RADIATION RESEARCH **155**, 397–401 (2001)
0033-7587/01 $5.00

The Bystander Effect in Radiation Oncogenesis: I. Transformation in C3H 10T½ Cells *In Vitro* can be Initiated in the Unirradiated Neighbors of Irradiated Cells

S. G. Sawant,[1] G. Randers-Pehrson, C. R. Geard, D. J. Brenner and E. J. Hall

Center for Radiological Research, Columbia University, New York, New York 10032

Sawant, S. G., Randers-Pehrson, G., Geard, C. R., Brenner, D. J. and Hall, E. J. The Bystander Effect in Radiation Oncogenesis: I. Transformation in C3H 10T½ Cells *In Vitro* can be Initiated in the Unirradiated Neighbors of Irradiated Cells. *Radiat. Res.* 155, 397–401 (2001).

It has long been accepted that radiation-induced genetic effects require that DNA be hit and damaged directly by the radiation. Recently, evidence has accumulated that in cell populations exposed to low doses of α particles, biological effects occur in a larger proportion of cells than are estimated to have been traversed by α particles. The end points observed include chromosome aberrations, mutations and gene expression. The development of a fast single-cell microbeam now makes it possible to expose a precisely known proportion of cells in a population to exactly defined numbers of α particles, and to assay for oncogenic transformation. The single-cell microbeam delivered no, one, two, four or eight α particles through the nuclei of all or just 10% of C3H 10T½ cells. We show that (a) more cells can be inactivated than were actually traversed by α particles and (b) when 10% of the cells on a dish are exposed to α particles, the resulting frequency of induced transformation is not less than that observed when every cell on the dish is exposed to the same number of α particles. These observations constitute evidence suggesting a bystander effect, i.e., that unirradiated cells are responding to damage induced in irradiated cells. This bystander effect in a biological system of relevance to carcinogenesis could have significant implications for risk estimation for low-dose radiation.

INTRODUCTION

It has been accepted dogma for over half a century that radiation-induced heritable damage required interaction of the radiation with DNA (*1, 2*), either by direct ionization or by the production of hydroxyl radicals in water molecules close to the DNA. However, over the past decade, a number of reports have appeared describing α-particle irradiations in which a larger proportion of cells showed biological damage than were estimated to have been hit by the α particles. This phenomenon, which has come to be known as the bystander effect, was first reported by Nagasawa and Little (*3*), who exposed cells to a low dose of α particles; 30% of the cells showed an increase in sister chromatid exchanges even though less than 1% were calculated to have undergone a nuclear traversal. Since then, reports of apparently the same phenomenon have appeared with biological end points including cell killing, micronucleus induction, mutation induction, changes in gene expression, increases in intracellular oxygen species, and increases in cell growth (*3–16*).

We report here a bystander experiment with two important features:

1. An *in vitro* oncogenic transformation system was used which is more pertinent to radiation carcinogenesis *in vivo.*
2. The use of the Columbia single-cell microbeam facility has made it possible to define precisely what proportion of cells are traversed by an exactly defined number of α particles, rather than relying on estimates of probabilities.

Several groups have developed single-cell microbeams, in which cells on a dish are irradiated individually by a predefined exact number of α particles, allowing the effects of individual particle traversals to be assessed (*17–21*). However, earlier microbeam irradiation systems were too slow to allow measurement of oncogenic transformation frequencies, because the low probabilities of transformation require that many cells ($\geq 10^5$) be irradiated individually. Specifically, the overall cellular throughput for the microbeam experiments described here was around 10^4 cells per hour, two orders of magnitude faster than earlier microbeam system throughputs. This increased microbeam throughput, made possible by developments in both hardware and software (*17*), now permits sufficient numbers of cells to be irradiated to assay quantitatively for *in vitro* oncogenic transformation.

The goal of this study was to compare induced oncogenic

[1] Author to whom correspondence should be addressed at Center for Radiological Research, Columbia University, 630 West 168th Street, New York, NY 10032.

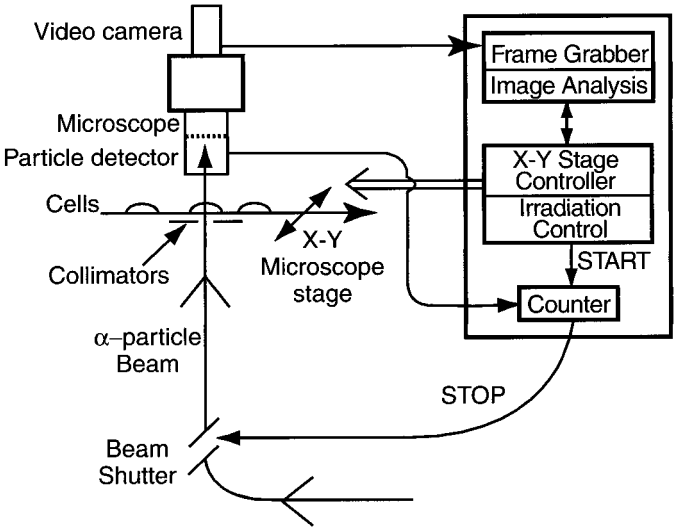

FIG. 1. Schematic of the Columbia microbeam system.

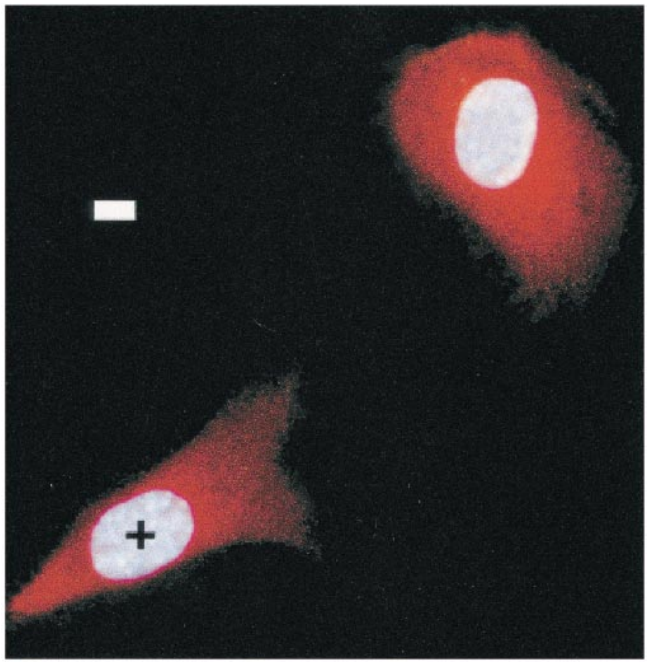

FIG. 2. Detail of C3H 10T½ cells from the automated microbeam image analysis system. Cells were stained with extremely low concentrations of the vital nuclear dye Hoechst 33342. The dish was moved under computer control, such that the centroid of every nucleus or every tenth nucleus (marked by the image analysis system as crosses) was sequentially situated under the microbeam for irradiation. For illustrative purposes, two unusually close cells that were also stained with fluorescent tetramethylrhodamine, which is preferentially taken up in the cytoplasm, are pictured. Scale bar represents 7 μm, illustrating the overall spatial precision of the α-particle microbeam of ±3.5 μm.

transformation frequencies in populations of cells, sparsely plated on dishes, in which (a) all cell nuclei were hit with precisely defined numbers of α particles, and (b) only a small proportion of the population was hit with the same numbers of α particles, while the rest received no direct radiation exposure.

MATERIALS AND METHODS

C3H 10T½ cells, which can be assayed quantitatively for oncogenic transformation (*22*), were used in this study. Transformed foci can be identified by their altered morphology, and prior studies have shown that such transformed cells produce malignant fibrosarcomas in immune-suppressed animals (*23*).

Microbeam Irradiation

A schematic of the Columbia microbeam system is shown in Fig. 1, and the irradiation procedure has been described in detail elsewhere (*17, 18*). Briefly, the cells were attached at low density to the thin bases (3.8 μm polypropylene) of 6.3-mm-diameter mini-wells. The average stopping power of the α particles traversing the cells was 90 keV/μm. 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 located the positions of all the cells and their nuclei on the dish (see Fig. 2). Next, the dish was moved under computer control such that the first cell nucleus was positioned over a highly collimated α-particle beam. The beam shutter was opened until the required number of α particles were detected (with a solid-state detector located above the cell) to have passed through the nucleus. In the present study, 5.3 MeV α particles accelerated by a Van de Graaff accelerator were used for the irradiations. The shutter was then closed, and the next cell was moved under the beam. The overall spatial precision of the beam, including positioning and beam spread, was about ±3.5 μm, which may be compared with the measured (*24*) average nuclear cross-sectional area of the cells of about 200 μm^2. Parallel experiments were performed in which every cell on the dish was irradiated with the same, defined numbers of α particles, and also in which cells were sham-irradiated—i.e., handled in an identical fashion except that the beam shutter was not opened.

The search-and-irradiate software can be instructed to expose any given proportion of the cells, selected at random, to any desired number of α particles. In this case, 10% or 100% of the cells were exposed to defined numbers of α particles through the nucleus.

Cell Culture

Before irradiation, C3H 10T½ mouse fibroblast cells from passages 10–12 were grown in Eagle's basal medium supplemented with fetal bovine serum with added iron and gentamicin. Twenty-four hours before exposure, 800–1600 exponentially growing cells were plated into the center of each of a series of 6.3-mm-diameter mini-wells. The attached cells were stained for 0.5 h with an extremely low concentration (50 n*M*) of the vital nuclear dye Hoechst 33342, enabling individual nuclei to be identified and located (see Fig. 2) with the optical image analysis system (*17*). Prior to irradiation, cells were washed with serum-free medium to avoid fluorescence from serum components. Irradiations were carried in the presence of a thin film of serum-free medium surrounding the cells.

After irradiation, the cells were trypsinized from the irradiation container (recovery rates were ~70%) and replated at a low density of about 300 viable cells per dish (*25*) 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 subjected to exactly the same conditions and were incubated for 2 weeks, after which the colonies were stained to determine plating efficiencies and surviving fractions of the control and irradiated cells.

Data analysis and estimation of confidence interval were performed using standard techniques (*26*) for a Poisson-distributed number of transformed cells, with two-sided comparisons made using Fisher's exact test (*27*).

TABLE 1
Results from Microbeam Bystander Studies

Percentage of cells irradiated	Percentage of α particles traversing each irradiated cell	Clonogenic surviving fraction	Percentage of dishes exposed	Percentage of surviving cells exposed[a]	Percentage of transformed clones produced[b]	Transformation frequency/10^4 surviving cells
0	0		26	9,600	0	0
10[c]	1[c]	0.98[c]	23[c]	8,500[c]	4[c]	4.7[c]
10[d]	1[d]	0.98[d]	85[d]	30,270[d]	7[d]	2.3[d]
10	2	0.95	27	10,000	7	7.0
10	4	0.89	25	9,000	7	7.8
10	8	0.75	18	8,500	9	10.6
0[e]	0		143	46,200	4	0.86
100[e]	1	0.83	105	42,700	5	1.2
100[e]	2	0.64	59	12,200	7	5.8
100[e]	4	0.41	18	6,600	5	7.6
100[e]	8	0.16	13	3,800	5	13.2

[a] Estimated, accounting for measured plating efficiency and clonogenic surviving fraction.
[b] No more than one transformed clone per dish was observed.
[c] Initial experiment only.
[d] Data combined from initial experiment and two subsequent repeats (see caveats in text).
[e] Data from ref. (*30*).

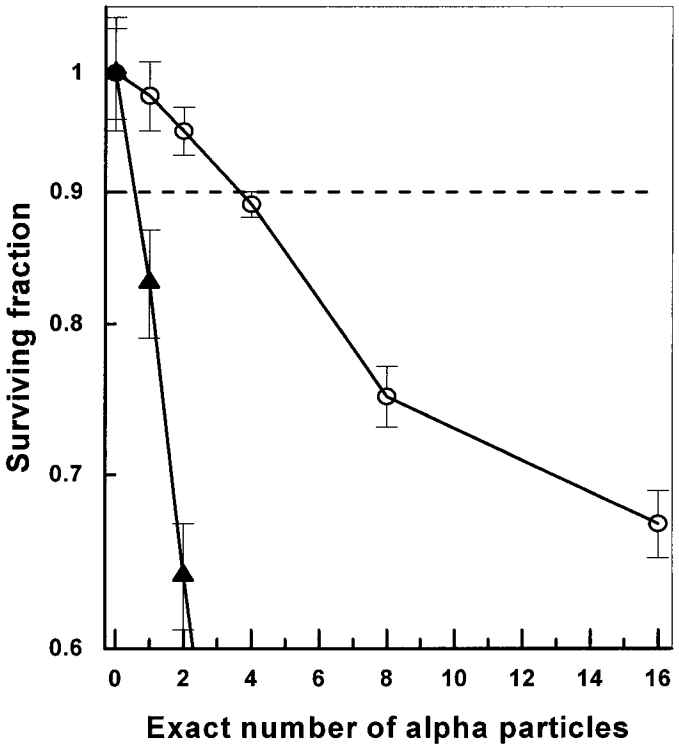

FIG. 3. Surviving fraction of cells resulting from nuclear traversals by 5.3 MeV α particles. Triangles represent to exposure of all cell nuclei on each dish to exact numbers of α-particle traversals using the microbeam system. Circles represent exposure of 1 in 10 cell nuclei on each dish to exact numbers of α particles; the dashed line indicates the 90% survival level—results of experiments using the 1-in-10 irradiation protocol that exhibit surviving fractions below this level reflect direct evidence of a bystander effect.

RESULTS

The results are shown in Table 1 and Figs. 3 and 4.

With regard to clonogenic survival, irradiation of 10% of the cells with large numbers of α particles (eight or more) resulted in clonogenic survivals of slightly, though significantly, less than 90%, using a χ^2 test (*28*). In other words, some cells were inactivated whose nuclei had not been traversed by α particles.

With regard to oncogenic transformation, when only 10% of the cells were exposed to exactly two or to more than two α particles, the resulting frequencies of induced oncogenic transformation were statistically indistinguishable from those induced when all of the cells were irradiated with the same number of α particles.

In our initial set of experiments (closed circles in Fig. 4), when 10% of the population was exposed to exactly one α particle, the rate of induced oncogenic transformation was significantly greater than that observed when all the cells were exposed to exactly one α particle. We subsequently performed two repeats of the part of the experiment in which 10% of the cells were exposed to exactly one α particle, which did not produce such enhanced effects, although no internal controls were assessed for these repeats. Based on a standard test for homogeneity between data sets (*29*), we were not able to pool the results of the later experiments with those of the original experiment, although these results and the combined results are shown for illustrative purposes in Fig. 4 and Table 1. The combined result suggests a possible increased transformation frequency of perhaps around a factor of two for the case when 10% of the cells were exposed to one α particle, compared to the case where 100% of the cells were exposed to one α particle; no statistical comparisons are possible for the combined results, however.

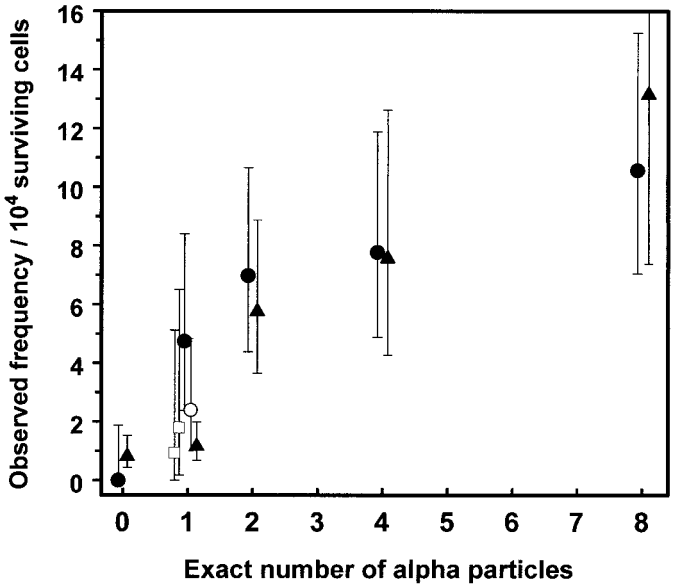

FIG. 4. Yield of oncogenically transformed cells per 10^4 surviving C3H 10T½ cells produced by nuclear traversals by 5.3 MeV α particles. Triangles represent to exposure of all cell nuclei on each dish to exact numbers of α particles, using the microbeam system. Solid circles represent exposure of 1 in 10 cell nuclei on each dish to exact numbers of α particles. Open squares represent subsequent repeats of the experiment in which 1 in 10 cell nuclei were exposed to exactly one α particle. Open circle represents combined data for all the experiments in which 1 in 10 cell nuclei were exposed to one α particle including these repeat experiments (with caveats described in the text). Standard errors (±1 SD) were estimated assuming an underlying Poisson-distributed number of transformed cells (*26*).

DISCUSSION

The observations discussed here are that (a) more cells can be inactivated by α particles than were actually traversed by α particles, (b) when 10% of the cells on a dish are exposed to two or more α particles, the resulting frequency of induced oncogenic transformation is indistinguishable from that when all the cells on the dish are exposed to the same number of α particles, and (c) there is a suggestion that, when 10% of the cells on a dish are exposed to exactly one α particle, the resulting frequency of induced oncogenic transformation could be greater than when every cell on the dish is exposed to exactly one α particle.

It is important to note that, while the C3H 10T½ cell system was chosen because the ease of quantification of results, the evaluations described here, particularly for fluences of one α particle per nucleus, are close to the lower limit for quantification.

Nevertheless, these observations provide evidence for a bystander effect, i.e., that unirradiated cells are responding to damage induced in irradiated cells. It should be noted that the cells were exposed at a low density, with essentially no overlap, and with typical nearest-neighbor separations of tens of micrometers. Other experiments (data not shown) in which transformations in C3H 10T½ cells were scored indicate that the nuclei of cells must be traversed for a bystander effect to be observed; α-particle traversals of cellular cytoplasm and medium do not produce this large effect.

If the results for *in vitro* oncogenic transformation are applicable *in vivo*, they could have significant consequences in terms of extrapolation of radiation risk to low doses, implying that the relevant target for radiation oncogenesis is larger than an individual cell, and that the risk of carcinogenesis would increase more slowly, if at all, at intermediate doses—an effect seen *in vivo* (*30*, *31*) as well as epidemiologically (*32*, *33*). Thus a simple linear extrapolation of radiation risk from intermediate doses (where they can be measured) to lower doses (where they must be inferred) would be of questionable validity, at least at high LET.

It is important to note that these results were obtained with a mouse fibroblast model system, since no quantitative oncogenic transformation system based on normal human cells has been developed. Caution must be used in applying conclusions drawn from such data to cancer risks in humans. However, this highly quantitative model system has reliably predicted epidemiologically observed trends of radiation-induced carcinogenesis in humans in the past (*34*, *35*).

Taken together, the observations discussed here provide evidence for a bystander effect and a framework to guide the quantitative modeling of bystander effects. The companion paper (*36*) suggests a quantitative model that is consistent with these results and discusses potential implications for risk assessment.

ACKNOWLEDGMENTS

Helpful discussions with Drs. Ray Sachs and Jack Little, as well as useful guidance from the reviewers and editors, are gratefully acknowledged. The Columbia University Radiological Research Accelerator Facility (RARAF) is an NIH-Supported Resource Center. This work was supported by grants RR-11623, CA-49062 and CA-37967 from the National Institutes of Health, and by grants DE-FG02–98ER62686 and DE-FG03-00-ER62909 from the U.S. Department of Energy.

Received: July 18, 2000; accepted: November 28, 2000

REFERENCES

1. R. E. Zirkle and W. Bloom, Irradiation of parts of individual cells. *Science* **117**, 487–493 (1953).
2. T. R. Munro, The relative radiosensitivity of the nucleus and cytoplasm of Chinese hamster fibroblasts. *Radiat. Res.* **42**, 451–470 (1970).
3. H. Nagasawa and J. B. Little, Induction of sister chromatid exchanges by extremely low doses of α particles. *Cancer Res.* **52**, 6394–6396 (1992).
4. A. W. Hickman, R. J. Jaramillo, J. F. Lechner and N. F. Johnson, Alpha-particle-induced p53 protein expression in a rat lung epithelial cell strain. *Cancer Res.* **54**, 5797–5800 (1994).
5. A. Deshpande, E. H. Goodwin, S. M. Bailey, B. Marrone and B. Lehnert, Alpha-particle-induced sister chromatid exchange in normal

human lung fibroblasts: Evidence for an extranuclear target. *Radiat. Res.* **145**, 260–267 (1996).

6. B. E. Lehnert and E. H. Goodwin, A new mechanism for DNA alterations induced by alpha particles like those emitted by radon and radon progeny. *Cancer Res.* **57**, 2164–2171 (1997).
7. C. B. Seymour and C. Mothersill, Delayed expression of lethal mutations and genomic instability in the progeny of human epithelial cells that survived in a bystander-killing environment. *Radiat. Oncol. Invest.* **5**, 106–110 (1997).
8. C. Mothersill and C. B. Seymour, Cell–cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: Evidence for release during irradiation of a signal controlling survival into medium. *Radiat. Res.* **149**, 256–262 (1998).
9. E. I. Azzam, S. M. de Toledo, T. Gooding and J. B. Little, Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. *Radiat. Res.* **150**, 497–504 (1998).
10. K. M. Prise, O. V. Belyakov, M. Folkard and B. D. Michael, Studies of bystander effects in human fibroblasts using a charged particle microbeam. *Int. J. Radiat. Biol.* **74**, 793–798 (1998).
11. S. A. Lorimore, M. A. Kadhim, D. A. Pocock, D. Papworth, D. L. Stevens, D. T. Goodhead and E. G. Wright, Chromosomal instability in the descendants of unirradiated surviving cells after α-particle irradiation. *Proc. Natl. Acad. Sci. USA* **95**, 5730–5733 (1998).
12. P. K. Narayanan, K. E. A. LaRue, E. H. Goodwin and B. E. Lehnert, Alpha particles initiate biological production of interleukin-8 in human cells. *Radiat. Res.* **152**, 57–63 (1999).
13. H. Nagasawa and J. B. Little, Unexpected sensitivity to the induction of mutations by very low doses of alpha-particle radiation: Evidence for a bystander effect. *Radiat. Res.* **152**, 552–557 (1999).
14. H. Zhou, G. Randers-Pehrson, C. A. Waldren, D. Vannais, E. J. Hall and T. K. Hei, Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc. Natl. Acad. Sci. USA* **97**, 2099–2104 (2000).
15. P. K. Narayanan, E. H. Goodwin and B. E Lehnert, Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Res.* **57**, 3963–3971 (1997).
16. R. Iyer and B. E. Lehnert, Factors underlying the cell growth-related bystander responses to alpha particles. *Cancer Res.* **60**, 1290–1298 (2000).
17. G. Randers-Pehrson, C. R. Geard, G. Johnson and D. J. Brenner, Technical characteristics of the Columbia University single-ion microbeam. *Radiat. Res.*, in press.
18. C. R. Geard, D. J. Brenner, G. Randers-Pehrson and S. A. Marino, Single-particle irradiation of mammalian cells at the Radiological Research Accelerator Facility: Induction of chromosomal changes. *Nucl. Instrum. Methods* **B54**, 411–416 (1991).
19. J. M. Nelson, A. L. Brooks, N. F. Metting, M. A. Khan, R. L. Buschbom, A. Duncan, R. Miick and L. A. Braby, Clastogenic effects of defined numbers of 3.2 MeV alpha particles on individual CHO-K1 cells. *Radiat. Res.* **145**, 568–574 (1996).
20. M. Folkard, B. Vojnovic, K. M. Prise, A. G. Bowey, R. J. Locke, G. Schettino and B. D. Michael, A charged-particle microbeam: I. Development of an experimental system for targeting cells individually with counted particles. *Int. J. Radiat. Biol.* **72**, 375–385 (1997).
21. Extended Abstracts: Proceedings of the 4th International Workshop: Microbeam Probes of Cellular Radiation Response. *Radiat. Res.* **153**, 220–238 (2000).
22. C. A. Reznikoff, J. S. Bertram, D. W. Brankow and C. Heidelberger, Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.* **33**, 3239–3249 (1973).
23. A. R. Kennedy, M. Fox, G. Murphy and J. B. Little, Relationship between x-ray exposure and malignant transformation in C3H10T½ cells. *Proc. Natl. Acad. Sci. USA* **77**, 7262–7266 (1980).
24. C. R. Geard, G. Randers-Pehrson, T. K. Hei, G. J. Jenkins, R. C. Miller, L. J. Wu, D. J. Brenner and E. J. Hall, Microbeam mediated cellular effects: Single α particle induced chromosomal damage, cell cycle delay, mutation and oncogenic transformation. In *Microdosimetry, an Interdisciplinary Approach* (D. T. Goodhead, P. O'Neill and H. G. Menzel, Eds.), pp. 327–330. Royal Society of Chemistry, Cambridge, 1997.
25. M. Terzaghi and J. B. Little, X-radiation-induced transformation in a C3H mouse embryo-derived cell line. *Cancer Res.* **36**, 1367–1374 (1976).
26. D. J. Brenner and H. Quan, Confidence limits for low induced frequencies of oncogenic transformation in the presence of a background. *Int. J. Radiat. Biol.* **57**, 1031–1045 (1990).
27. R. A. Fisher, *Statistical Methods for Research Workers.* Oliver and Boyd, Edinburgh, 1934.
28. S. Siegel and N. J. Castallan, *Nonparametric Statistics for the Behavioral Sciences.* McGraw-Hill, New York, 1988.
29. M. Zelen, The analysis of several 2 × 2 contingency tables. *Biometrika* **58**, 129–137 (1971).
30. R. C. Miller, G. Randers-Pehrson, C. R. Geard, E. J. Hall and D. J. Brenner, The oncogenic transforming potential of the passage of single alpha particles through mammalian cell nuclei. *Proc. Natl. Acad. Sci. USA* **96**, 19–22 (1999).
31. R. J. M. Fry, P. Powers-Risius, E. L. Alpen and E. J. Ainsworth, High-LET radiation carcinogenesis. *Radiat. Res.* **104 (**Suppl.**)**, S188–S195 (1985).
32. F. T. Cross, Evidence of lung cancer from animal studies. In *Radon and its Decay Products in Indoor Air* (W. W. Nazaroff and A. V. Nero, Eds.), pp. 373–404. Wiley Interscience, New York, 1988.
33. National Research Council, Committee on Health Risks of Exposure to Radon, *Health Effects of Exposure to Radon (BEIR VI).* National Academy Press, Washington, DC, 1999.
34. D. J. Brenner, The significance of dose rate in assessing the hazards of domestic radon exposure. *Health Phys.* **67**, 76–79 (1994).
35. D. J. Brenner, R. C. Miller, Y. Huang and E. J. Hall, The biological effectiveness of radon-progeny alpha particles. III. Quality factors. *Radiat. Res.* **142**, 61–69 (1995).
36. D. J. Brenner, J. B. Little and R. K. Sachs, The bystander effect in radiation oncogenesis: II. A quantitative model. *Radiat. Res.* **155**, 402–408 (2001).