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X-ray–Induced Breakage and Rejoining of Human Interphase Chromosomes

Abstract. *A method was developed for the high-resolution measurement of breaks in prematurely condensed chromosomes at the G_1 phase of the cell cycle. The dose response for fragments (breaks) produced immediately after x-irradiation of confluent cultures of normal human cells was linear down to 10.9 rad (0.109 Gy) and extrapolated to zero effect at zero dose. The curve had a slope of 0.063 breaks per cell per rad, which is at least an order of magnitude greater than that for breaks scored in the same cells after they have progressed to mitosis following subculture. When incubated at 37°C half of the breaks disappeared in 2 hours. A slower, perhaps nonrejoining component was apparent at later incubation times. The initial rate of break rejoining was similar to the rate of increase in survival after incubation because of the repair of potentially lethal damage and is also in close agreement with recently reported values for the rejoining of double-strand breakage in DNA.*

The issue of risk assessment from exposure to ionizing radiation centers around oncogenic, mutagenic, and teratogenic effects in humans exposed to low doses of radiation. Since precise measurement of the effects of low doses in humans or mammals is not possible, we must rely on extrapolations of mathematical models that describe dose-effect data for much higher doses. Unless such models are based on sound biophysical principles the issue cannot begin to approach a satisfactory settlement. It is possible that a dose-response relation fits the data well at higher doses, but deviates appreciably from extrapolated values at low doses. For example, at high doses the effects may be governed by the response of a resistant subpopulation of cells, whereas at low doses they may be governed by a relatively small sensitive subpopulation. Since chromosomal breaks and rearrangements may play a crucial role in both mutagenesis and oncogenesis following exposure to ionizing radiation (*1*, *2*), studies of their dose-effect and rejoining kinetics are germane to the biophysical principles we seek.

In 1970 Johnson and Rao (*3*) discovered that the fusion of mitotic and interphase cells resulted in the premature condensation of interphase chromosomes. Shortly thereafter, Waldren and Johnson (*4*), and Hittleman and Rao (*5*), and others, used this technique to study the breakage of chromosomes immediately after they occurred, and the rejoining of these breaks during interphase. However, several problems prevented the adoption of this approach for high-resolution studies at low doses. Since the prematurely condensed interphase chromosomes (PCC's) and their fragments often overlap and intermingle with the mitotic chromosomes, some are easily obscured. For low-dose studies the uncertainty associated with the detection of

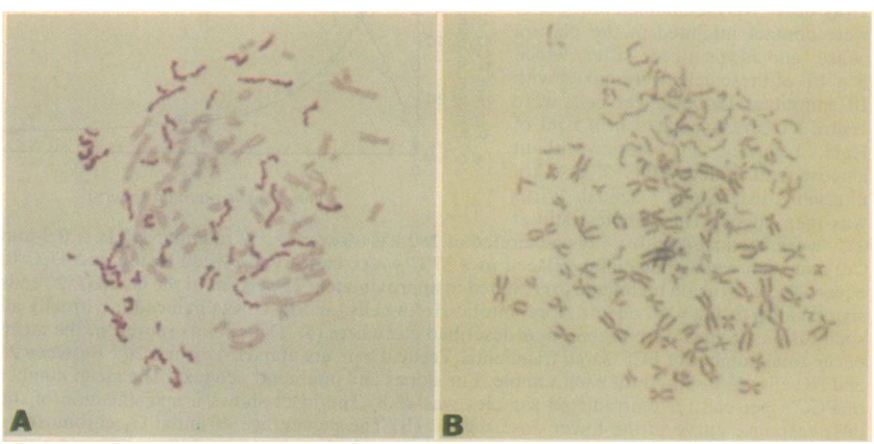

Fig. 1. (A) Prematurely condensed G_1 chromosomes (dark magenta) of an AG1522 human cell fused with a mitotic HeLa cell (light pink chromosomes) which had been grown for several generations in F12 medium supplemented with 10 percent fetal bovine serum and containing 5×10^{-6} mole of BrdU per liter. The cell was stained with Giemsa after treatment with Hoechst 33258 dye and exposure to near-ultraviolet light. (B) Prematurely condensed G_1 chromosomes of an AG1522 cell fused with a mitotic HeLa cell and stained with Giemsa in the conventional way. In some cases (not shown) the PCC's were more segregated toward one edge of the cell, but it is still not possible to tell whether a fragment is lost among the mitotic chromosomes.

one or a few fragments introduces an unacceptably large error which biases the measurements toward spuriously low fragment numbers. Further, interphase cells inevitably contaminate the synchronized mitotic populations used to induce PCC and these must be identified to avoid scoring the wrong fusion products.

We overcame this problem (*6*) by using a relatively simple technique based in principle on the Giemsa staining properties of 5-bromodeoxyuridine (BrdU)-substituted chromosomes, discovered by Perry and Wolff (*7*). The mitotic cells used to induce PCC were derived from cultures grown continuously in BrdU, and the fusion products were stained with Giemsa, after treatment with Hoechst 33258 dye and exposure to near ultraviolet light. With this procedure the BrdU-substituted mitotic chromosomes stained very lightly while the PCC's from the interphase (G_1) cells (which did not contain BrdU) stained a dark blue or magenta color (Fig. 1A). Thus, the PCC's and their fragments were much easier to score than when conventional Giemsa staining was used (Fig. 1B).

Using this technique we measured the frequency of breaks produced in noncycling G_1 cells immediately after x-irradiation, over a range of doses from 10.9 to 600 rad (0.109 to 6.00 Gy). We also compared the rejoining rate of x-ray–induced G_1 chromosome breaks with the rate of repair of potentially lethal damage (PLD).

Normal human fibroblast-derived AG1522 cells (NIA Cell Repository) were chosen for these experiments because they are relatively homogeneous with respect to chromosome number ($2n = 46$) and because they show "contact inhibition" in monolayers (*6*). A large spread in chromosome number per cell would lead to a large statistical uncertainty in determining whether breaks had been produced in a given cell or whether that cell contained more chromosomes to begin with. The second point is important because populations of cycling cells would be heterogeneous with respect to the radiosensitivity of individual cells (*8*). Noncycling cells have been used by others for the study of break rejoining in PCC's after treatment by drugs (*9*) or x-rays (*10*).

The frequency of total PCC's and fragments per cell, measured immediately after irradiation, for doses ranging from 10.9 to 600 rad, is shown in Fig. 2A. The data were fitted by least squares regression to a straight line having a slope of 0.0599 ± 0.0003 fragments (breaks) per cell per rad and an intercept of 45.92 ± 0.08 fragments. The line fitted to the low dose data alone is shown in the inset and has a slope of 0.0627 ± 0.0002 fragments per cell and an intercept of 45.78 ± 0.01 fragments. This break frequency per rad is of the same order as that reported for log phase G_1 HeLa cells by Waldren and Johnson after doses of 200 to 1800 rad. Hittleman and Rao (*5*) found that the break frequency per rad is much lower in G_2 PCC's, which agrees well with the lower x-ray sensitivity to cell killing in early to mid-G_2 reported by Griffiths and Tolmach (*9*).

We measured the frequency of metaphase chromosome aberrations after plateau phase AG1522 cells were given 330-rad x-rays, immediately subcultured, and allowed to progress to mitosis. Cells were collected with Colcemid over 6-hour intervals between 24 and 48 hours after irradiation. During this first wave of cell division only 20 percent of the cells reached mitosis compared to approximately 50 percent for unirradiated controls. The total frequency of chromosome-type aberrations per cell ranged from 1.3 at the earliest collection interval to 0.83 at the last collection interval. Virtually no chromatid-type aberrations were seen. This same dose produced approximately 20 breaks per cell as measured by PCC analysis, immediately after irradiation.

Our data for breaks in PCC's were obtained by scoring 25 to 80 cells per sample. The difference in means for the 0- and 10.9-rad samples (0.72 net fragments) was highly significant ($\chi^2 = 15.09$;

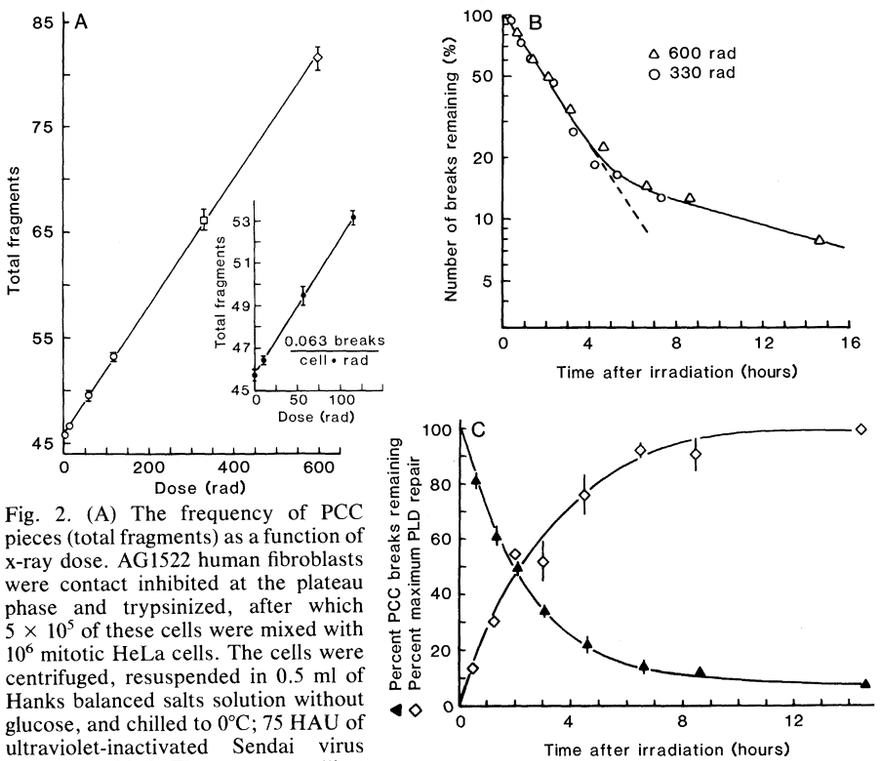

Fig. 2. (A) The frequency of PCC pieces (total fragments) as a function of x-ray dose. AG1522 human fibroblasts were contact inhibited at the plateau phase and trypsinized, after which 5×10^5 of these cells were mixed with 10^6 mitotic HeLa cells. The cells were centrifuged, resuspended in 0.5 ml of Hanks balanced salts solution without glucose, and chilled to 0°C; 75 HAU of ultraviolet-inactivated Sendai virus was then added. The samples, still at 0°C, were irradiated with x-rays generated at 280 kV (dose-rate: 59 rad/min; HVL = 0.3 mm Cu) and then were immediately placed in a 37°C water bath to allow cell fusion and PCC to occur. The cell fusion technique (*6*) resulted in approximately 25 percent of the G_1 AG1522 cells successfully fusing with one or more mitotic HeLa cells, and PCC was induced in virtually all such fusions. Slides were prepared as described elsewhere (*6*). Data points represent the mean of the total number of PCC's and fragments. Vertical bars are standard errors (*20*). Between 25 and 80 cells were scored for each sample. For slopes and intercepts see text. The mean number of PCC's per cell for unirradiated samples was 45.8. The inset shows a magnification of the dose-response curve in the lower dose region. (B) The percentage of initial G_1 chromosome breaks (net fragments) remaining as a function of incubation time after 330- or 600-rad x-rays [beam characteristics; same as (A)]. Plateau phase AG1522 cells were irradiated and incubated at 37°C for various time periods up to 14 hours, after which they were removed by trypsinization and fused with mitotic HeLa cells for PCC analysis as described in (A). A two-component exponential curve of the form $F = Ae^{ct} + Be^{dt}$ was fitted to the data. The best fit parameters were $A = 0.934 \pm 0.054$, $B = 0.081 \pm 0.058$, $c = -0.0073 \pm 0.0007$, and $d = 0.00005 \pm 0.00104$. For the 600-rad series, parallel samples were also plated to assay for survival by colony formation (see C). (C) Break rejoining data for the G_1 chromosomes exposed to 600 rad shown in (B) were replotted on linear-linear coordinates, together with results from the parallel cell survival measurements.

$P < 0.005$). Assuming breaks are produced randomly, that the break frequency per rad is 0.063, and a distribution of PCC's per cell for zero dose controls similar to that measured in these experiments, we used a Monte Carlo approach to calculate theoretical PCC + fragment distributions. The results indicate that chromosome breakage could be detected for doses as low as 3 to 5 rad by scoring about 250 cells per sample. If the dose-response were linear above 3 rad, it may not be necessary to measure the response for doses much lower than this. A macroscopic x-ray dose of about 0.5 rad would, on average, result in one electron track crossing the nucleus of each cell (*11*). Since the tracks are produced randomly, the proportion of nuclei traversed by 0, 1, or 2 electron tracks would be approximately 0.37, 0.37, and 0.18, respectively. For lower doses, a larger and larger proportion of cell nuclei would receive no dose (track) at all. Those that did would all receive (on average) the same dose since the porportion receiving two or more tracks would diminish very rapidly. Therefore, unless interactions among neighboring or surrounding cells influence the response, if 0.5 rad produces an effect, and the effect is linear above 0.5 rad, the dose response must also be linear from 0 to 0.5 rad. Establishment, beyond question, of the early-stage dose-effect kinetics of single-break production at low doses would solidify the biophysical basis for interpreting and predicting dose-effect relations at low doses for the more complicated, and perhaps more important, chromosome exchanges that require two breaks for their initiation.

The repair of potentially lethal damage (PLD) is an operationally defined phenomenon (*12*) and is similar, if not identical, to "liquid holding recovery" discovered many years ago in microorganisms (*13*). In general, incubating cells under certain suboptimal growth conditions for a few hours after irradiation allows some cells to survive, presumably by repair of damage, which otherwise would have died. We conducted experiments to compare the rate at which G_1 chromosome breaks were rejoined after irradiation with the rate of increase in cell survival due to this repair of PLD. X-irradiated cultures were maintained in contact-inhibited monolayers for various periods of time before subculturing and plating to assay for survival. As shown in Fig. 2B, half of the breaks rejoined in about 2.0 hours. A much slower, or perhaps nonrejoining component was evident at the longer incubation times. After a dose of 2000 rad, Johnson and co-workers (*14*) found an initial rejoining half-time of about 2.5 hours for cycling G_1 HeLa cells, also accompanied by a slower component at later times. Hittleman and Pollard (*15*) observed no decrease in chromatid gaps or breaks in cycling G_2 CHO cells during the first 30 minutes after 312 rad, then a decrease to about half the initial value after a total of 60 minutes. The increase in AG1522 cell survival as a function of incubation time in plateau phase monolayers after 600 rad is shown in Fig. 2C. Also shown are the data for G_1-chromosome break rejoining measured in parallel samples in the same experiment. Survival is plotted as a percentage of the maximum increase that occurred. The curves are mirror images, both crossing the 50 percent level after about 2 hours.

Chromosomal damage is an important component of the damage leading to cell death (*16*), and double-strand breaks (dsb) in DNA are probably the lesions responsible for chromosome breaks (*17*). The production of dsb's in the DNA of Ehrlich acites tumor cells is linear in the 500 to 2×10^5 rad range and corresponds to about 0.41 dsb per cell per rad (*18*). If a similar frequency applied for G_1 AG1522 cells, this number could be compared with the 0.063 chromosome breaks per cells per rad reported here. It is possible, therefore, that only about 15 percent of the dsb's are immediately expressed as breaks in prematurely condensed G_1 chromosomes. Studies on the rejoining rate of x-ray–induced dsb's in the DNA of mammalian cells revealed an exponential rejoining with a half-time of about 2 hours, measured over a period of 4 hours (*18*). This value is in close agreement with our estimate of the rejoining rate of breaks in human G_1 chromosomes. These observations are inconsistent with the model of radiation action proposed by Chadwick and Leenhouts (*19*).

The induction of PCC enables one to measure the breakage and rejoining of chromosomes at low radiation doses without the perturbing influence of processes associated with cell cycle progression to mitosis, which may modify their expression or eventual assessment. Those chromosome breaks that do not rejoin, rejoin incorrectly, or rejoin with other breaks to form exchanges, and that also allow the cell to survive and proliferate, are likely to be most important from the standpoint of mutagenesis and oncogenesis. An understanding of processes involved in these early stages of break induction and rejoining is important, not only as a guide toward establishing sound biophysical models for predicting risks from low level radiation exposure, but also for what it may reveal about processes involved in radiation mutagenesis and oncogenesis.

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