Unexpected Sensitivity to the Induction of Mutations by Very Low Doses of Alpha-Particle Radiation: Evidence for a Bystander Effect
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RAPID COMMUNICATION

Unexpected Sensitivity to the Induction of Mutations by Very Low Doses of Alpha-Particle Radiation: Evidence for a Bystander Effect

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We examined the induction of HPRT mutations in CHO cells exposed to low fluences of $^{238}$Pu α particles from a specially constructed irradiator. The dose–response relationship was linear over the dose range of 5 cGy–1.2 Gy. However, unexpected sensitivity, leading to a significantly higher frequency of mutations than would be predicted by a back extrapolation from the data for higher doses, was observed in the dose range below 5 cGy, where the mean number of α-particle traversals per nucleus was significantly less than one (0.05–0.3). The frequency of mutations induced by a single α particle traversing the nucleus of a cell was increased nearly fivefold at the lowest fluence studied. The data are consistent with the conclusion that the enhanced efficiency of each nuclear traversal at low particle fluences is the result of mutations arising in nonirradiated, bystander cells.

INTRODUCTION

There has been considerable recent concern about the potential carcinogenic effects of very low doses of α-particle radiation, because of the exposure of the general population to radon in homes. It has been estimated that as much as 15% of all lung cancer may be caused by exposure to residential radon (1, 2). Such estimates are derived from a back extrapolation of the data from underground miners exposed to higher radiation doses, assuming a linear, no-threshold dose–response relationship. Such a linear extrapolation to very low doses is not inconsistent with the results of a meta-analysis of eight case–control epidemiological studies of exposure to residential radon (3). However, the marked scatter among the data points and large potential errors make it unlikely that the precise shape of the dose–response curve at such low doses can be determined from epidemiological studies alone.

There is a significant difference, however, between the nature of the effects at the cellular level of such high- and low-dose exposures. For the higher radiation doses received by many of the underground miners, every cell nucleus in the bronchial epithelium would be expected to be traversed by at least one and probably multiple α particles, whereas for the doses associated with exposure to residential radon, only a very small fraction of the cells will be traversed by an α particle each year; most of the cells will receive no direct radiation exposure (2). As it has been assumed previously that only those cells in which the nucleus is actually traversed by an α particle are damaged and potentially mutated, the use of a linear dose–response relationship to estimate risk at these very low fluences seemed reasonable. An increase in the fluence or dose should lead to a proportional increase in the number of cells hit and potentially mutated.

It is well known that α-particle radiation is significantly more efficient than X rays in killing cells and inducing chromosomal aberrations, gene mutations and oncogenic transformation (4–9). It is thought that these biological effects are the result of unrepaird or misrepaired DNA double-strand breaks; the apparently increased efficiency of such DNA breaks induced by high-LET radiation is likely because their complexity is greater (10–13). Linear dose–response relationships have generally been observed for the induction of mutations by α particles (4–7). However, such studies have usually been carried out over the dose range in which most of the cells received one or multiple nuclear traversals. It has been difficult to measure the induction of genetic changes in cell populations where only a small fraction of the cells are traversed by an α particle, particularly...
in the case of mutations where the frequencies observed are very low.

In earlier studies, we examined the induction of sister chromatid exchanges (SCE) in CHO cells exposed to extremely low fluences of α particles (14). We made the unexpected observation that an enhanced frequency of SCE was induced in 30 to 45% of the cells in a population exposed to only 0.03 to 0.25 cGy of α-particle radiation, doses at which only 0.07 to 0.6% of the nuclei should have actually been traversed by an α particle. This finding was later confirmed in human diploid cells (15). These studies provided the first evidence to suggest that damage signals may be transmitted from irradiated to neighboring nonirradiated cells in a population, leading to the occurrence of genetic changes in these nonirradiated cells. This phenomenon has been termed the bystander effect. However, the significance of SCE in the genesis of cancer remains unclear.

The present investigation was designed to examine the mutagenic effect of α-particle radiation at very low fluences in which only a fraction of the cells were traversed by an α particle. This approach was made possible by the use of a specially constructed irradiator which allows the accurate delivery of very low fluences of α particles to cultured cells (16) as well as the use of the CHO cell HPRT mutation system, in which the background mutation frequency is very low and large numbers of cells can be irradiated, thus allowing the measurement of very low mutation frequencies with reproducible accuracy. The results provide evidence for unexpected sensitivity of cells to the mutagenic effects of very low doses of α-particle radiation, yielding a significantly higher frequency of mutations than would be predicted by a back extrapolation from the data for higher doses. We propose that this reflects the occurrence of mutations in bystander cells.

**MATERIALS AND METHODS**

**Cell Culture**

Chinese hamster ovary (CHO) cells with a stable, near diploid chromosome number were cultured at 37°C in humidified 5% CO₂ atmosphere with Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated (56°C for 30 min) bovine calf serum (HyClone), penicillin (50 U/ml), and streptomycin (50 mg/ml). To reduce the level of spontaneous HPRT mutants, the cells were grown in HAT medium (2 × 10⁻⁴ M hypoxanthine, 2 × 10⁻⁵ M aminopterin, and 1.75 × 10⁻⁵ M thymidine) for 3 days prior to the experiments. Synchronization of cell populations in the G₁ phase of the cell cycle was accomplished by the isoleucine depletion method (17). Briefly, 10⁶ cells from stock cultures were seeded on 1.5-μm-thick Mylar-based dishes coated with fibronectin to facilitate attachment. When the growth of cultures reached nearly 30% confluence, the culture medium was removed and replaced with isoleucine-deficient α-MEM containing 5% of 3X dialyzed fetal calf serum. The cultures were incubated in this medium for an additional 30 h. At this point, they had reached approximately 80% confluence (1.5 × 10⁶ cells/Mylar dish); this monolayer was irradiated with α particles.

**Irradiation**

For α-particle irradiation, the Mylar dishes were placed over a Mylar window in the exposure well of a specially constructed irradiator that provides a uniform source of well-characterized 3.7 MeV α particles (16). The source consists of 296 MBq of ²³⁸PuO₂ electrodeposited onto one side of a 100-mm-diameter stainless steel disk. The cells are irradiated from below in a helium environment, and the α particles traverse a reciprocating collimator before reaching the Mylar window. The target-to-source distance is 42 mm in helium gas, 6 mm in air, and 3 mm in Mylar. Dose was controlled by a timer and precision photographic shutter, which allows irradiation with doses as low as 0.01 mGy with high accuracy. The calculated entry dose rate at the cell surface is 9.9 cGy/min based on a mean stopping power of 110 keV/μm (16). X irradiation was carried out with a Philips 100 kV X-ray generator (HVL 2.6 mm aluminum) at a dose rate of 70.6 cGy/min in air.

**Cell Survival**

The surviving fraction was determined by a standard colony formation assay. Briefly, cells from each culture were suspended and seeded at low density in fresh medium in replicate P-100 dishes immediately after irradiation. These dishes were incubated for 7–10 days, then fixed with 95% ethanol and stained with 0.1% crystal violet; macroscopic colonies were counted and the surviving fraction was calculated.

**Mutation Assay**

The cells in 8–10 replicate Mylar dishes for each dose were suspended by trypsinization immediately after α-particle irradiation and transferred to a similar number of P-100 plastic petri dishes containing nonselective medium. The cells were cultured for 8–10 days to allow for phenotypic expression as described previously (18). The cells from each dish were then seeded in 10 new P-100 plastic petri dishes at a density of 2 × 10⁶ cells and cultured in selective medium containing 6-thioguanine (5 μg/ml) for 10–12 days. HPRT mutant colonies were thus scored in a total of 80–100 dishes for each dose in a given experiment; mutation frequencies were calculated based on the number of mutant colonies scored and the cloning efficiency at the time of seeding in selective medium.

**RESULTS**

Survival curves for CHO cells irradiated in the G₁ phase are shown in Fig. 1. The D₅₀ was 1.96 Gy for X irradiation and 39 cGy for irradiation with α particles; the extrapolation numbers were 1.6 and 1.1, respectively. These results yield a relative biological effectiveness (RBE) based on the ratio of the slopes of approximately 5 for α-particle radiation. The cloning efficiency of nonirradiated CHO cells in these experiments was 95–100%, and survival at doses below 10 Gy was greater than 95% as reported previously (19).

The dose–response relationship for the induction of HPRT mutations by α-particle radiation over the dose range of 5 cGy to 1.2 Gy is shown in Fig. 2, where it is compared with that for X radiation. The number of α-particle traversals per nucleus associated with the various mean doses is shown on the horizontal axis. The dose–response curve for α-particle exposure appears to be linear over this dose range. The RBE is approximately 18 compared X rays in the dose range up to 1 Gy, based on the ratio of the slopes.

The induction of mutations by α particles in the range of 0 to 10 cGy, in which only a fraction of the nuclei were...
traversed by an α particle, is shown in Fig. 3. The dose–response curve is clearly nonlinear in this dose range, appearing biphasic with the transition point at approximately 3 cGy. Estimation of the first and second slopes of the biphasic curve yield a mutation rate of $0.3 \times 10^{-5}$ and $0.1 \times 10^{-5}$ mutants per centigray, respectively. This second slope is the same as that measured from the higher-dose curve in Fig. 2.

The data in Figs. 2 and 3 have been replotted in Fig. 4, where the mutation frequency per α-particle track traversing a nucleus is plotted as a function of dose. The numbers in parentheses represent the estimated mean number of α-particle traversals per nucleus for each dose. As can be seen, the curve is flat over the dose range of approximately 5–10 cGy and higher, consistent with the linear dose–response relationship at higher doses shown in Fig. 2. As the α-particle fluence decreases, however, the curve rises, reaching a 4.6-fold enhancement in the mutation frequency per nuclear track at the lowest dose studied (0.83 cGy). At this point, the mutation frequency per track is 71 times the background level (Fig. 4).

**DISCUSSION**

The results of the present study indicate linearity for the dose–response relationship for the induction of mutations by α-particle radiation at doses above approximately 5 cGy (Fig. 2). This finding is consistent with the results of Hei and coworkers (20), who used a microbeam source to specifically irradiate the nuclei of cells with a precise number of α particles. They found a linear dose–response relationship for the induction of mutations in A$_t$ cells over the range of one to eight particles per cell, consistent with earlier reports for other cell systems (4–6). Interestingly, these workers also found that α-particle irradiation targeted specifically to the cytoplasm could induce mutations, though at a significantly lower frequency than direct irradiation of the nucleus (21). They hypothesized that this phenomenon might be the result of the generation of reactive oxygen species in the cytoplasm that were translocated to the nucleus, producing mutations as a consequence of oxidative damage.

Of particular note in the present study are the results shown in Fig. 3 indicating a significant change in the slope of the dose–response curve with α-particle fluences considerably less than one traversal per nucleus, with a transition point at about 3 cGy. As is shown in Fig. 4, the efficiency with which a single α-particle track traversing the nucleus of a cell can induce a mutation increases nearly fivefold at the lowest dose studied (0.83 cGy), where only 5% of the cells will be hit. This finding is of interest in relation to our previous studies of the induction of SCE and chromosomal aberrations by low doses of α-particle radiation (14,
FIG. 3. Dose–response curve for the induction of HPRT mutations by α particles in the dose range of 0.83 to 10 cGy. The results represent data derived from four to six separate experiments ±1 SEM. The background mutation frequency in these experiments was \((0.14 \pm 0.02) \times 10^{-5}\).

The kinetics of the induction of SCE differed significantly from that for mutations, in that the frequency rose rapidly at very low fluences, reaching a plateau below 1 cGy, after which no further increase in their frequency occurred with increasing dose, though a decline occurred at higher doses. The dose–response curve for the induction of chromosomal aberrations (19), on the other hand, was biphasic, with increased sensitivity at low doses and a transition point in the same general dose range as that found for mutations (Fig. 3).

Possible explanations for this phenomenon could include the existence of a small population of cells that were particularly sensitive to the mutagenic effects of radiation, or perhaps to the induction of some type of repair process by low doses such as that described by Joiner and his colleagues for cell killing (22). Such mechanisms would be plausible for exposure to low-LET radiation, by which all cells receive a low uniform dose of radiation and an incremental increase in exposure results in a comparable increase in dose to all of the cells. Such explanations, however, would seem unlikely for exposures to low fluences of α particles, by which only a small fraction of the cells are directly exposed to radiation. In the present cell system, any nucleus traversed by an α particle will receive 17.4 cGy; an incremental increase in exposure will result in a larger fraction of the cells receiving the same dose. Thus, if a sensitive subpopulation did exist or if some kind of a repair process was induced by 17.4 cGy, the effects should be comparable whether 5 or 50% of the cell nuclei were actually traversed by an α particle, the range within which the transition point in the curve occurs (Fig. 3).

It is possible that the enhanced efficiency with which each α-particle track induces mutations at very low doses (Fig. 4) could represent an increasing contribution of irradiation of the cytoplasm, thus effectively increasing the target size in the directly irradiated cells in the population. This would appear unlikely for two reasons. First, the microbeam studies of Hei and coworkers (20, 21) indicate that irradiation of the cytoplasm is much less effective than irradiation of the nucleus in inducing mutations. One traversal of the cytoplasm per cell increased the frequency of mutations by about 2-fold (21), whereas we observed that a mean of one traversal of the nucleus per cell yielded a 20-fold enhancement in the mutation frequency over background levels (Fig. 2). The mutation frequency per nuclear track increased to 71 times background with a fluence yielding a mean of one traversal of the nucleus per 20 cells (Fig. 4). Second, the nucleus of CHO cells is very large relative to the cytoplasm. Based on transmission electron microscopy measurements of CHO cells grown on a Mylar surface under conditions similar to those employed in the present experiments (23), the cross section is such that an increase in target size of only 2-fold would be expected if traversals of the cytoplasm were included and the entire cell was considered to be the target. Our data thus support the conclusion that the enhanced efficiency of each traversal at low fluences is the result of mutations arising in bystander cells.

Evidence for a bystander effect in irradiated cell populations was first derived from studies of SCE (14, 15). Lehner and his coworkers (24–26) subsequently presented evidence that the effect was mediated by the release of cytokines or other factors into the culture medium that increased the production of reactive oxygen species in
bystander cells. Enhanced cytotoxic effects have also been reported to occur in bystander cells (27, 28), as has a higher frequency of apoptotic and micronucleated cells in cultures irradiated with a microbeam of helium-3 particles (29). Finally, significant changes in gene expression have been observed in bystander cells in confluent cultures of normal human diploid fibroblasts irradiated with very low fluences of α particles, as studied by Western blotting and in situ immunofluorescence microscopy (30). This phenomenon appears to involve gap junction-mediated intracellular communication. Of particular interest is the observation that the TP53 damage response pathway is activated in bystander cells (30). It is tempting to speculate that activation of this pathway along with up-regulation of oxidative metabolism might contribute to the occurrence of genetic changes in these bystander cells.

Whatever the mechanism, the increased sensitivity to the induction of mutations by very low fluences of α particles, as shown in Fig. 3, could be significant in terms of the estimation of the potential carcinogenic risk in human populations. This finding needs to be confirmed and extended to even lower doses, and it must be determined whether it may occur in target cell populations in vivo.

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