

## Adaptive Response and the Bystander Effect Induced by Radiation in C3H 10T $\frac{1}{2}$ Cells in Culture

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This paper discusses two phenomena of importance at low doses that have an impact on the shape of the dose–response relationship. First, there is the *bystander effect*, the term used to describe the biological effects observed in cells that are not themselves traversed by a charged particle, but are neighbors of cells that are; this exaggerates the effect of small doses of radiation. Second, there is the *adaptive response*, whereby exposure to a low level of DNA stress renders cells resistant to a subsequent exposure; this reduces the effect of low doses of radiation. The present work was undertaken to assess the relative importance of the adaptive response and the bystander effect induced by radiation in C3H 10T $\frac{1}{2}$  cells in culture. When the single-cell microbeam delivered from 1 to 12  $\alpha$  particles through the nuclei of 10% of C3H 10T $\frac{1}{2}$  cells, more cells were inactivated than were actually traversed by  $\alpha$  particles. The magnitude of this bystander effect increased with the number of particles per cell. An adaptive dose of 2 cGy of  $\gamma$  rays, delivered 6 h beforehand, canceled out about half of the bystander effect produced by the  $\alpha$  particles. © 2001 by Radiation Research Society

### INTRODUCTION

Risk estimates for radiation-induced cancer are readily available for doses in the range 0.2 to 2.5 Sv from the epidemiological study of the Japanese survivors of the A-bombs (1, 2). However, these relatively large acute exposures represent much more radiation than any received occupationally or by the general public from medical procedures or from nuclear power.

Standard-setting bodies such as ICRP and NCRP have

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formulated a policy whereby risks at low doses (where they cannot be observed directly) are inferred from observed risks at high doses by extrapolation, using a linear, no-threshold model (3, 4). This is described as “prudent and conservative”, but there is much discussion concerning its validity.

Two conflicting phenomena appear to be of importance at low doses and have the potential to have an impact on the shape of the dose–response relationship, and therefore on this linear extrapolation. First, there is the bystander effect, the term used to describe the biological effects observed in cells that are not themselves traversed by a charged particle, but are neighbors of cells that are. The phrase “bystander effect” has been borrowed from the gene therapy field, where it usually refers to the killing of several types of tumor cells by targeting only one type of cell within a mixed population (5). Experiments in mammalian monolayer cell cultures irradiated by a low flux of  $\alpha$  particles, however, were able to show effects in neighboring cells that were not directly traversed by a particle or its secondary electrons. These effects include a significant increase in sister chromatid exchanges (6, 7), up-regulation of TP53 expression (8, 9), up-regulation of oxidative metabolism (10), chromosomal instability (11), and involvement of gap junctions with protein modulation in bystanders (9). More recent experiments involving the Columbia microbeam, which allows a known fraction of cells to be traversed by an  $\alpha$  particle, have clearly demonstrated a bystander effect for mutation (12) and for oncogenic transformation in C3H 10T $\frac{1}{2}$  cells (13).

Second, there is the adaptive response, whereby exposure to a low level of DNA stress resulting, for example, from a low dose of radiation renders cells resistant to a subsequent exposure. The first reproducible experiments to show an adaptive response to low doses of radiation were reported as a reduction in the number of chromosome aberrations in human lymphocytes (14). Subsequent adaptive response studies showed a reduction of micronuclei and sister chromatid exchange in Chinese hamster V79 cells (15, 16), a reduction of mutation frequency in human lymphocytes (17, 18), a reduction and an altered spectrum of mutants in human–hamster hybrid A<sub>1</sub> cells (19), and a re-

duction in micronucleus formulation in human lymphocytes (20).

These two low-dose phenomena are conflicting in the sense that they operate in opposite directions. The bystander effect tends to *exaggerate* the effect of low doses, by communicating damage from hit to non-hit cells, while the adaptive response confers *resistance* to a subsequent dose by a low initial priming dose.

Here we attempt to assess the relative magnitude of these two phenomena using cells cultured *in vitro*.

## MATERIALS AND METHODS

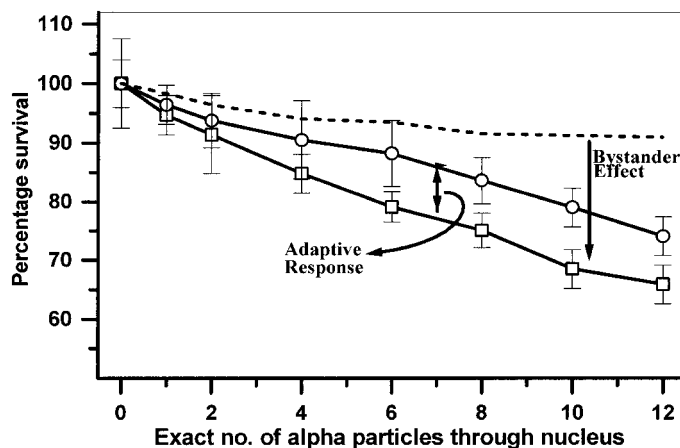
Mouse C3H 10T $\frac{1}{2}$  fibroblast cells from passage 9 were grown in Eagle's basal medium supplemented with 10% heat-inactivated fetal bovine serum and gentamicin.

For experiments involving the microbeam, about 1,000–1,200 exponentially growing cells were plated into the center of each of a series of 6.3-mm-diameter miniwells. The attached cells were stained for 0.5 h with an extremely low concentration (50 nM) of the vital nuclear dye Hoechst 33342, enabling individual nuclei to be identified and located with the optical imaging analysis system. After cells were allowed to attach overnight, half of the dishes were exposed to 2 cGy of 250 kVp X rays from a Westinghouse Coronado X-ray machine, operating at 2 mA, with 0.2 mm copper and 1 mm aluminum added filters; the absorbed dose rate was calculated to be 5.5 cGy/min. Six hours later, cells were prepared for irradiation with the microbeam. The cells were washed with serum-free medium to avoid fluorescence from serum components, and irradiations were carried out in the presence of a thin film of serum-free medium surrounding the cells. The computer was programmed so that 1 in 10 cells, selected randomly, was exposed to various numbers of  $\alpha$  particles, from 1 to 12. After irradiation, the cells were trypsinized and replated at a low density of about 300 viable cells per dish (21) into 100-mm culture dishes. The cells were incubated for 7 weeks with fresh culture medium every 12 days, before being fixed and stained to identify morphologically transformed type II and III foci, as described elsewhere (22). In parallel, dishes were plated with about 30 viable cells that had been subject to exactly the same conditions, and were incubated for 2 weeks, after which the cells were stained to determine plating efficiencies and surviving fractions of the control and irradiated cells.

Data from three to four independent experiments were pooled. All data for clonogenic survival and transformation are presented as a mean with a standard error of the mean. The statistical significance of differences between the surviving fractions of control and adaptive groups was tested by Student's *t* test. Data analysis was performed using standard techniques (23) for a Poisson-distributed number of transformed cells, with two-sided comparisons made using Fisher's exact test (24).

### Microbeam Irradiation

The Columbia microbeam has been described in detail elsewhere (25, 26). Briefly, the cells were attached at low density to the thin bases (3.8  $\mu$ m polypropylene) of 6.3-mm-diameter miniwells. The average stopping power of the  $\alpha$  particles traversing the cells was 90 keV/ $\mu$ m. The individual nuclei (including mitotic cell nuclei) were identified and located with an optical image analysis system. For each dish, a computer/microscope-based image analysis system first automatically locates the positions of all the cells and their nuclei on the dish. Next, the dish is moved under computer control such that the first cell nucleus is positioned over a highly collimated  $\alpha$ -particle beam. The beam shutter is opened until the required number of  $\alpha$  particles are detected (with a solid-state detector located above the cell) to have passed through the nucleus. The shutter is then closed, and the next cell is moved under the beam. The overall spatial precision of the beam, including positioning and beam spread, is



**FIG. 1.** The adaptive response and the bystander effect for cell survival in C3H 10T $\frac{1}{2}$  cells. The dotted line shows the percentage of cells that would be expected to survive when 10% of the cells are exposed to various numbers of  $\alpha$  particles calculated from the survival curve for all cells irradiated. The squares show survival for various numbers of  $\alpha$  particles, from 1 to 12, traversing 10% of the cell population. The extent to which this falls below the dotted line is an indication of the magnitude of the bystander effect. The circles show survival for cells exposed to 2 cGy of  $\gamma$  rays, 6 h before exposure to various numbers of  $\alpha$  particles traversing 10% of the population. The extent to which the circles are above the squares reflects the adaptive response.

about  $\pm 3.5$   $\mu$ m, which may be compared with the measured (26) average nuclear cross-sectional area of the cells of about 200  $\mu$ m<sup>2</sup>. The search-and-irradiate software can be instructed to expose any given proportion of the cells, selected at random, to any desired number of  $\alpha$  particles. In this case, 10% or 100% of the cells were exposed to defined numbers of  $\alpha$  particles directed at the centroid of the nucleus. Alpha particles (5 MeV) accelerated by a Van de Graaf accelerator were used for the irradiations.

## RESULTS AND DISCUSSION

The results of the experiments to compare the adaptive response to the bystander effect for cell survival are shown in Fig. 1. The upper dotted line in the figure shows the calculated estimated surviving fraction when 10% of the cells are hit in the absence of either an adaptive response or a bystander effect; it is calculated by applying the survival curve in Fig. 2 (where all cells are hit by various numbers of  $\alpha$  particles) to the 10% of cells that are actually hit. When only 10% of cells are hit, the surviving fraction would not be expected to fall below 90%. The actual results of irradiating 10% of the cells are shown by the solid squares. The surviving fraction falls progressively with more  $\alpha$  particles traversing each nucleus, eventually reaching 70%; this demonstrates a substantial bystander effect. The solid circles show the corresponding data for cells that had received 2 cGy of X rays 6 h prior to microbeam irradiation. The extent to which the solid circles are above the solid squares reflects the magnitude of the adaptive response on cell survival. These experiments clearly demonstrate what has come to be termed an adaptive response in C3H 10T $\frac{1}{2}$  cells. In the experiment designed to inves-

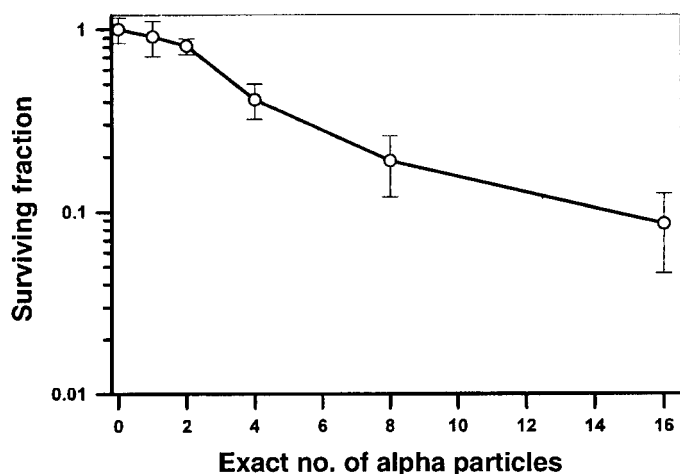


FIG. 2. Clonogenic survival resulting from nuclear traversals by 5.3 MeV  $\alpha$  particles. Each data point (mean  $\pm$  SE) on the line refers to surviving fraction of cells when all cell nuclei on each dish were exposed to exact numbers of  $\alpha$ -particle traversals using the microbeam system.

investigate the interaction of the adaptive response with the bystander effect, it is remarkable that the adaptive response resulting from such a low dose of X rays (2 cGy) can cancel out about half of the bystander effect generated by the high-LET  $\alpha$  particles.

Pre-exposure of C3H 10T $\frac{1}{2}$  cells to low-dose  $\gamma$  radiation was shown to reduce the oncogenic transformation frequency when the cells were subsequently exposed to a 4-Gy challenge dose (27). The data from the experiments to demonstrate the effect of an adaptive response on bystander cells with oncogenic transformation as a biological end point are summarized in Table 1. Three separate experiments were performed to investigate the effect of a priming dose of 2 cGy of X rays on the transformation frequency induced by irradiating 10% of the cell population with eight  $\alpha$  particles directed through the nucleus. The priming dose of 2 cGy has a large, statistically significant effect (increase) in the clonogenic survival, while the decrease in transformation frequency shows a trend but is not statistically significant. An inspection of the raw data indicates that the 2-cGy priming dose had little effect on the transformed foci; the change in the fraction of dishes with foci

is a reflection of the change in clonogenic survival resulting from the priming dose. The possibility cannot be ruled out that cells with increased survival may be refractory to transformation.

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TABLE 1  
Adaptive Response in Bystander Cells for Radiation-Induced Transformation

Treatment	No. of dishes (3 repeat experiments)	Total no. of cells irradiated	Clonogenic survival	No. of transformed foci	Fraction of dishes with foci
8 $\alpha$ particles through 10% of cells	11	45,864	0.19 $\pm$ 0.02	3	0.31 $\pm$ 0.03
	10			3	
	9			7	
2 cGy X rays + 8 $\alpha$ particles through 10% of cells	18	64,612	0.29 $\pm$ 0.01	4	0.26 $\pm$ 0.03
	16			4	
	19			6	

Note. Because of the time required to irradiate each cell individually with  $\alpha$  particles, these data represent three experiments pooled. Consequently the number of cells per dish during irradiation varied slightly, but averaged 1,163.

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