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● *Original Contribution*

DELAYED APPEARANCE OF LETHAL AND SPECIFIC GENE MUTATIONS IN IRRADIATED MAMMALIAN CELLS

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We have examined the occurrence of lethal and 6-thioguanine resistant (hprt locus) mutants among the progeny of irradiated CHO and BALB/3T3 cells. The expression of lethal mutations, as measured by a reduced cloning efficiency in the progeny cell population, was detected up to 30 mean population doublings after x-radiation. Preliminary evidence indicates that the expression of mutations at the hprt locus may also be delayed for at least 6–7 population doublings. These results suggest that radiation can induce genetic instability in cells, resulting in an increased rate of spontaneous mutations which persists for many generations of cell division. These findings are discussed in terms of their possible influence on the response of irradiated tumor cell populations *in vivo*.

X-irradiation, Mutagenesis, hprt locus, CHO cells, BALB/3T3 cells.

INTRODUCTION

It is well known that exposure to ionizing radiation can induce mutations in mammalian cells. Such mutations may result from a number of mechanisms ranging from single base pair changes to complex chromosomal rearrangements including translocations and deletions. All have the effect of producing an irreversible change in the coding sequence of a specific gene or set of genes. Classical mutation theory holds that the actual mutation or change in coding sequence occurs during semi-conservative DNA replication over the damaged template or during processing of DNA damage by enzymatic repair processes. Thus, most mutations should occur within the first round of cell division following radiation exposure.

In their earliest studies, Puck and Marcus (11) showed not only that radiation kills mammalian cells by inhibiting their ability to divide successfully, but that lethally irradiated cells could undergo several rounds of cell division before mitosis ceased. This finding was later confirmed and extended by Elkind and his co-workers (3). By use of time-lapse cinematographic techniques, Thompson and Suit (14) followed all of the descendants of irradiated cells for seven generations after exposure. From the resultant pedigrees, they showed that considerable damage was expressed amongst surviving (colony-forming) cells in terms of the death of their progeny. This death appeared to have occurred randomly among the progeny cells, though it was often not evident until several generations after radiation. These studies showed clearly that the expression of cytotoxicity may be delayed for several generations of cell replication. This phenomenon has recently been ascribed to an increased rate of "lethal mutations" occurring among the progeny of the original irradiated cells (10, 12).

In the present report, we present results which indicate that an increased rate of lethal mutations, as manifested by a reduced cloning efficiency among the progeny of irradiated cells, can be detected up to 30 rounds of cell division after irradiation. Preliminary evidence is also presented indicating that the appearance of specific gene mutations in irradiated cells may also be delayed, again occurring among progeny cells after a number of rounds of cell division. Together, these results suggest that radiation may induce genetic instability in cells, leading to the occurrence of an increased rate of spontaneous mutations over many generations of replication. This phenomenon would result in a delay in the expression of these cellular effects of radiation.

METHODS AND MATERIALS

Chinese hamster ovary (CHO) cells and mouse BALB/3T3 cells were grown and maintained as previously described (5, 9). They were irradiated with 100 kV X rays at a dose rate of 65 cGy/min from a constant potential*

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* Phillips MG100.

industrial X ray generator operated at 9.6 mA with 1 mm Al filtration. The methodology for survival experiments, as well as the various protocols used for the detection and measurement of lethal mutations, are described in detail elsewhere (5).

The standard technique for measuring mutations to 6-thioguanine resistance (hprt locus) in CHO cells has been described (9). Briefly, cells are irradiated in mass cultures ($\sim 10^6$ cells) in 75 cm^2 flasks, maintained for 8 days expression time post-irradiation with serial subcultivation at 1:10 dilutions when they become confluent, then suspended and seeded with 5 μg/ml 6-thioguanine at a density of 200,000 cells in replicate 100 mm Petri dishes. However, the protocol followed in the experiments described herein differed significantly. Cells irradiated in mass cultures were immediately subcultivated into 40 replicate 25 cm^2 flasks at a density of 300 viable cells per flask. Subcultivation at this low density was designed to preclude any immediately mutated cells from being seeded in the flasks. These flasks were returned to the incubator and the cells allowed to proliferate until they reached confluence (about 15 population doublings). Five days later, the confluent cultures were exposed to trypsin, and 200,000 cells from each flask were seeded into a single 100 mm Petri dish with 6-thioguanine (total 40 dishes). Thioguanine resistant mutant clones were scored 14 days later. Control cultures were handled in an identical fashion.

The 5 days in confluence prior to selection were allowed for maximal expression of the mutants which arose during proliferation. We have previously shown that mutations to 6-thioguanine resistance in human diploid fibroblasts are efficiently expressed in confluent cultures without subcultivation during the expression period (6). In similar experiments with irradiated CHO cells, expression was complete within 5 days; the mutant fraction remained unchanged with expression times up to 10 days prior to selection.

RESULTS

The results of experiments are presented in Table 1, in which the cloning efficiency (surviving fraction) of the progeny of CHO cells irradiated with various doses was measured 15 mean population doublings after exposure. In these experiments, a sufficient number of cells was seeded into replicate flasks for each radiation dose to yield approximately 300 viable colony-forming cells per flask. The cells were irradiated 24 hr later with 0 to 1000 cGy; the flasks were then returned to the incubator and the cells allowed to grow to confluence. At this point, they were subcultured to low density in 100 mm Petri dishes in order to measure the cloning efficiency of the cell population. As seen in Table 1, there was a progressive decline in the cloning efficiency of the progeny cell population with increasing radiation dose; following exposure to 1000 cGy, the surviving fraction among the progeny cells after 15 rounds of cell division was only 59% of that of non-irradiated controls maintained under similar conditions.

Table 1. Survival of progeny of CHO cells assayed: 15 mean population doublings after irradiation*

Dose (cGy)	Cells seeded per flask†	Cells/flask at confluence	Surviving fraction ± SE at subculture‡
0	450	9.9×10^6	1.03 ± 0.05
100	600	9.5×10^6	0.95 ± 0.03
200	700	9.2×10^6	0.91 ± 0.04
400	1100	9.1×10^6	0.83 ± 0.06
600	1700	10.5×10^6	0.85 ± 0.07
800	3800	9.5×10^6	0.70 ± 0.10
1000	14,500	10.6×10^6	0.59 ± 0.06

* Results are mean ± SE of 4 independent experiments.

† Number of cells seeded designed to yield approximately 300 viable (colony forming) cells per flask.

‡ Surviving fraction in irradiated groups as measured by cloning efficiency is corrected for the cloning efficiency of nonirradiated cells (0 dose group) treated by the same protocol.

The results of such experiments are shown graphically in Figure 1 for both CHO cells and for mouse 3T3 cells. The lower curves in both cases are for cells subcultured to measure survival immediately after irradiation, whereas the upper curves are the survival curves for the progeny cells subcultured 13–15 mean population doublings after irradiation. The decreased cloning efficiency among the progeny of irradiated cells is interpreted to reflect a persistent increase in the frequency of lethal mutations and cell death.

Table 2 presents the results of experiments in which CHO cells seeded and irradiated at low density were suspended and diluted after growth to confluence, then reseeded at 300 viable cells per dish to allow them to undergo an additional 15–16 mean population doublings. These cells thus underwent a total of 31 mean population doublings post-irradiation prior to measuring survival. As seen in Table 2, there was a small but significant reduction in the cloning efficiency of the progeny in the 1000 cGy group; this effect was observed consistently in three separate experiments.

These results are summarized in Figure 2, in which the surviving fraction among the progeny population is presented in terms of the mean number of population doublings post-irradiation (800 cGy) for both cell types. These data were derived from experiments such as those shown in Table 1, as well as experiments in which a variable number of irradiated cells were seeded in replicate flasks to allow differing numbers of mean population doublings to occur prior to confluence and assay for the surviving fraction (5).

Similar experiments were carried out to determine whether an increased rate of specific gene mutations might also persist amongst the progeny of irradiated CHO cells. In these experiments, cells irradiated with 400 cGy were suspended and seeded into 40 replicate 25 cm^2 flasks in numbers to allow 300 viable cells in each flask. These

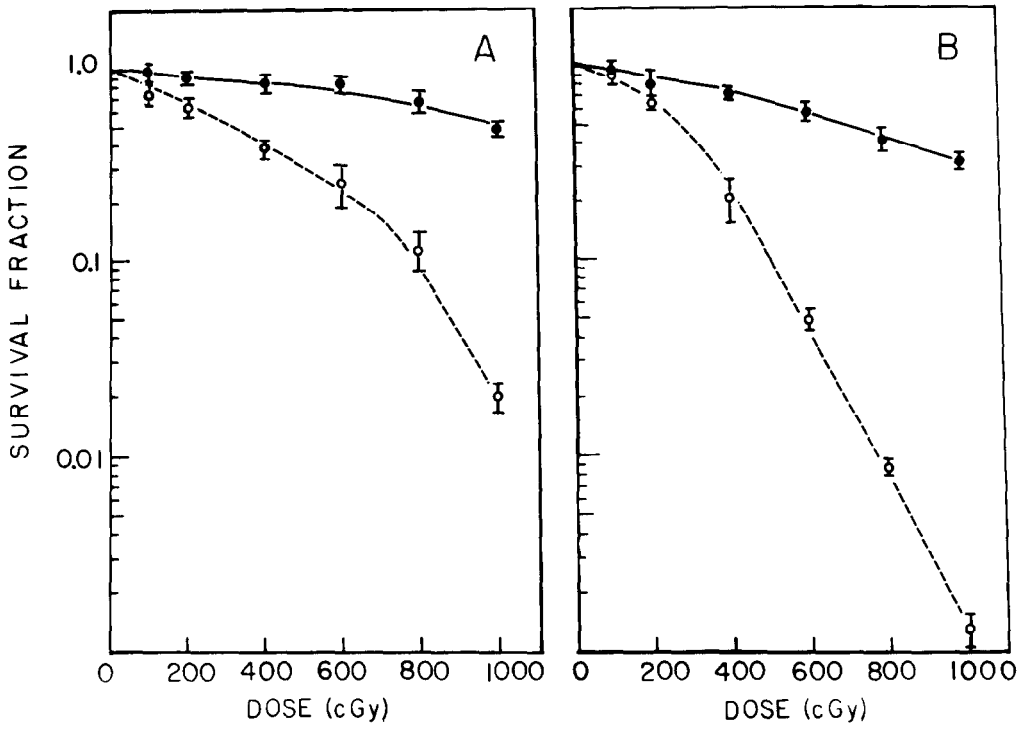

Fig. 1. Expression of lethal mutations in the progeny of irradiated cells. The survival curves represented by the open symbols and dashed lines are for cells assayed immediately after irradiation. The survival curves represented by solid symbols and lines are for the progeny of the irradiated cells assayed after many rounds of cell division. Panel A: CHO cells assayed approximately 15 mean population doublings postirradiation. Panel B: BALB/3T3 cells assayed approximately 13 mean population doublings post-irradiation.

cells were allowed to grow to confluence; 5 days later they were subcultured at a density of 200,000 cells per 100 mm Petri dish in selective medium in order to estimate the number of 6-thioguanine resistant mutant cells in each flask.

When the induction of mutations by 400 cGy was measured by the standard technique (see Methods and Materials) in this strain of CHO cells, a mutant fraction on the order of 4×10^{-5} (one mutant per 25,000 cells) was observed (8), as compared with 0.3×10^{-5} in untreated cultures. If mutations occurred only within the first round of cell division after irradiation, the appearance of a mutant in a population of 300 irradiated cells would be a rare occurrence (probability 1.2×10^{-2}); Perhaps one or two of the 40 flasks in each experiment would contain a mutant cell. In this case, proliferation of cells in the mutant clone should have yielded a large number of mutants at confluence in the occasional flask initially seeded with a mutant cell.

Table 2. Survival of progeny of CHO cells assayed: 31 mean population doublings after irradiation*

Dose (cGy)	Cells seeded per flask†	Cells/flask at confluence	Surviving fraction ± SE at subculture‡
0	450/450	7.7×10^6	0.99 ± 0.07
800	3800/600	7.2×10^6	0.97 ± 0.01
1000	14,500/600	7.3×10^6	0.88 ± 0.04

* Results are mean ± SE of 3 independent experiments.
† Cell number seeded initially/after first subculture.
‡ Surviving fraction at second subculture (after second round of proliferation to confluence).

In reality, quite a different result was observed. In four preliminary experiments, 46% of the flasks contained mutants in the irradiated group, whereas the number of mutant cells observed per flask varied from 1 to 240. In the control groups, 22% of flasks contained mutants with 1–28 mutant cells per flask. This finding suggests that mutants arose among the progeny of the irradiated cells during their growth to confluence.

The pooled results of these preliminary experiments are presented graphically in Figure 3. From the distribution of mutant clone sizes among the replicate flasks, as determined by the number of 6-thioguanine resistant cells observed, the post-irradiation generation at which each mutation occurred was estimated and a mutant fraction determined based on the number of cells present in the flasks at that generation. The results are compared with the distribution of mutations observed in non-irradiated control cultures maintained under similar conditions. This calculation of mutant fractions assumes a single mutational event in each flask and is thus only approximate; it will be refined in future experiments. However, the results shown in Figure 3 suggest that the rate of oc-

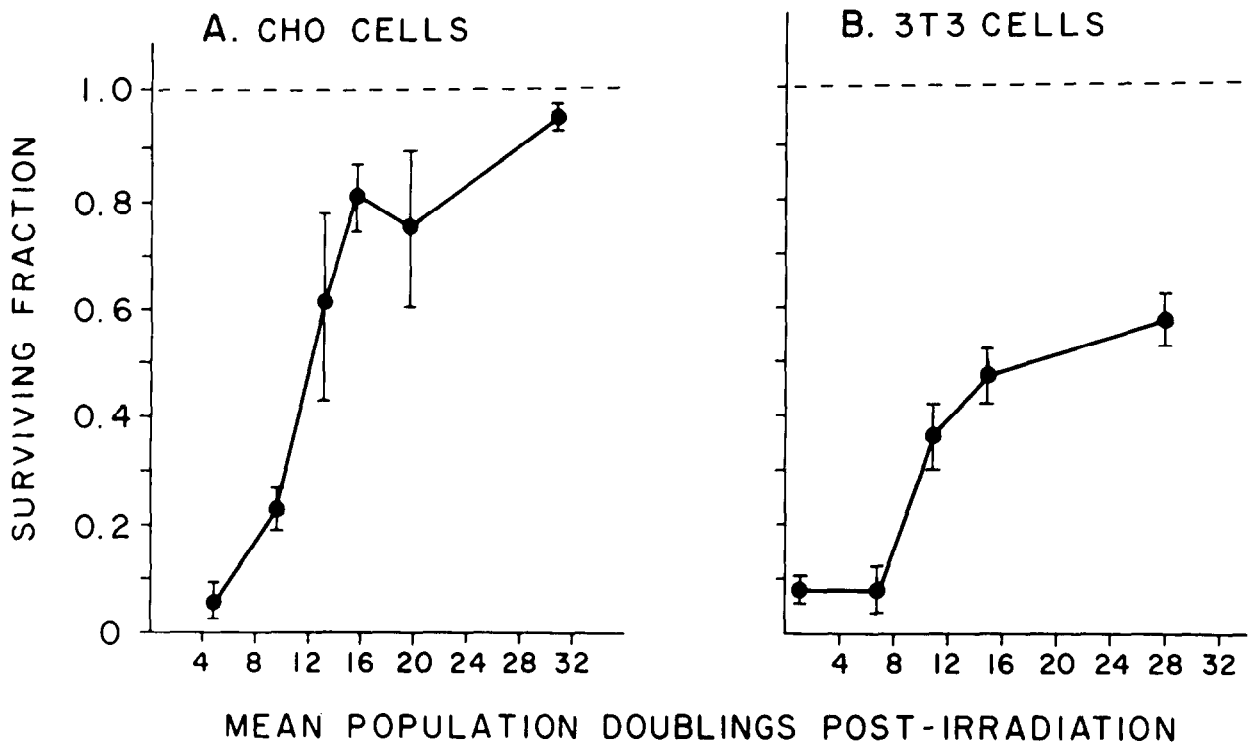

Fig. 2. Persistence of expression of lethal mutations in the progeny of cells irradiated with 800 cGy, expressed as the decrease in their cloning efficiency as compared with nonirradiated cells treated identically. Results are plotted as a function of the number of generations postirradiation. Panel A: CHO cells. Panel B: 3T3 cells.

currence of hprt mutants among the progeny of irradiated cells remains significantly elevated over background levels for at least 6–7 generations of cell division post-irradiation.

DISCUSSION

Taken together, these results indicate that the expression of radiation damage that results in cell killing or specific gene mutations may be delayed for a significant period of time after radiation, and be manifest only in the progeny of the initial irradiated cells after a number of rounds of cell division. The expression of "lethal mutations" during proliferation at long times after irradiation was first described by Seymour *et al.* (12). The fact that we observed this phenomenon in several different types of experiments (5) suggests that it is not an artifact of a specific experimental protocol. The delayed appearance of specific gene mutations has also been described in mammalian cells by Stomato and co-workers (13), and Fabre (4) observed that radiation-induced recombinational activity persisted and was transmitted through many generations of cell replication in Saccharomyces cerevisiae. Our results are also consistent with previous findings on the oncogenic transformation of mouse cells (7, 8); the actual transforming event appears to be a rare one which occurs at random among the progeny of the irradiated cells after many rounds of cell division.

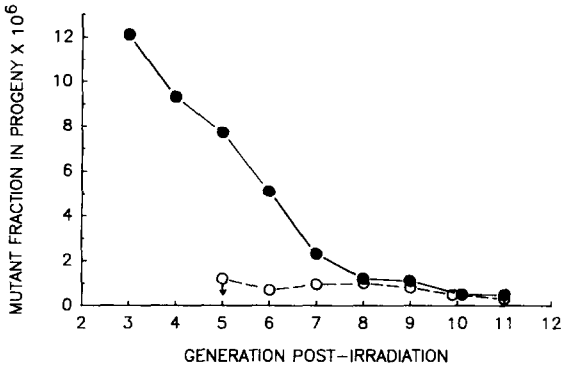

Fig. 3. Persistent appearance of 6-thioguanine resistant mutants (hprt locus) in the progeny of irradiated cells expressed as the mutant fraction observed in the progeny population 3 to 11 generations after irradiation. The upper curve (solid circles) is for cells irradiated with 400 cGy whereas the lower curve (open circles) represents the mutant fractions observed in nonirradiated controls handled in an identical fashion. Arrow means mutant fraction is less than indicated value.

The mechanism for this persistent genetic instability in irradiated cell populations remains to be elucidated. One possibly is that persistent, unrepaired DNA damage is transmitted to daughter cells over a number of generations of replication, eventually being converted into a mutation. Another explanation is that radiation induces a process which leads to a persistent elevation in the spontaneous mutation frequency in the progeny of the irradiated cells. Known inducible processes such as virus reactivation in mammalian cells and the SOS repair mechanisms in E. coli are generally repressed within the first round of cell

division following exposure to the mutagen (1, 2). This does not appear to be the case for recombinational processes in yeast (4); indeed, Francis Fabre (4; and unpublished data, written communication) has found that the rate of gene conversion remains elevated for at least 10 generations in yeast cells irradiated with ^{60}Co gamma rays. However, no such induced recombinational or DNA repair process has as yet been clearly identified in mammalian cells. A reduced fidelity of DNA replication or repair among a subset of irradiated cells would have a similar effect.

The occurrence of a persistently increased rate of cell death among the progeny of irradiated cells after as many as 15–30 rounds of cell division could have significant implications in terms of the response of tumor cells *in vivo* to radiation therapy. Clinicians have long since known that the response of tumors to fractionated irradiation may be considerably delayed. This phenomenon has generally been ascribed to the complex kinetics of cell proliferation in tumors which may contain a large fraction of quiescent cells. The delayed expression of cytotoxicity among the proliferating cell population could well contribute to this effect. A better understanding of the underlying mechanism might allow the development of a new approach to the modulation of the cytotoxic effects of ionizing radiation in tumor cells.

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