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Application of the Premature Chromosome Condensation Assay in Simulated Partial-Body Radiation Exposures: Evaluation of the Use of an Automated Metaphase-Finder

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Abstract. The premature chromosome condensation (PCC) assay has been proposed as a useful and rapid end point for biological dosimetry following accidental high-dose radiation overexposures. A major benefit of the PCC assay is that it does not require cells to divide for evaluation of cytogenetic damage. The PCC assay was performed on isolated human peripheral lymphocytes exposed in vitro to doses from 1 to 9 Gy of 250 kVp x-rays. The dose-response relationships of the frequency distribution and the yield of PCC fragments in cells were determined after one day of repair at 37°C. A Q_{pcc} approach, which involves the analysis of the yield of excess PCC fragments in damaged cells, was used to establish a dose-response calibration curve. This method is identical in concept to the Q_{dr} technique introduced by *Sasaki* [1] for partial-body exposure dose-estimates using asymmetrical chromosome aberrations (i.e., dicentrics and rings) in metaphase spreads of human lymphocytes. A simulated in vitro test of a partial-body exposure to a 6-Gy dose was performed. The results from this test provided dose estimates of 5.3 ± 0.6, 4.7 ± 0.6, 5.0 ± 0.6 and 4.7 ± 0.8 Gy for the 20, 30, 50 and 75 percent component of 6-Gy irradiated cells, respectively. An automated metaphase-finding system was evaluated for use with the PCC assay. This system helped to locate PCC spreads among the mitotic inducer Chinese hamster ovary (CHO) metaphase spreads, thereby facilitating rapid scoring of samples. We conclude that the measurement of excess PCC fragments in Giemsa-stained preparations provides useful biological dosimetry information on the size of the irradiated fraction in cases of acute radiation exposures. Use of Q_{pcc} analysis is recommended for partial-body exposures to determine dose estimates for the irradiated fraction. Automated metaphase finding significantly enhances the speed of the PCC assay.

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Introduction

The premature chromosome condensation (PCC) technique, initially introduced by *Johnson* and *Rao* [2], involves either ultraviolet light-inactivated Sendai virus [2, 3] or polyethylene glycol (PEG) [3, 4] assisted fusion of mitotic "inducer" cells with "test" interphase cells. A mitotic factor from the mitotic inducer cells is believed to cause a dissolution of the nuclear envelope and subsequent premature condensation of interphase DNA. In the case of a nonirradiated and resting G_1 human lymphocyte, this process permits visualization of 46 single-chromatid-like structures, which appear along with the conventional metaphase chromosomes of the mitotic inducer cell following appropriate hypotonic swelling, fixation and staining.

Ionizing radiation causes an increase in the number of PCC fragments in cells. The PCC assay has been used to examine the effects of radiation quality and the kinetics of repair following exposure of mammalian cells to low- and high-linear energy transfer (LET) radiations [5-9]. *Bedford* and *Goodhead* [10] showed that alpha particles (3.2 MeV, 128 keV/μm)

produced a relative biological effectiveness (RBE) of 2.2 for the early production of excess PCC fragments in noncycling HF19 human diploid fibroblasts. *Pantelias* and *Maillie* [11] demonstrated that even though rat peripheral blood lymphocytes have undergone significant repair by one day following whole-body exposure to 3 Gy x-rays, elevated yields of excess PCC fragments can still be detected for several days postirradiation.

The PCC assay has been suggested as a valuable end point for use as a biological indicator for radiation doses in cases of accidental radiation exposures [4, 12]. Since the PCC technique does not require the stimulation of resting cells to proliferate, radiation injury in cells that otherwise would not proceed into mitosis can be visualized. A significant advantage of the PCC technique is that it is rapid and affords the opportunity for cytogenetic laboratories to provide results within one day of receipt of a peripheral blood sample.

Dose inhomogeneity is a major factor to consider in biodosimetry studies. Relying primarily upon the use of the measurement of dicentrics and rings in metaphase spreads from stimulated human lymphocytes, dose inhomogeneity has been addressed by several approaches, including the contaminated Poisson [13, 14] and Q_{dr} [1, 13] methods. *Natarajan* and colleagues recently demonstrated that the PCC assay, compared with the dicentric and micronuclei assay, provides a more accurate prediction of small fractions of partial-body exposures [15].

Searching for metaphases represents the major time-consuming and tedious part for a cytogenetic laboratory that scores chromosome aberrations [16]. The use of an automated metaphase-finding system offers significant benefit in the study of rogue cells [17], in the survey of populations of occupationally irradiated workers, and in cases of dose estimations for radiation accidents involving partial-body exposures [16]. Reduction in labor costs associated with the scoring of large number of samples represents the major benefit. At present, it is not known whether any of the commercially available metaphase-finders would require adaption for use in the PCC assay, where PCC spreads represent a small fraction (<10%) of the mitotic inducer cell spreads.

We evaluated the application of the PCC assay for estimation of the irradiation fraction and dose to the irradiated fraction in simulated partial-body exposures as well as the feasibility of using an automated metaphase-finding system to expedite locating PCC spreads for analysis.

Materials and Methods

Mitotic Cells

Mitotic Chinese hamster ovary (CHO) cells, which were used as mitotic-inducer cells in the PCC assay, were obtained by a modified mitotic-cell collection protocol suggested by *E. A. Blakely* of Lawrence Berkeley Laboratory, CA. This protocol is based on colchicine-induced inhibition of cell progression in metaphase, followed by repeated sequential mitotic "shake-off" procedures of the colchicine-treated CHO cells.

CHO cells were maintained as stock cultures in complete growth media consisting of α-minimum essential medium (α-MEM) supplemented with fetal bovine serum, sodium bicarbonate and antibiotics; cells were grown at 37°C in an atmosphere of 5% CO_2/95% air with biweekly subculturing. In the mitotic collection experiments, exponentially growing cells were plated into tissue-culture flasks so that after one day the flask was subconfluent. Typically, several preshakes were performed to dislodge dead, loosely-attached or floating cells. Mitotic cells were then harvested from a total of three 3.5 h collection intervals with complete medium containing 2 μg/ml of colchicine (mitotic medium), pelleted by centrifugation (4°C), and resuspended in mitotic medium (4°C); then a cell count was performed. Typically, the mitotic index of this cell suspension ranged from 0.93 to 0.97, based on microscopic examination of more than 1,000 Geisma-stained cells in smears. Approximately 1×10^6 mitotic cells were then transferred to cyrovials (Nunc Cryotubes, Intermed, Roskilde, Denmark) in mitotic medium containing 10% dimethyl sulfoxide. Cells in cryovials were then placed in a freezing container (Cryo 1°C freezing container, Cat. No. 5100-0001, Nalgene Corp., Rochester, NY) that was transferred to a mechanical freezer (–70°C) for a controlled-rate freezing (i.e., –1°C/min). After 24 h the cryovials were transferred to a liquid-nitrogen storage container until later use in the PCC-assay.

Lymphocyte Culture

Whole blood from healthy human donors was collected into standard blood collection

tubes containing ethylenediamine tetraacetate acid (EDTA) (Becton-Dickinson, Rutherford, NJ). The informed consent form used in this study was approved by the Uniformed Services University of the Health Sciences, Human Use Committee, Bethesda, MD. Lymphocytes were isolated from whole peripheral blood using Ficoll (Histopaque 1077, Sigma Chemical Co., St. Louis, MO) gradients, washed with phosphate buffered saline (PBS), and suspended in complete growth medium (KaryoMax Bone Marrow Karyotyping Medium, GIBCO BRL, Gaithersburg, MD). Cell cultures were typically aerated in complete culture media in tissue-culture flasks for at least 30 min at 37°C prior to either sham treatment or exposure to radiation.

Irradiation Procedure and Dosimetry

Cells suspended in culture media in tissue-culture flasks were irradiated on a rotating Plexiglas holder with a Phillips industrial x-ray machine (Phillips GMBH, Hamburg, Germany) at room temperature. The irradiation conditions were a dose rate of 1 Gy/min with an effective energy of 83 keV (source-to-surface distance was approximately 55 cm, 250 kVp at 12.5 mA, filtration with 0.20 mm Cu and 1.0 mm Al) [18]. Dosimetry was determined using ion chambers placed in tissue-culture flasks filled with tissue-equivalent plastic [19]. Nominal doses were reported; however, for each experiment, the individual dose measurements obtained were within 5% of the nominal dose.

Cells, following radiation procedures, were typically transferred to sterile centrifuge tubes and maintained at 37°C for various incubation intervals as indicated.

PCC Assay

The lymphocytes were fused with CHO mitotic cells in the presence of polyethylene glycol (PEG) (MW 1450; Sigma Chemical Co., St. Louis, MO) by a modified method of *Pantelias* and *Maillie* [4] for the induction of PCC. Briefly, approximately two million mitotic cells were mixed with lymphocytes isolated from 3-4 ml of blood in a 15 ml round-bottomed glass test tube and washed with PBS. After centrifugation at 200 *g* for 8 min, the supernatant was discarded and the pellet was dried by inverting the tube over a blotting paper. The pellet was floated, being careful to keep the pellet intact, with the addition of 0.2 ml of 50% PEG (weight/volume) using a 1ml syringe. Subsequently, 2 ml of PBS was added, drop by drop in a 3 min interval, to dilute PEG; then cells were centrifuged at 200 *g* for 8 min. The pellet was resuspended in 0.4 ml of complete growth medium (KaryoMax). Colchicine (50 µl of a 10 µg/ml stock solution) and magnesium chloride (50 µl of 20 mM stock solution) was added to the cell suspension. This cell suspension was then incubated in a water bath at 37°C for 30 min. Chromosome spreads were prepared on acid-cleaned, grease-free glass slides following hypotonic treatment in 1% sodium citrate and fixation in 1:3 acetic methanol by the standard air-drying technique. Slides were stained in 4% Giemsa in distilled water for counting the number of PCC fragments under a light microscope at 1500× magnification.

Statistical Evaluation

The analysis of the yield of PCC fragments in cells for the experimental conditions described included the determination of the mean ± SE and evaluation of the frequency distribution using the σ^2/y and μ *Papworth* test [20]. Using the *Papworth* test, values of $\sigma^2/y \geq 1$ and $\mu \geq 1.96$ indicate overdispersion.

Student's *t*-test was used to compare the yield of Q_{pcc} for the partial-body studies and a x^2 test was performed to compare observed dose estimates with those expected.

Automated Metaphase-Finder

Slides with PCC samples were evaluated with an automated metaphase-finding system (LAI Metafind, Loats Associates Inc., Westminster, MD). This system consisted of a standard cytogenetic microscope equipped with 15 slide capacity stage, motorized x-, y-, and z-axis computer-controlled positioning with specially adapted autofocus capabilities. Spread acquisition was obtained using a color camera and color digitizer board. Image display was accomplished using a 1024-line resolution video monitor. The system was controlled with customized software for automated spread localization, quality control checks and verification on a 486/66 personal computer.

Results and Discussion

The analysis of radiation-induced damage was performed in nonstimulated human lymphocytes by the PCC assay [4, 11, 12, 21]. Table I shows the results of repeated dose-response studies following exposure to 250 kVp x-rays

Table I. Results from 250 kVp x-ray dose-response studies measuring excess PCC fragments in human lymphocytes after one day of repair[a]

Dose, Gy	n	Excess PCC Fragments, Mean ± SE	σ^2/y, Ratio ± SE	μ
0	79	1.62 ± 0.32	5.12 ± 0.16	25.81
1	53	4.75 ± 0.69	5.36 ± 0.20	22.29
3	45	7.13 ± 0.69	3.04 ± 0.21	9.60
6	81	13.18 ± 0.78	3.67 ± 0.16	16.94
9	41	25.0 ± 1.78	5.11 ± 0.22	18.39

[a]Experimental conditions are described in the legend of Figure 1. Distribution analysis of the number of excess PCC fragments was analyzed as described by *Papworth* [20] using the σ^2/y and overdispersion parameter (μ). A μ value >1.96 indicates the distribution is significantly overdispersed.

and one day of repair (37°C). The number of excess PCC demonstrated a progressive increase, with dose, from 1.62 ± 0.32 at 0 Gy to 25 ± 1.78 at 9.0 Gy. These data were fitted to a linear model with a slope of 2.3 ± 0.2 excess PCC/Gy and intercept of 1.22 ± 0.63 excess PCC/Gy (r^2 = 0.968). Similar linear dose-response relationships have been observed for x-rays using human lymphocytes for doses up to 8 Gy after 1 to 24 h [12, 21].

The dose-response data of x-ray induced excess PCC fragments were also subjected to a distribution analysis using the σ^2/y and μ overdispersion test [20] (Table I). At all radiation doses the number of excess PCC fragments revealed significant overdispersion as indicated by μ values >1.96. Again the observations by *Pantelias* and *Maillie* [12] corroborate these findings since they also report significant overdispersion of lymphocyte PCC data following exposure to x-rays.

Since the distribution of PCC fragments is non-Poisson, a method that does not rely on the data demonstrating Poisson distributions was used to estimate either the irradiated fraction or dose to the irradiated fraction. We introduced the Q_{pcc}

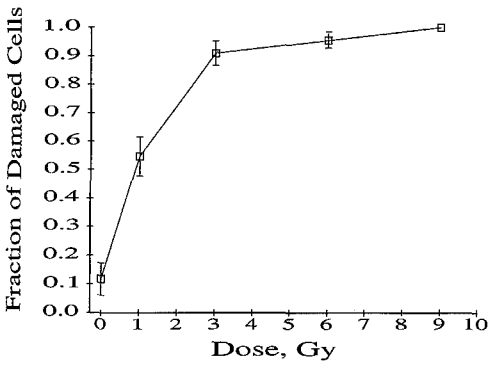

Fig. 1. Dose-response relationship for the fraction of damaged cells. Isolated human lymphocytes were exposed to 0 to 9 Gy doses of 250 kVp x-rays. Following one day of repair at 37°C, the effect of radiation on the induction of DNA damage in lymphocytes was evaluated using the PCC assay. Damaged cells represent cells with ≥48 PCC fragments per cell. Symbols reflect the mean fraction with bars representing standard errors. Data was pooled from three independent experiments.

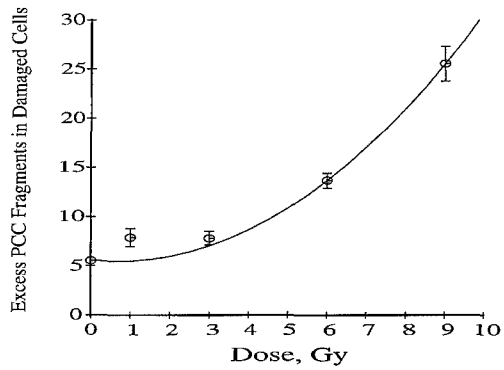

Fig. 2. Dose-response relationship for the yield of excess PCCs in damaged lymphocytes using the Q_{pcc} method. Experimental conditions are as described in the Figure 1 legend. Symbols and bars reflect the mean ± SE of excess PCC fragments in damaged cells. The curve represents a fit of the data to a weighted linear-quadratic model.

method, which is identical in concept to the Q_{dr} approach of *Sasaki* [1], and is applicable to the PCC assay for partial-body dose estimates. Damaged cells were defined as those with ≥48 PCC fragments or a suitable number in excess of nonirradiated cells. Confirmation that this approach provided a reliable indication of the size of the irradiated fraction was evidenced by the relationship of the dose dependency of the fraction of damaged cells as shown in Figure 1. These data show that the fraction of damaged cells rapidly increased with dose of radiation delivered, reaching a plateau of ≥90% damaged cells by the 3 Gy dose. These findings support the use of this approach to readily estimate partial-body fractions at high doses (>3 Gy). Recently *Darroudi* and colleagues reported successfully using this approach to accurately estimate the irradiated fraction in a partial-body primate exposure study [22].

The Q_{pcc} method also permits evaluation of the dose to the irradiated fraction. The dose dependency for the yield of excess PCC fragments in damaged cells is illustrated in Figure 2. The dose dependency of excess PCC fragments in damaged cells was fitted to a linear quadratic relationship ($Y = 5.96 + 0.046D + 0.235D^2$; D = dose in Gy, Y = excess number of PCC fragments in damaged cells) for radiation dose up to 9 Gy. This data can be used as a Q_{pcc} dose-response calibration curve to estimate dose to the irradiated fraction in simulated partial-body exposure studies.

Using in vitro 6 Gy irradiated cells, a simulated partial-body exposure study was performed. Irradiated and nonirradiated lymphocytes were mixed, following the irradiation protocol, and were incubated for one day at 37°C. The PCC assay was performed to address the potential role of interphase cell death, efficiency of PEG-mediated fusion and chromosome condensation in determining estimates of the irradiated fraction and dose to the irradiated fraction of cells in a mixed-cell culture. The fraction of damaged cells and the estimated radiation doses for different fractions of irradiated cells for this study are shown in Table II. The measured fraction of damaged cells correlated with the irradiated fraction, indicating no apparent decreased efficiency of the PCC assay on damaged cells. Using the Q_{pcc} method, the dose to the irradiated fraction was estimated based on the mean number of excess PCCs and the

Table II. Results from a mixed-cell culture study examining the ability of the PCC assay to determine the fraction of irradiated cells and dose to the irradiated fraction of cells.[a]

Mixed Culture		Sample Size		% >48 PCCs		Q_{pcc} Analysis					
% 0 Gy	% 6 Gy	Total no.	No. >48 PCCs	% (SE)	Excess PCCs in Damaged Cells, Mean (SE)	P, *t*-test	Estimated Radiation Doses, Gy Equivalent (SE)	x^2	DF	P, x^2 test
100	0	34	6	18 (6.6)	—	—	—	—	—	—
80	20	40	14	35 (7.6)	11.6 (1.9)	.267	5.3 (0.6)	.082	1	.77
70	30	40	14	35 (7.8)	10.1 (1.7)	.049	4.7 (0.6)	.282	1	.60
50	50	40	24	60 (7.8)	11.0 (1.4)	.071	5.0 (0.6)	.167	1	.68
25	75	40	32	80 (6.4)	10.8 (1.4)	.042	4.7 (0.8)	.282	1	.60
0	100	81	78	96 (2.1)	13.7 (2.1)	—	6.0 (0.2)	—	—	—

[a]Experimental conditions for the mixed-culture study were as described in the Results and Discussion section. The excess number of PCC fragments in damaged cells was compared with the results from the 100% 6-Gy sample using Student's *t*-test. Estimated radiation doses were determined by using measured values of excess PCC fragments and the Q_{pcc}-standard dose-response curve. The resultant predicted dose-equivalent values were compared with the expected value of 6-Gy using the x^2 test. The x^2 *P* value for the pooled results was 0.94.

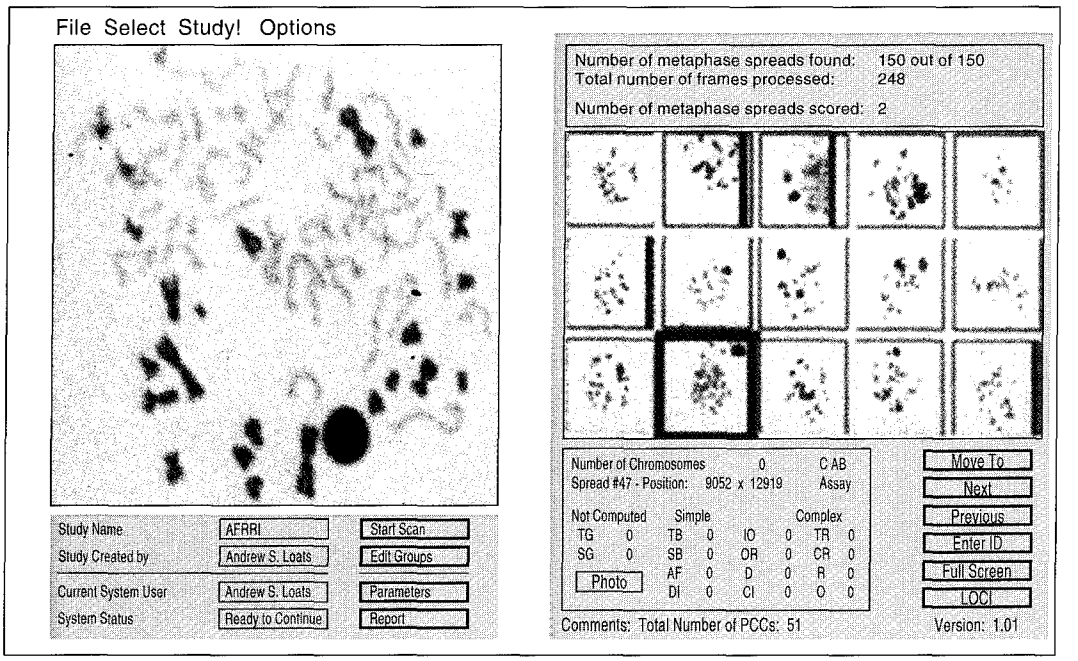

Fig. 3. Photograph of a video monitor image demonstrating the use of automated metaphase-finding system for locating PCC spreads. The customized metaphase software uses gray-scale morphometric algorithms to isolate candidate metaphase spreads, based on geometric shape, spread density and texture measurements. Candidate spreads are displayed in a postage-stamp format for user review of spread quality or, as in this case, for identification of PCC spreads for analysis. User-chosen spreads are automatically located on the slide and displayed at high power for analysis on a high-resolution monitor.

Q_{pcc}-dose-response calibration curve. Dose estimates ranged from 4.7 to 5.3 Gy for the 0% to 75% irradiated volume and were not significantly different from those expected at the 6 Gy dose (pooled Chi^2 $P = 0.94$).

Investigators earlier addressed the problem of cell selection, interphase death and mitotic delay leading to the underestimation of the radiation dose in the event of partial-body exposure by the conventional dicentric assay [23, 24]. For x-rays from 1 to 10 Gy and ratios of irradiated and nonirradiated blood from 1:1 to 1:9, agreement was found between calculated and applied radiation doses and fractions exposed by use of the contaminated Poisson method on dicentric data by *Hilali* and colleagues [14]. However, this approach is very dependent on scoring of a large number of samples (>500 metaphase spreads), which can be difficult in the case of accidental high-dose radiation overexposures.

Our studies demonstrated the potential advantages of the PCC assay to aid in overcoming some of the complex problems (e.g., mitotic delay, interphase death and failure of stimulation) that interfere with dose estimation in the lymphocyte dicentric assay. At very high radiation doses (>9 Gy) with significant reductions in lymphocyte peripheral blood counts, the PCC assay may provide an underestimation of the radiation dose to the irradiated fraction without correcting the results for loss of heavily damaged cells from the circulating peripheral blood pool.

Rapid localization of the PCC spreads on the slide is desired to expedite the visual enumeration of PCC fragments. Use of an automated metaphase-finding system can facilitate the localization and scoring of the PCC spreads, since the yield of PCCs is relatively low compared to the mitotic inducer cells and lymphocytes.

Using the search program of the metaphase-finding software under a low-power (10×) objective, PCCs were readily detected amidst the metaphase spreads and were displayed in a postage-stamp mode following automatic

scanning of the slides (Fig. 3). The metaphase-finding software also conveniently repositions the microscope stage to permit reexamination of specific PCCs at higher magnification by selecting identified postage-stamp images of PCC spreads. Typical slide-scanning times of ≤20 min per slide, to obtain a threshold of 150 metaphase spreads, were observed. The automated finding of candidate metaphase spreads significantly increased the speed of scoring PCCs.

Efforts are under way to automate the counting of the PCC fragments in our laboratory. *Pantelias* and colleagues [25] recently applied C-banding techniques to the PCC assay, permitting the scoring of dicentrics and centric rings in these preparations. Similar capabilities can be obtained using in situ DNA hybridization methodology [7, 26] applied to PCC samples. Application of these chromosome-painting techniques to permit measurement of chromosome aberrations typically associated with radiation injury and the use of automated scoring systems can assist cytogeneticists in providing biodosimetry information rapidly to clinicians treating radiation victims following acute exposures.

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