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Prematurely Condensed Chromosome Rings after Neutron Irradiation of Human Lymphocytes

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PCC-rings/Dose-effect curves/High LET irradiation neutrons/Calyculin A/Human lymphocytes.

Calibration curves for fission spectrum neutrons and other high LET radiations are scarce in cytogenetic dosimetry and particularly for Prematurely Condensed Chromosome Rings (PCC-ring). Here we analyzed the behavior of the PCC-ring frequency and PCC index after neutron irradiation in a broad dose interval from 1 to 26 Gy. PCC-rings were induced in lymphocytes with Calyculin A. 6455 PCC cells in G1, G2/M and M/A stages were analyzed. The best fitting between the frequency of PCC ring (Y) and the Dose (D) was obtained with the equation $Y = (0.059 \pm 0.003)\ D$. The saturation of the PCC-ring was observed after around 4 Gy, but it was still possible to analyze cells exposed up to 26 Gy. The distribution of rings by cell follows Poisson or Neyman type distribution for all doses. This PCC-ring dose effect curve can be used in case of accidental overexposure to neutron radiation, allowing a dose assessment in a reliable way. Additionally, the PCC index seems to be well correlated with radiation dose and decrease in a dose dependent manner from 13% in non exposed sample down to 0.2%. This observation allows the possibility to perform a quick classification of victims exposed to high doses of both gamma and neutron radiations. The PCC assay can then be used for both neutron dose estimation up to 4 Gy and for the rapid classification of victims exposed to higher doses. This assay could be included in the multiparametric approach developed to optimize the medical treatment of radiation victims.

INTRODUCTION

Since 50 years biological dosimetry is used to assess the doses in cases of accidental exposures and in order to support legal decisions in exposed workers. The dicentric assay has been mainly used for its good properties even if it presents some limitations specifically when the doses are very high. The PCC–R was proposed to solve the problem of conventional cytogenetic for high doses.[1)] This assay was first applied after the Tokaimura accident where very high doses of neutron and gamma were delivered. However at that time no corresponding PCC ring dose effect curve was available.

At the present time few laboratories have established PCC-R curves for a large dose range of Gamma radiation.[2–4)] The potentially application of this assay to a mass casualty accident where it is essential to have a fast and reliable triage method for crude assessment of radiation dose has also been tested.[3)] The authors concluded in a good efficiency of the PCC assay to estimate high doses when the triage mode is applied.

In 70's decade, various laboratories carried out *in vitro* determinations of calibration curves for fission spectrum neutrons and other high LET radiations by the dicentric assay and these data were reviewed and resumed by Bender and a cytogenetic working group.[5)] In almost all laboratories linear relationships were obtained, as would be expected from high LET irradiated dominated by single-track events. The alpha coefficients agree rather well, with a mean of 0.77 ± 0.13 for a dose range between 0.04 and 3 Gy[5)] for fission neutrons . More recently, α coefficients values of 0.68 with doses between 0.75 to 2.5 Gy (fission neutrons $\bar{E} = 0.71$ MeV; $y_F = 18.0$ keV/m; dose rate = 0.25 Gy/min) and values of 0.63 and 0.68 with doses between 0.1 and 1.0 Gy (neutrons of $\bar{E} = 2.1$ MeV) were obtained by other laboratories[6,7)] and a linear quadratic relationship for dicentric and rings was reported in the interval between 0–4 Gy.[8)]

In previous paper published by our group, we first reported a neutron PCC-R curve.[2)] This curve has an insufficient goodness of fitting ($r^2 = 0.90$) because no doses lower than

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5 Gy were used and also because the data obtained has shown fast saturation. In spite of these, the convenience of PCC-R technique was evidenced as valuable cells were obtained for doses as high as 26 Gy.[2)]

For this reason, we decided to realize a second experimental series to constrict the range of doses making emphasis on doses under 5 Gy. The aim of this second experimental series to know the behaviour of the indicator PCC-R in that region and its usefulness in dose estimations. The data from the two sets were analyzed together, because both experimental sets were made under the same condition, which is, using the same scorer, the same reactor configuration and the same protocols of assay.

Additionally, was evaluated the possibility of the PCC index as a biomarker for "stratification of patients in to dose categories" to increase the possibilities for a fast classifications of overexposure people using the PCC technique.

MATERIALS AND METHODS

Blood sample exposure

Peripheral blood from three healthy individuals was drawn on heparinized tubes. Blood sample was collected in accordance with French law (L. 2004-800) on bioethics. Informed consent was obtained for each donor. Blood was immediately exposed to fission neutrons at the SILENE facility (Valduc, France). A chain reaction was produced in an experimental reactor resulting in 1.04•1017 fissions. To limit the gamma exposure a lead shield was placed around the reactor. Irradiation lasted 234 seconds and the mean energy of the neutron spectrum is 0.49 MeV. The samples were exposed to 1.0, 1.7, 3.8 or 6.0 Gy of neutron irradiation. To obtain such doses, the blood tubes were located at different distance from the core. Following irradiation, blood samples were maintained at 37°C for 2 hours allowing repair mechanisms to take place. 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 1.

Cell culture

Lymphocytes were cultured as described previously.[2)] Briefly, a culture of 48 hours was set up with RPMI 1640 media (Life Technologies, Cergy Pontoise, France), supplemented with 20% (v/v) foetal calf serum, 1% (v/v) phytohemagglutinin(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 hypotonic solution of KCl (0.075 M) was used for 7 minutes at 37°C following by 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 at 45%, temperature 22°C and ventilation controlled. The slides were then stained with Giemsa.

Table 1. Frequencies of total PCC rings/cell, distribution of PCC rings and PCC index in lymphocytes exposed to different doses of neutrons. Both σ2/Y and u values were calculated to test if the distribution of aberrations follows a Poisson distribution.

Total Dose (Gy)	Gamma Dose (Gy)	Neutron Dose (Gy)	PCC cells scored	PCC rings	PCC rings/cell (mean ± S)	Distribution					σ2/Y	u	Dist.	PCC index (%) (mean ± SD)
						0	1	2	3	≥ 4				
0	0	0	1500	0	0.00 ± 0.00[a]	1500	0	0	0	0	–	–	–	12.7 ± 1.4[a]
0	**0**	**0**	**1000**	**0**	**0.00 ± 0.00[b]**	**1000**	**0**	**0**	**0**	**0**	**–**	**–**	**–**	**14.2 ± 1.3[b]**
1.0	0.2	0.8	1513	61	0.04 ± 0.02[a]	1452	61	0	0	0	0.96	1.10	P	10.9 ± 0.8[a]
1.7	0.2	1.5	1246	118	0.09 ± 0.01[a]	1131	112	3	0	0	0.96	1.08	P	6.3 ± 1.0[a]
3.8	0.4	3.4	948	243	0.25 ± 0.01[a]	736	185	23	4	0	1.03	0.71	P	4.5 ± 1.1[a]
5.4	**0.7**	**4.7**	**1200**	**292**	**0.24 ± 0.02[b]**	**952**	**214**	**27**	**4**	**3**	**1.15**	**3.63**	**NN**	**3.2 ± 1.6[b]**
5.6	**0.7**	**4.9**	**1025**	**296**	**0.29 ± 0.03[b]**	**773**	**214**	**32**	**6**	**0**	**1.05**	**1.14**	**P**	**3.3 ± 1.8[b]**
6.0	0.5	5.5	1248	378	0.30 ± 0.03[a]	931	263	49	3	2	1.07	1.71	P	3.3 ± 1.6[a]
9.4	**1.0**	**8.4**	**972**	**317**	**0.33 ± 0.03[b]**	**714**	**209**	**40**	**8**	**1**	**1.12**	**2.57**	**N***	**1.9 ± 0.4[b]**
12.7	**1.1**	**11.6**	**211**	**68**	**0.32 ± 0.06[b]**	**153**	**49**	**8**	**1**	**0**	**1.01**	**0.06**	**P**	**0.4 ± 0.03[b]**
26.1	**1.7**	**24.4**	**156**	**51**	**0.33 ± 0.07[b]**	**114**	**34**	**7**	**1**	**0**	**1.07**	**0.64**	**P**	**0.2 ± 0.02[b]**

[a]Values (mean ± SD) were obtained from the irradiated blood of three different subjects. [b]Values (mean ± SD) were obtained from the irradiated blood of two different subjects. In bold, data from experimental serie 1.[2)] P: Poisson, N: Neyman type A, NN: neither Poisson neither Neyman * Chi2 Goodness-of-Fit to Neyman: 0.3056.

Scoring criteria

The scoring was performed by the same operator according the same criteria previously published.[2] In brief, were recorded as ring any visible hole (with or without visible centromere) in G1, G2 or M stages of the cell cycle (see Fig 1). Cells with more than 46 elements or 92 in late M phase were scored. Couple of rings in partners (separated or united by the centromere) in metaphase cells were considered like one ring. At least 100 rings or 1500 PCC cells for each radiation dose were scored. The frequencies of PCC-R were evaluated as the ratio between rings scored and total observed cells. A light microscope at × 60 magnification (SA, Nikon, Japan) was used. Pictures were taking using Metasystems software coupled to a microscope.

PCC index was expressed as a percentage of PCC cells among all nuclei observed in lymphocytes exposed to different doses of neutron radiation. The S-PCC cells were not included in the PCC index.

Statistics

Dose-effect relationships were fitted according to a linear model using DoseEstimate software.[9] The significance of slope (alpha coefficient) was tested by a t-test and the goodness of fit was tested by Chi-square test.

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. In case of overdispersion the Neyman type A distribution was tested for difference between means of two samples with different variances using the software program NETA.[10] The difference between donors was tested using regression comparison method.

RESULTS AND DISCUSSION

Table 1 shows 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. This table includes the results of two experimental series performed by the same team under the same conditions (i.e. the same scorer, the same irradiation conditions and reactor configuration).

When comparing the frequencies of PCC rings among donors we did not find significant differences to any dose, therefore the donors were pooled to calculate the calibration curve. The data were expressed as the mean ± SD from two subjects in the experimental serie 1 and three subjects in serie 2.

Figure 2 shows the relationship between PCC-R frequencies in lymphocytes and different doses of neutrons exposure for two sets of data. In the first experimental series, marked in bold, a total of 4564 cells were analyzed. This series included doses of 5.4; 5.6; 9.4; 12.7 and 26.1 Gy.[2] When only doses from 5.4 to 26.1 Gy are considered a flat curve is observed corresponding to a saturation. Moreover the goodness of fit of the curve is insufficient ($r^2 = 0.90$).[2] To correct this and to evaluate the slope of the curve at doses below 5 Gy a second experimental series with more experimental points in the low dose region was conducted, considering moreover that exposures involving neutron irradiation may be of different magnitudes and the low doses between 1–5 Gy might cause damages comparable to high doses of gamma radiation. In the second series [present work], 6455 cells were analyzed. Figure 2 shows the linear phase of the neutrons dose response obtained in the interval from 0 up to 3.8 Gy in the experimental series 2.

The use of extended dose interval from 1 Gy to 26.1 Gy and the possibilities to obtain scorable cells in this interval allow us a more precise characterization of the dose

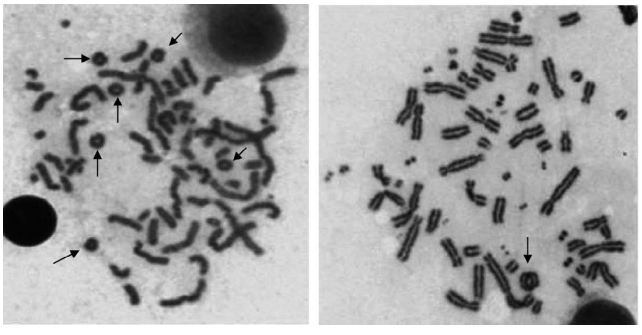

Fig. 1. Left photomicrography: Six rings in a PCC cell following 5 Gy neutrons exposure. Right microphotography: Double ring in PCC cell following 0.8 Gy neutrons exposure scored as one PCC ring.

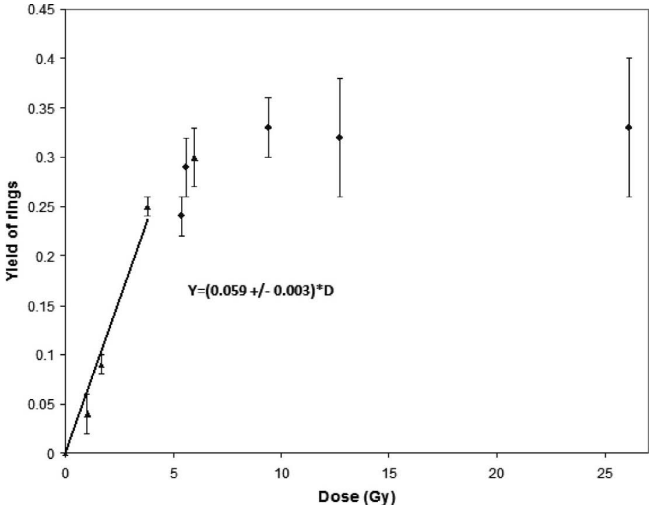

Fig. 2. Relationship between PCC-R frequencies in lymphocytes and doses of neutrons exposure. The data were expressed as the mean ± SD from two subjects (•)[2] and three subjects (▲). The linear phase of the neutrons dose response allows the dose estimation from the yield of PCC rings.

response and the saturation process. In previous works, using high LET radiation and conventional cytogenetic assay, the dose interval tested was between 0–3 Gy,[5–7] perhaps by difficulties associated with cell division capability after high dose and high LET radiation. An exception is the 0–8 Gy interval reported by Wang, where it was not possible to adjust the relationship upper than 4 Gy probably due to the saturation of dicentrics at high doses.[8] According to our results, at the 3.8 Gy dose the frequency of PCC rings per cell is 0.25 ± 0.01 and this figure remains stable for increasing doses up to 5.4 while it increases slowly to reach the frequency of of 0.33 ± 0.07 for the 26.1 Gy dose point (see Table 1 and Fig. 2). Regarding high doses and low LET irradiation, no evidences of saturation phenomenon of PCC-R frequency has been reported for doses lower than 20 Gy.[2–4]

The best fitting of the data from 0 to 3.8 Gy was obtained with a linear model Y = (0.059 ± 0.003)•D, (t = 19.7, p < 0.05) as expected from high LET radiations. The value of the alpha coefficient (0.059 ± 0.003) is an order smaller than the alpha coefficients (0.63–0.8) for neutrons curves appeared in the literature with dicentrics[5] which correspond to the difference of an order in the formation of rings and dicentrics.[5–7] If we compare the linear coefficient of our dose effect. PCC-R curves (gamma versus neutron), the coefficient of neutron radiation is 2.8 times the one of gamma rays which is equivalent to a Relative Biological Effectiveness (RBE) of 2.8 for neutron radiations. This is quite similar than the RBE described for high LET radiations.[11]

With high LET radiations there is a high tendency to see an overdispersion of dicentrics.[12] For fission neutron radiation literature is conflicting.[13] In our work, the distribution of rings per cell follows in general a Poisson distribution except for the 9.4 Gy dose (Neyman type distribution) and 5.4 Gy dose (NN, neither Poisson neither Neyman) (Table 1), this Neyman distribution tends towards the generalized Poisson distribution with increasing sample size to test the difference between means of two samples with different variances.[14] Like us, other author has also found a Poisson distribution of dicentrics.[6] As there is no other available data about distribution of PCC-rings after neutrons exposure, it is difficult to conclude whether the distribution should be Poisson or not. If the Poisson distribution is confirmed, this approach could also be used to assess heterogeneous exposures where deviation from Poisson distributions are used.[15]

As can be seen from Table 1 and Fig. 3, the PCC index decreased from 13% to 0.2% in a dose dependent fashion. We studied the possibility of using PCC index as a criteria for "stratification of patients into dose categories" or fast classification of levels of overexposure. This potential of PCC index has been marked in previous reports[16] as a simple method for rapid decision making of therapy for radiation accident victims exposed to high levels of radiation. A broad criterion for neutrons dose estimation was summarized in Fig. 3. According to the criteria three groups of doses were discriminated easily, 0–1 Gy, > 1–10 Gy and > 10 Gy. Although due to the saturation process, precise dose estimation by the PCC ring is not longer possible for extremely high-dose neutron exposures, a PCC index value less than one strongly suggests that the neutrons radiation dose is over 10 Gy.

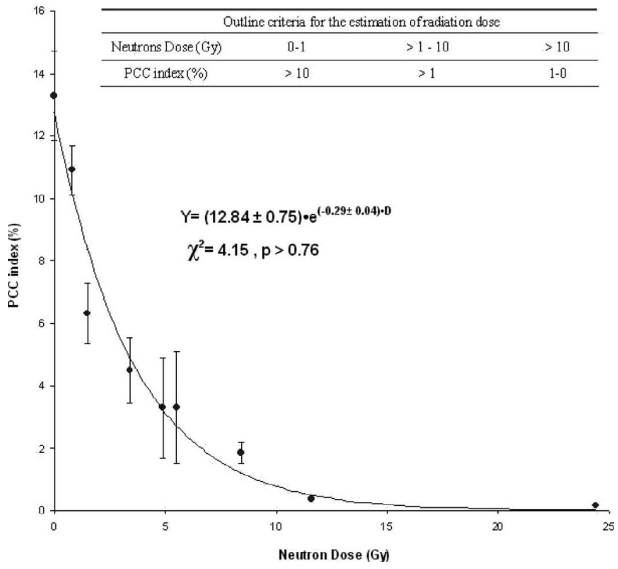

Fig. 3. Relationship between PCC index in lymphocytes and doses of neutrons exposure. An outline criteria for rapid dose discrimination is presented.

In conclusion, the PCC-ring dose effect curve could be used in case of accidental overexposure to neutron radiation. A precise dose estimation can be obtained up to 4 Gy. If the exposure is higher, a rapid classification of the victims according to the level of exposure could be performed based on the PCC index. The PCC could be considered an additional tool in biological dosimetry.

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