enough dose measurement. A more appropriate technique is to measure ring chromosomes in stimulated lymphocytes. Dose-effect relationships were obtained by plotting the frequencies of Premature Chromosome Condensation (PCC)-rings in PCC lymphocytes obtained by chemical induction with Calyculin A *in vitro*, irradiated with doses between 5 to 25 Gy. Cells were exposed either to neutron or to gamma rays and the corresponding dose effect curves are presented in this paper for the first time in literature. For the elaboration of these curves, 9 675 PCC cells in G1 G2 and M/A stages were analysed. The results were fitted to a lineal model in gamma irradiation up to 25 Gy. For neutron irradiation the data was fitted to a lineal model up to 10 Gy, and then dose saturation was observed. In conclusion, with this technique it is possible to set up dose effect curves up to 25 or 10 Gy according to the gamma or neutron radiation.

INTRODUCTION

Premature Chromosome Condensation-ring (PCC-R) in Giemsa stained lymphocytes has become an attractive method for biological dosimetry particularly for high dose exposure. This method proposed by Kanda[1)] combines the possibilities of the efficiently premature chromosome condensation induction by the okadaic acid or the Calyculin A with the simplicity of the Giemsa staining.[2)] This method overcomes the three major problems of the conventional biological dosimetry by dicentric analysis at high doses: (i) the lymphopenia due to cell death reducing the number of lymphocytes available; (ii) the radio-induced cell cycle arrest causing low mitotic index; (iii) the dicentrics production saturation at high doses reducing the precision of the dose estimation. Therefore the mitotic index is low and the number of cells available to have a statistically significant result based on at least 100 cells scored is very difficult to achieve at such high doses. In addition, as the increase rate of dicentrics with the dose is less pronounced the dose estimation is less precise.[1)] PCC chemically induced is efficiently obtained even in cells exposed *in vitro* up to 40 Gy dose radiation.[3)] The Tokai-mura accident showed the possibilities to apply this technique in cases exposed up to around 20 Gy of gamma irradiation.[1)] However in this accident exposure radiation was a mix of neutron and gamma rays and no corresponding PCC ring dose effect curve was available. Up to now just one gamma rays PCC-R curve was published but without any coefficient calculation to be used to estimate a dose. In the present work this technique was applied *in vitro* for fission neutron radiation with doses up to 25 Gy. Furthermore, the relevance of this technique was confirmed in the interval of 5–25 Gy for gamma radiations.

MATERIALS AND METHODS

Blood sample exposure

Peripheral blood was drawn with heparinized syringes from two healthy individuals, a set of tubes was immediately irradiated with ^{60}Co gamma rays exposure at 5, 7.5, 10, 20 and 25 Gy (0.5 Gy.min^{-1}). Irradiations were performed at 37°C, the exposure time being 10 minutes for the lowest dose and 50 minutes for the highest one. The ^{60}Co gamma ($E\gamma$ = 1.25 MeV) exposures were performed on ICO-4000 facility at Fontenay-aux-Roses (France). For reproducible positioning, a PMMA (poly méthacrylate de méthyle) sample holder was added inside the water bath. Kerma rate was

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determined using a PTW ionization chamber irradiated in the conditions used for the samples: water bath, sample holder and sample location. Dosimetry was expressed in terms of tissue Kerma. Uncertainty on Kerma rate of 4% at 2σ.

Two other sets of blood samples were exposed during the same experiment to fission neutrons at the SILENE facility (Valduc, France) at the following doses: 5, 7.5, 10, 20 and 25 Gy. A chain reaction was produced in an experimental reactor resulting in 3.8×10^{17} fissions. A lead shield was placed around the reactor in order to limit the gamma exposure. Irradiation lasted 234 seconds and the mean energy of the neutron spectrum is 0.49 MeV. The photon and the neutron dose components of the SILENE radiation field were estimated respectively using alumina oxide powder and silicon diode as passive dosimeters, they are reported in Table 2.[4)]

Following irradiation, blood samples were maintained at 37°C for 2 hours allowing to act the cellular reparation mechanisms.

Cell culture

Lymphocytes were cultured for 48 hours in RPMI 1640 media (Life Technologies, Cergy Pontoise, France), supplemented with 20% (v/v) foetal calf serum, 1% (v/v) phytohemaglutinin (Life Technologies, Cergy Pontoise, France), 1% (v/v) Hepes, and 50 IU penicillin, 50 µg/ml streptomycin. Colcemid (Life Technologies, Cergy Pontoise, France) (0.05 µg/ml) was added 24 hours after cultures started and Calyculin A (Calbiochem, France, 50 nM) was added for the last hour. The cultured cells were treated with a hypotonic solution of KCl (0.075M) for 7 minutes at 37°C and fixed in three changes of fixative (methanol: acetic acid, 3:1 v/v). Finally the fixed cells were dropped onto slides in a Thermotron equipment (ADGENIX, France) with humidity controlled 45%, temperature 22°C and ventilation controlled. The slides were then stained with Giemsa.

Scoring criteria

The incidence of rings in PCC cells into G1, G2 and M/A with more than 46 elements or 92 in late M phase were scored. Two operators participated to the scoring according the same criteria. A ring with a visible hole (with or without visible centromere) in any stage of the cell cycle and a couple of rings in partners (separated or united by the centromere) in metaphase cells was considered like one ring. When possible, at least 100 rings or 500 PCC cells for each radiation dose were scored. The frequencies of PCC-R in all stages of cellular cycle were evaluated as the ratios between rings scored and total observed cells. The proportion of cells in each class was analyzed. A 'class' corresponds to the number of rings in one cell, ie. class 0 is "no rings", class 1 "one ring by cell", class 2 "two rings by cell", class 3 "three rings by cell", class 4 "four rings by cell". The distribution is therefore the number of cells in each class. PCC-R were observed with a light microscope at x60 magnification (SA, Nikon, Japan). A Metasystem coupled to a microscope Nikon was used to take the pictures.

Statistics

The frequency confidence interval was calculated assuming a Poisson distribution of aberrations in the cells. The u test was used to test whether dispersions of aberrations can be described by a Poisson distribution.[5)] When the distribution of aberration follows a Poisson statistical law, there is only 5% likelihood that the u value exceeds |1.96 |. Another indicator of dispersion from Poisson distribution can be evaluated using the variance to mean ratio (σ^2/Y), since one characteristic of Poisson distribution is that the variance is equal to the mean.

The difference between, donors was tested using a Wilcoxon paired test.

Dose-effect relationships were fitted according to a linear model based on the maximum likelihood method described in Edwards,[6)] and in Papworth.[7)]

RESULTS AND DISCUSSION

Figure 1 shows some examples of Giemsa stained G1, G2, and M/A PCC cells induced by Calyculin A after ^{60}Co gamma rays exposure or neutrons exposure. Calyculin A concentration (50 nM) was chosen according to Kanda *et al.*[8)] At this concentration the percentage of PCC cells among irradiated lymphocytes was high (around 20%, data not shown), and the chromosomes were suitable for morphologic analysis. To higher Calyculin A concentrations the number of PCC cells increases, but the yield of PCC rings falls probably because the over-treatment resulting in diffuse and short chromosomes which made chromosome analysis difficult. Another chemicals like okadaic acid was used by Kanda to induce PCC with a similar PCC index dose-dependent relationship.[8)] Nevertheless for a similar PCC index with both chemicals the Calyculin A concentration was 20 times lower. For our experiments we have chosen arbitrary one chemical, for some comparisons between both chemicals refer to Kanda publication.[8)]

Table 1 and Table 2 show the number of PCC cells scored, the frequency of PCC-rings, the cell distribution of PCC-rings with their associated σ^2/Y and u values together with the PCC-index expressed as a percentage of PCC cells among all nuclei observed in lymphocytes exposed to different doses of ^{60}Co gamma and neutron radiations.

The PCC index decreased from 15.1% to 4.3% in ^{60}Co gamma rays exposures and from 13.25% to 0.51% for neutron exposures. At 5 Gy we obtained a PCC index of 12.2%, slightly lower than 17% obtained by Kanda with X Rays[8)] but very similar to the values found by Gotoh[2)] who obtained a G2 PCC cells induction of around 11% with 5 Gy of ^{60}Co

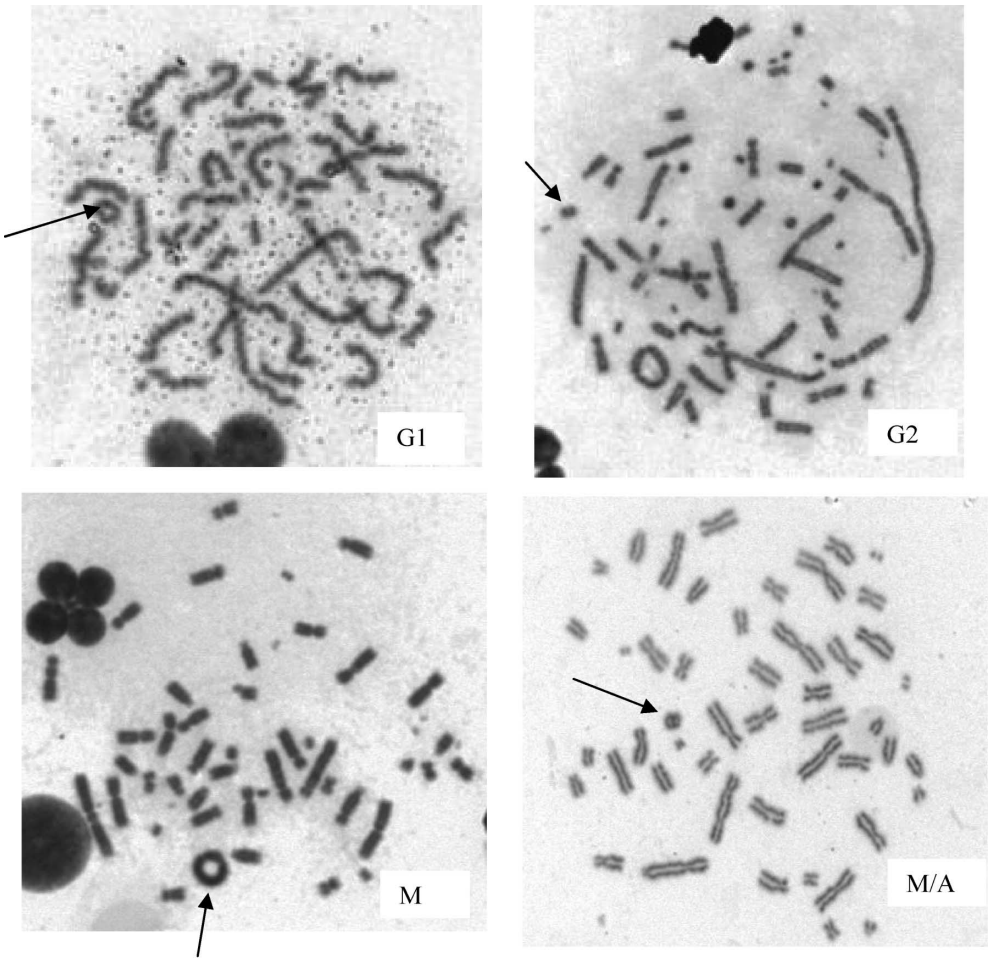

Fig. 1. Upper left photomicrography: G1 PCC with a single ring following 7.5 Gy ^{60}Co gamma rays exposure. Upper right photomicrography: G2 PCC with a single ring following 25 Gy ^{60}Co gamma rays exposure. Lower left photomicrography: Single ring in M PCC following 10 Gy neutrons exposure. Lower right photomicrography: Double ring in M/A PCC following 5 Gy ^{60}Co gamma rays exposure scored as one PCC ring.

Table 1. Frequencies of total PCC rings/cell, distribution of PCC-rings and PCC index in lymphocytes exposed to different doses of ^{60}Co gamma rays exposure. Both σ^2/Y and u values were calculated to test if the distribution of aberrations follows a Poisson distribution.

Dose (Gy)	PCC cells scored	PCC rings	PCC rings/cell	Distribution					σ^2/Y	u	PCC index
				0	1	2	3	4			
0	1000	0	0.00 ± 0.00	0	0	0	0	0	–	–	15.1%
5	1000	107	0.11 ± 0.02	897	100	2	1	0	0.99	–0.33	12.2%
7.5	1100	168	0.15 ± 0.02	948	137	14	1	0	1.05	1.19	10.8%
10	876	212	0.24 ± 0.03	695	154	24	2	1	1.10	2.07	7.8%
20	638	277	0.43 ± 0.04	417	173	41	6	1	1.04	0.66	6.0%
25	497	248	0.50 ± 0.04	304	145	42	5	1	1.01	0.18	4.3%

gamma rays exposures.

A total of 9675 cells were analyzed, 5111 for ^{60}Co gamma rays exposures and 4564 for neutrons exposures. For each curve two blood donors were analysed. When comparing the frequencies of PCC rings among donors we did not find significant differences to any dose of the curve, neither for ^{60}Co gamma rays exposures nor for neutrons exposures (data not shown), therefore the two donors were pooled to calculate a

Table 2. Frequencies of total PCC rings/cell, distribution of PCC-rings and PCC index in lymphocytes exposed to different doses of neutrons. Both σ^2/Y and u values were calculated to test if the distribution of aberrations follows a Poisson distribution.

Total dose Dose (Gy)	Dose gamma (Gy)	Dose neutron (Gy)	PCC cells scored	PCC rings	PCC rings/cell	Distribution					σ^2/Y	u	PCC index
						0	1	2	3	4			
0	0	0	1000	0	0.00 ± 0.00	0	0	0	0	0	–	–	13.25%
5.4	0.7	4.7	1200	292	0.24 ± 0.02	952	214	27	4	3	1.15	3.63	6.4%
5.6	0.7	4.9	1025	296	0.29 ± 0.03	773	214	32	6	0	1.05	1.14	4.2%
9.4	1.0	8.4	972	317	0.33 ± 0.03	714	209	40	8	1	1.12	2.57	3.0%
12.7	1.1	11.6	211	68	0.32 ± 0.06	153	49	8	1	0	1.01	0.06	0.54%
26.1	1.7	24.4	156	51	0.33 ± 0.07	114	34	7	1	0	1.07	0.64	0.51%

single curve for each radiation quality.

The distribution of rings followed a Poisson distribution for almost all ^{60}Co gamma rays exposures and neutrons exposures. Only for 10 Gy in the ^{60}Co curve and 5.4 and 9.4 Gy in the neutron curve the u values exceeded ± 1.96. We would have expected an overdispersion of rings for the highest neutron doses which was not observed. However in this radiation type a saturation of the yield of rings per cell was noted. Indeed when the distributions of aberrations were compared between all neutron doses we obtained the same proportion of cells in each class. The comparison with the literature is difficult as most publications dealt with dicentrics distributions and not rings. In addition for ^{60}Co gamma rays exposure no dicentrics overdispersion was described in the literature above 5 Gy.[9] Nevertheless, for fission neutron radiation literature is more conflicting[10] even if with high LET radiations there is a high tendency to see an overdispersion of dicentrics.[11]

One of the main interest of distribution studies is to evaluate dose heterogeneity. Indeed when the irradiation is homogeneous a Poisson distribution of aberrations in cells is expected. It should be an interesting focus of this technique to evaluate individuals irradiated partially in small areas of the body. But referring to Kanda *et al.*[12] publication, less information are available with the ring distribution than with the dicentrics one.

Figure 2 shows dose-effect relationship for the *in vitro* induction of PCC-R in lymphocytes to doses of ^{60}Co gamma rays exposure up to 25 Gy. The frequencies of PCC-R were fitted to the doses and the fitted values of the constant C and of the α coefficient of the linear function are also presented on the graphic. The shape of the curve is different from Kanda's curve[1] as they observed a three modal curve: one linear part form 0 to 10 Gy followed by a progressive saturation phase from 10 to 20 Gy and ended by a plateau phase from 20 to 40 Gy. In our case, it is not clear whether a beginning of a saturation was observed at 25 Gy (Fig. 2), higher radiation doses would be required to validate this point.

The frequencies of PCC-rings obtained in this work for

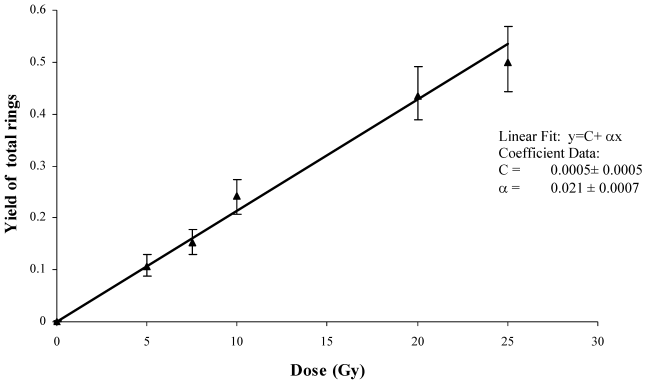

Fig. 2. Relationship between PCC-R frequencies in lymphocytes and different doses of ^{60}Co gamma rays exposure.

^{60}Co gamma rays exposure were 5 times lower than those obtained by Kanda for X Rays,[1] even though our scoring criteria where carefully selected to match the one described in Kanda's report.[1] This discrepancy could be due to a difference in the condition of hypotonic treatment and air drying. Our less spreading conditions could have caused the small rings difficult to be analyzed, which resulted in reduced frequency of a total PCC-ring. As discrepancies from one study to another are observed it is important to establish the dose effect curve in the same experimental situation as the dose estimation was performed. By this way, variations on the dose estimation can be reduced.[13]

Figure 3 shows dose-effect relationship for *in vitro* induction of PCC-R in lymphocytes to different doses of neutrons. By contrast with gamma rays, the observed frequencies of PCC-R had a linear increase with the doses up to 10 Gy, and then a saturation was observed. The data of the linear part was fitted to a linear model with values of the α coefficient of 0.042. Here again there is no other PCC-curves for neutrons to compare with. Apparently the saturation of PCC rings curves can occur at different doses for different kinds of radiation. In our work the saturation started from 10 Gy for neutron radiation (Fig. 3). Furthermore, this saturation

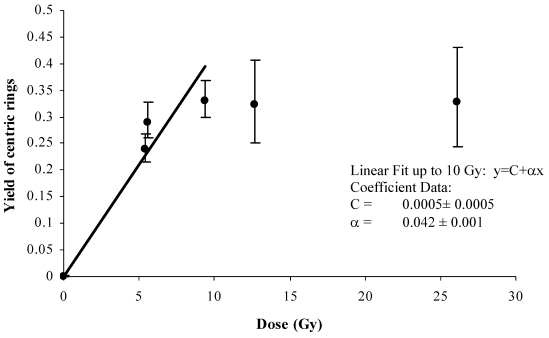

Fig. 3. Relationship between PCC-R frequencies in lymphocytes and different doses of neutrons exposure.

was observed for a PCC-R frequency of 0.3 per cell whereas with the gamma radiation we have observed a PCC-R frequency of 0.5 per cell. This is the consequence of the difference of radiation quality. With neutrons, the damages are more localized and the cell is not able to repair. Furthermore, the saturation is often linked to an under dispersion of chromosomal aberrations and more particularly dicentrics. Here the underdispersion for neutron radiation is not marked but still exists. If the saturation is the result of cell killing, as we are looking at rings in PCC cells whatever the cell cycle we don't see the effect of mitosis cell death but just the effect of interphase death. Therefore the reduction in the distribution is less pronounced.

Such curves are useful in case of accidental overexposure to high doses as in the Tokai-mura accident. It was the first case where this technique was used to estimate a dose in a real situation. The yields of PCC-rings were measured in the patient and reported on the X-rays (200kV) curve published by Kanda *et al*. in 1999,[1)] resulting in an equivalent gamma dose estimation. However the exposure was mostly due to neutron radiations but such curves were not available at that time.

If we compare the linear coefficient of our two dose effect curves, the fitted linear coefficient of neutron radiation is double the one of gamma rays which is equivalent to a Relative Biological Effectiveness (RBE) of 2 for neutron radiations. This is lower than what is described for lower doses, but is in accordance with the RBE value that was taken in Murata publication (2002) when doing the physical dose reconstruction of the Tokai-mura as an RBE value of 1.7 was used.[15)]

In conclusion, this technique is suitable to estimate high doses of radiation up to 25 Gy of gamma rays and up to 10 Gy of neutrons. It overcomes two major limits of the conventional technique at high doses which are the saturation of dicentrics yield and the low mitotic index. Therefore the dose effect curves established could be used in case of accidental overexposure to ionizing radiation resulting of high doses of gamma rays or neutrons radiations such as in nuclear power station. They supplement the panel of dose effect curves available in a biological dosimetry laboratory. Such laboratories should have all the available tools to be able to estimate doses whatever the accidental situation.

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