Induction of a bystander mutagenic effect of alpha particles in mammalian cells

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Ever since the discovery of X-rays was made by Röntgen more than a hundred years ago, it has always been accepted that the deleterious effects of ionizing radiation such as mutation and carcinogenesis are attributable mainly to direct damage to DNA. Although evidence based on microdosimetric estimation in support of a bystander effect appears to be consistent, direct proof of such extranuclear/extracellular effects are limited. Using a precision charged particle microbeam, we show here that irradiation of 20% of randomly selected A_L cells with 20 alpha particles each results in a mutant fraction that is 3-fold higher than expected, assuming no bystander modulation effect. Furthermore, analysis by multiplex PCR shows that the types of mutants induced are significantly different from those of spontaneous origin. Pretreatment of cells with the radical scavenger DMSO had no effect on the mutagenic incidence. In contrast, cells pretreated with a 40 μM dose of lindane, which inhibits cell–cell communication, significantly decreased the mutant yield. The doses of DMSO and lindane used in these experiments are nontoxic and nonmutagenic. We further examined the mutagenic yield when 5–10% of randomly selected cells were irradiated with 20 alpha particles each. Results showed, likewise, a higher mutant yield than expected assuming no bystander effects. Our studies provide clear evidence that irradiated cells can induce a bystander mutagenic response in neighboring cells not directly traversed by alpha particles and that cell–cell communication process play a critical role in mediating the bystander phenomenon.

Epidemiological studies of uranium mine workers and experimental animal studies suggest a positive correlation between exposure to alpha particles emitted from radon and its progeny and the development of lung cancer (1–4). The mechanism(s) by which alpha particles cause lung cancer has not been elucidated, although a variety of genetic lesions, including chromosomal damage, gene mutations, induction of micronuclei, and sister chromatid exchanges (SCE), have been associated with the DNA-damaging effects of alpha particles (5–9).

For over a century since the discovery of X-rays, it has always been accepted that the deleterious effects of ionizing radiation such as mutation and carcinogenesis are attributable mainly to direct damage to DNA. Although the differential biological effects of nuclear versus cytoplasmic irradiation has been of interest to biologists and geneticists for decades, not much is known about the potential interaction between the two types of cellular damages. However, there is recent evidence to suggest that extranuclear or extracellular targets may also be important in mediating the genotoxic effect of irradiation (8–13). It was found, for example, that very low doses of alpha particles induced clastogenic responses (principally SCE) in both Chinese hamster ovary (CHO) and human fibroblast cultures at levels significantly higher than expected based on microdosimetric calculation of the number of cells estimated to have been traversed by a particle (8, 9). In CHO cells irradiated with low dose of alpha particles where $<1\%$ of the cells were actually traversed by a particle, an increase in SCE was observed in $>30\%$ of the cells (8). Subsequently, based on microdosimetric analysis, it was estimated that the potential target size for this SCE-induced effect would require an area 350 times the typical size of a CHO nucleus (9). The additional responding cells that received no irradiation were "bystanders" of either directly hit cells or resulted from agents released from the irradiated medium (8, 10). Subsequent studies suggested that reactive oxygen species might contribute to the induction of SCE among the bystander cells (11). Enhanced expression of the p53 tumor suppressor gene in bystander cells has also been reported in immortalized rat lung epithelial cells and human diploid fibroblast cells irradiated with alpha particles (12, 13). Although evidence in support of a bystander effect appears to be consistent, clear and unequivocal proof of a mutagenic effect has not been available.

Using a precision charged particle microbeam, we showed recently that irradiation of cellular cytoplasm with either a single or an exact number of alpha particles resulted in mutation in the nucleus while inflicting minimal toxicity, and that free radicals mediate the mutagenic process (14). The results with the well established free radical scavenger dimethyl sulfoxide (DMSO), and the thiol depleting drug buthionine S-R-sulfoximine provide further support of the idea that reactive oxygen species, particularly hydroxyl radicals, modulate the mutagenic response of cytoplasmic irradiation. More recently, Prise *et al.* (15) reported that a single human fibroblast irradiated with five alpha particles from a microbeam induced a significant increase in micronuclei among neighboring cells, although no mechanistic explanations were provided in this study as to how a single irradiated cell mediated a bystander response. Using human–hamster hybrid A_L cells, we report here that irradiated cells can induce a bystander mutagenic response in neighboring cells not directly traversed by alpha particles, and that signal transduction pathways, other than hydroxyl radical-mediated oxidative stress, play a critical role in mediating the bystander effect.

Materials and Methods

Cell Culture. Human–hamster hybrid A_L cells that contain a standard set of Chinese hamster ovary-K1 chromosomes and a single copy of human chromosome 11 were used in the study (16). Chromosome 11 encodes a cell surface marker that renders A_L cells sensitive to killing by specific monoclonal antibody E7.1 in the presence of rabbit serum complement (Covance, Denver, PA). Monoclonal antibody specific to the *CD59* (formerly called *S1*) antigen was produced from hybridoma culture as described (16, 17). Cells were maintained in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum, 25 μg/ml gentamycin, and

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Abbreviations: SCE, sister chromatid exchanges; CHO, Chinese hamster ovary.

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2× normal glycine (2×10^{-4} M) at 37°C in a humidified 5% CO_2 incubator, and were passaged as described (18–20).

Irradiation Procedure. Approximately 500 exponentially growing A_L cells were inoculated into each of a series of microbeam dishes constructed by drilling a 1/4-inch hole in the center of 60-mm diameter non-tissue-culture dishes (14, 19). A 3.8-μm-thick polypropylene film was epoxied over the bottom of the hole, creating a miniwell that was then coated with Cel-Tak to enhance cell attachment. Two days after plating, when the number of attached cells reached an average of 2,000 per dish and covered ≈80% of the growth surface, with ≈70% of the attached cells in contact with neighboring cells, the nuclei of attached cells were stained with a 50 nM solution of Hoechst 33342 dye for 30 min. An image analysis system was used to determine the centroid of each nucleus. The nucleus of a fixed proportion of A_L cells, chosen at random, was then irradiated with an exact number of alpha particles (14, 19). After irradiation, cells were maintained in the dishes for 3 days before being removed by trypsinization and replated into culture flasks. After incubation for an additional 4–5 days, the cells were trypsinized and reinoculated into plates for mutation studies as described (18–20).

Dose Response for Cytotoxicity. Irradiated and control cells in a series of miniwells were trypsinized immediately after irradiation and were replated into 100-mm diameter Petri dishes for colony formation. As described previously, we routinely recovered >98% of the attached cells from each miniwell for analysis (14, 19). Cultures were incubated for 7–12 days, at which time they were fixed with formaldehyde and were stained with Giemsa. The number of colonies was counted to determine the surviving fraction as described (18–20).

Quantification of Mutations at the *CD59* locus. To determine mutant fractions, 5×10^4 cells were plated into each of six 60-mm dishes in 2 ml of growth medium and were incubated for 2 hr for attachment, at which time 0.3% *CD59* antiserum and 1.5% (vol/vol) freshly thawed complement were added to each dish as described (21). The cultures were further incubated for 7–8 days and were fixed and stained, and the number of $CD59^-$ mutant colonies was scored. Controls included identical sets of dishes containing antiserum alone, complement alone, or neither agent. Each culture derived from each treatment dose was tested for mutant yield for two consecutive weeks to ensure full expression of mutations. The mutant fraction at each dose (MF) was calculated as the number of surviving colonies divided by the total number of cells plated after correction for any nonspecific killing on the plating efficiency due to complement alone.

PCR Analysis of Mutant Spectrum. Cloning of $CD59^-$ mutants and PCR analysis of mutant spectrum were performed as described (19–21). In brief, independently derived colonies were isolated by cloning and were expanded in cultures, and DNA was extracted by using a high salt method (22). To ensure that all mutants analyzed were independently generated, irradiated cells from each microbeam dish were processed individually. In the few cases in which clones recovered from each dish were fewer than expected, cells from 2–3 dishes were pooled and processed together for mutation assay as individual flasks. Irradiated A_L cells recovered from each microbeam dish were plated out for mutagenesis studies as described above. In most cases, only one and at times no more than two $CD59^-$ mutants were isolated from each irradiated population for mutant spectrum analysis. Five DNA marker genes on chromosome 11 (Wilms' tumor, Parathyroid Hormone, Catalase, RAS, and Apolipoprotein A-1) were chosen for multiplex PCR analysis because of their mapping positions relative to the *CD59* gene, which encodes the *CD59* antigen (16, 17, 23), and the availability of PCR primers for the coding regions of these genes (24–26). PCR amplifications were performed for 30 cycles by using a DNA thermal cycler model 480 (Perkin–Elmer/Cetus) in 20-μl reaction mixtures containing 0.2 μg of the *Eco*RI-digested DNA sample in 1× Stoffel fragment buffer, all four dNTPs (each at 0.2 mM), 3 mM $MgCl_2$, 0.2 mM each primer, and 2 units of Stoffel fragment enzyme (19, 21). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. After the last cycle, the samples were incubated at 72°C for an additional 20 min, were electrophoresed on 3% agarose gels, and were stained with ethidium bromide.

Prediction of the Expected Yield of Mutants. To predict mutant yields in experiments in which a fixed fraction of the cells were irradiated with an exact number of alpha particles through the nuclei, we use a mathematical analysis to a combination of results from experiments in which cells were irradiated and assayed in homogenous groups, assuming no bystander effect. We define: N is the number of cells in the irradiated dish; S is the number of clonogenic cells after irradiation; P is the number of progeny at the time when mutation is assayed; M is the number of mutants counted; K is the number of alpha particles delivered to the nuclear centroids; f is the fraction of cells that are irradiated; and F is the fraction of cells that are progeny of irradiated cells at the time of assay.

The present experiment is described as

$$N_K = f \times N \quad [1]$$

$$N_0 = (1 - \mathrm{f}) \times N, \quad [2]$$

where the subscripts show the number of alpha particles delivered. After irradiation, the number of unirradiated survivors is expected to be

$$S_0 = N_0 \times \mathrm{PE} \quad [3]$$

The plating efficiency (PE) is derived from sham-irradiated control. The number of irradiated survivors is expected to be

$$S_K = N_K \times \mathrm{PE} \times \mathrm{SF}_K \quad [4]$$

where the survival fraction (SF) is derived from an experiment in which 100% of the cells were irradiated with k alpha particles. The fraction of cells that are progeny of the irradiated cells in the culture after the expression period is assumed to be the same as the fraction that survived initially, so

$$F = S_K/(S_K + S_0) \quad [5]$$

Aliquots of 5×10^4 cells are assayed for mutation as described. The number of progeny of irradiated and unirradiated cells in each aliquots is

$$P_K = F \times 5 \times 10^4 \quad [6]$$

$$P_0 = (1 - \mathrm{F}) \times 5 \times 10^4 \quad [7]$$

Assuming no bystander effects, the number of mutants in each aliquot arising from irradiated and unirradiated cells is expected to be

$$M_K = P_K \times \mathrm{MF}_K \quad [8]$$

$$M_0 = P_0 \times \mathrm{MF}_0 \quad [9]$$

where the mutant fractions (MF) are derived from experiments in which 100% of the cells were irradiated through the nucleus or sham irradiated respectively. The predicted mutant fraction in the present experiment, assuming no bystander effect, is therefore

$$\mathrm{MF} = (M_0 + M_K)/5 \times 10^4 \quad [10]$$

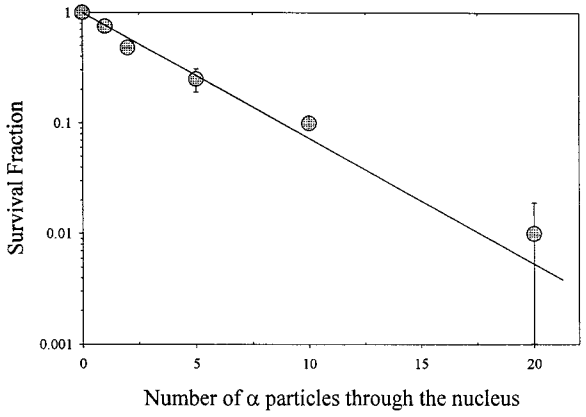

Fig. 1. Survival of A_L cells irradiated with an exact number of alpha particles in the nucleus. Data were pooled from three to four independent experiments. Error bars represent ± SEM.

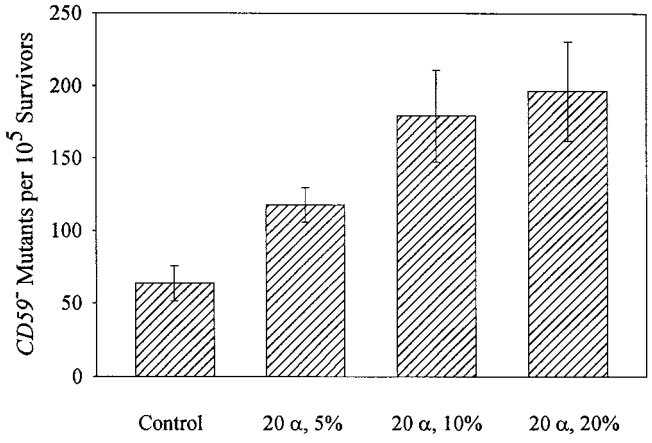

Fig. 2. Mutant fraction obtained from populations of A_L cells in which 0, 5, 10, or 20% of whose nuclei were traversed by 20 alpha particles. Data were pooled from three to eight independent experiments. Error bars represent ± SEM.

Treatment with DMSO. To examine the role of reactive oxygen species in mediating bystander mutagenesis, cells were treated with 8% DMSO 10 min before and 10 min after the irradiation or with 0.2% DMSO for 24 hr before irradiation, continued through the 7-day expression period. DMSO at the doses used in these experiments was nontoxic and nonmutagenic but had been shown to be an effective free radical scavenger (14, 27, 28). After treatment, cultures were washed, trypsinized, and replated for determination of survival and mutation as described.

Treatment with Lindane. The role of cell–cell communication in the bystander genotoxicity of alpha irradiation was investigated by treating A_L cells with a 40 μM dose of lindane for 2 hr before and 3 days after the irradiation. Lindane, a γ-isomer of hexachlorocyclohexane, has been shown to be an effective inhibitor of cell–cell communication (29). After treatment, cultures were washed, trypsinized, and replated for analysis of survival and mutagenesis as described above.

Statistical Analysis. All data for cell killing and mutation were calculated as means and standard errors of the mean. Determinations of the statistical significance of survival fractions and induced mutant fractions between treated groups and controls were made by Student's t test. Differences in the mutation spectra for *CD59*$^-$ mutants between treated group and control were analyzed by χ^2 analysis. A P value of 0.05 or less between groups was considered to be significant.

Results

Lethality of Alpha Particles Traversal through the nucleus of A_L Cells. Fig. 1 shows the dose-response for clonogenic survival of A_L cells irradiated through the nucleus with an exact number of alpha particles. The average plating efficiency of non-irradiated A_L cells grown on polypropylene under the experimental conditions used here was ≈10%. The survival data were fit by a log-linear curve with no shoulder. The mean lethal dose D_0, defined as the dose that reduced survival to 0.37 ($1/e$) in the log-linear portion of the curve, was ≈3.6 particles. It is clear from these data that most of the cells survived to form colonies after exposure of their nuclei to a single particle. This result is consistent with our previous finding (19). The surviving fraction after irradiation with 20 alpha particles was 0.01 ± 0.01. In the mutation experiment, 20% of the 2,000 cells in each microbeam dish were irradiated with 20 alpha particles. From Eqs. **2** and **3**, we expect the number of non-irradiated survivors to be $S_0 = (1 - 0.2) \times 2{,}000 \times 0.1$ or 160 ± 16. Similarly, the number of irradiated survivors from Eqs. **1** and **4** is predicted to be $S_{20} = 0.2 \times 2{,}000 \times 0.1 \times 0.01$ or 0.4 ± 0.4 cells per dish. The fraction of irradiated survivors in the culture from Eq. **5** is, therefore, $F = 0.4/(0.4 + 160)$ or $(2 \pm 2) \times 10^{-3}$. This means that 99.8% of the progeny are from unirradiated cells. The uncertainty is dominated by the uncertainty in the surviving fraction following 20 alpha particles.

Mutagenicity of Alpha Particle Traversals Through the Nucleus. We have reported the mutagenic effects of exact numbers of alpha particles up to eight particles per nucleus in the A_L cell assay (19). Using the same procedures, the mutant fraction, when 20 alpha particles traversed the nucleus, was 130 ± 38 per 10^5 survivors. The error was the SEM of three independent experiments. Although this yield is less than that from eight alpha particles, it is consistent with broad beam irradiation at high doses (21). The background mutant fraction of the A_L cell population used in the present experiments was 64 ± 15 per 10^5 survivors.

Bystander Mutagenesis in A_L Cells in Which a Fixed Proportion of Randomly Selected Cells Each Received 20 Alpha Particles Through Their Nucleus. The relatively high mutagenic sensitivity of the A_L cell system made it possible to assess the bystander mutagenic potential of alpha particles. Using a precision charged particle microbeam and image analysis system, we irradiated 20% of randomly selected A_L cells with 20 alpha particles each, such that the clonogenic survival fraction was reduced to <0.01. Under the experimental conditions, ≈70% of the cells were in direct contact with an irradiated cells. The results for mutation after irradiation with 20 alpha particles, along with the survival results can be combined to predict the number of mutants expected in the present experiments, assuming no bystander effect. The number of mutants in an aliquot of 5×10^4 cells resulting from unirradiated cells predicted from Eqs. **7** and **9** is $M_0 = (1 - 0.2 \times 0.01) \times 5 \times 10^4 \times (64 \pm 15) \times 10^{-5}$ or 32 ± 8. Similarly, using Eqs. **6** and **8**, we predict that the number of mutants resulting from the progeny of the cells irradiated with 20 alpha particles to be $M_{20} = (2 \pm 2) \times 10^{-3} \times 5 \times 10^4 \times (130 \pm 38) \times 10^{-5}$ or 0.13 ± 0.14. The predicted result is that 99.6% of the mutants found in the absence of bystander effects are from spontaneous mutagenic events among the unirradiated cells. The predicted mutant fraction is MF = 64 ± 15 per 10^5 progeny in the absence of a bystander effect. As shown in Fig. 2, the measured mutant fraction when 20% of cells were irradiated with 20 alpha particles each was 196 ± 34 per 10^5 progeny, a 3-fold higher than expected yield assuming no bystander effect. The results suggest that

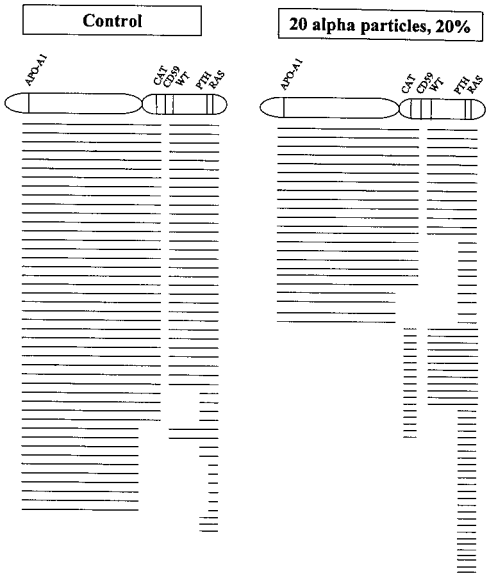

Fig. 3. Mutational spectra of *CD59*$^-$ mutants isolated from unirradiated populations or from populations in which 20% of the cells had been irradiated with 20 alpha particles through their nuclei. Each line depicts a single mutant. Blank spaces depict missing markers on chromosome 11 as determined by multiplex PCR.

unirradiated cells acquire the mutations indirectly. In other words, irradiated cells clearly induce a bystander mutagenic response in neighboring cells not directly traversed by alpha particles.

If irradiated cells generate substances that induce mutation in neighboring, non-irradiated cells, then mutant yields in the latter would be expected to decrease when fewer cells were irradiated. To test this prediction, 5 and 10% of the cells were randomly irradiated through their nuclei with 20 alpha particles each. The expected yield as calculated above, assuming no bystander effect, should be almost the same as the background mutation yield. As shown in Fig. 2, the actual mutant fraction obtained when 5% of cells were irradiated with 20 alpha particles each was 118 ± 12 per 10^5 progeny, a value significantly higher than the expected value of 64 mutants per 10^5 survivors assuming no bystander effect ($P < 0.05$). A similar finding was also observed when 10% of the population was randomly irradiated to result in a measured mutant fraction of 179 ± 32 per 10^5 progeny ($P < 0.05$). The difference in bystander mutant fractions between 10 and 20% of cells traversed through their nuclei by 20 alpha particles each was not statistically different.

Analysis of Mutant Spectrum. To determine the types of mutation associated with the *CD59*$^-$ phenotype in bystander A_L cells, we isolated individual independent clones and applied multiplex PCR to determine the presence or absence of five chromosome 11 markers located on either side of the *CD59* gene. The primers and PCR conditions were selected to amplify only the human genes and not their CHO cognates (19, 21, 30). Previous studies have shown that a small segment of the human chromosome 11 near the RAS gene is required for survival of *CD59*$^-$ mutants. The obligate presence of this region identified here by the presence of RAS probe in all of the mutants provides a convenient internal PCR control (29). A total of 108 mutants, including 47 spontaneous ones, were analyzed. As shown in Fig. 3, 30 of 47 or 63% of spontaneous *CD59*$^-$ mutants had retained all of the markers. In contrast, 82% of the mutants from populations in which 20% of the cells were irradiated with 20 alpha particles each had lost at least one additional marker, which included 28% complex mutations. The difference in spectrum between the two types of mutants was highly significant ($P < 0.01$). Furthermore, the spectrum of mutants generated in the present study was significantly different from that induced by cytoplasmic irradiation, which consisted mainly of small alterations involving only the *CD59* gene (14). The difference in spectrum suggests that different mutagenic mechanisms are involved in the two processes.

Table 1. Effects of the free radical scavenger DMSO on mutant yield in A_L cells in which 20% of them were irradiated with 20 alpha particles each through their nuclei

Irradiation	DMSO, %	Mutant fraction per 10^5 survivors
0	0	63 ± 20
0	0.2	41 ± 12
0	8	61 ± 10
20 α, 20%	0	210 ± 30
20 α, 20%	0.2	203 ± 27
20 α, 20%	8	224 ± 39

DMSO when used at 0.2% was added to the cells 24 hr before irradiation and was removed after 7 days of incubation. DMSO, 8%, was present for 20 min, 10 min before and 10 min after irradiation (14). Data were pooled from three independent experiments.

Bystander Mutagenicity of Alpha Particles Is Not Affected by DMSO. Reactive oxygen species such as superoxide anion, hydroxyl radicals, and hydrogen peroxides are the intermediates formed during oxidative metabolism. The antioxidant DMSO has been shown to be an effective radical scavenger, particularly of hydroxyl radicals, and it can protect mammalian cells against the toxic and genotoxic effects of variety of agents such as ionizing radiation, asbestos fibers, and arsenic in which oxyradicals are known to mediate their biological effects (31, 32, 33). Table 1 shows that, in cells pretreated with 0.2% DMSO 24 hr before irradiation and maintained in it throughout the expression period, the bystander mutation frequency was like that in cells without DMSO treatment. Similarly, treatment with 8% DMSO 10 min before and 10 min after irradiation, which reduced the mutagenic response caused by cytoplasmic irradiation (14), did not affect the bystander mutation fraction in the present experiments (Table 1). DMSO treatment by itself was nontoxic and nonmutagenic to A_L cells under the experimental condition used in the present study.

Is the Bystander Mutagenicity of Alpha Particles Mediated by Cell–Cell Communication? To explore the possible mechanisms involved in bystander mutagenic effects, experiments were performed to investigate the contribution of cell–cell communication between irradiated and non-irradiated cells using lindane as described (13, 29). Lindane by itself at the dose used was neither toxic nor mutagenic to A_L cells (data not shown). As shown in Fig. 4, in cells pretreated with a 40 μM dose of lindane 2 hr before irradiation and maintained in it for 3 days after irradiation, the mutant fraction was decreased significantly to 97 ± 16 per 10^5 progeny ($P < 0.05$), but at a level slightly higher then that of lindane control (64 ± 15).

Discussion

Most of the genetic effects induced in mammalian cells by ionizing radiation have been shown to result from direct damage to nuclear DNA or via "quasi-direct" effects mediated by water molecules associated with it (34–37). Thus, when a proportion of cells is exposed to alpha-particle irradiation, biological effects would be expected only in those cells whose nuclei are physically traversed by alpha particles. Presumably, no effects are to be

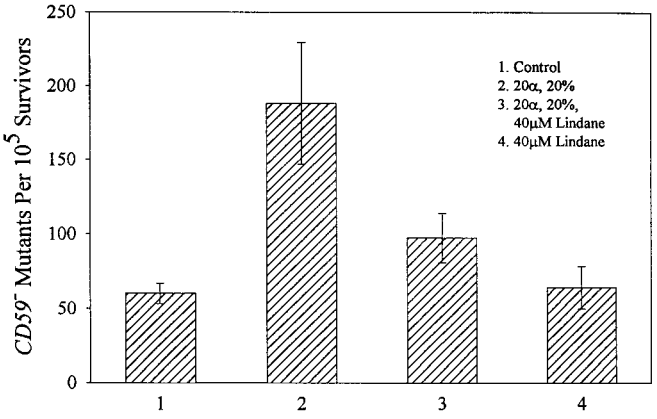

Fig. 4. Effect of lindane treatment (40 μM, 2 hr before and 3 days after irradiation) on mutant yields in A_L cells 20% of which had been irradiated with 20 alpha particles through their nuclei. Data were pooled from three independent experiments. Error bar represents ± SEM.

expected in the unirradiated cells in the population. However, there is also evidence, much of it based on statistical considerations, that indicates that irradiated mammalian cells can produce and release substances that cause genetic damage in co-cultivated but unirradiated cells. Early evidence for this bystander effect came from studies in which the frequency of SCE in populations of cells exposed to low fluences of alpha particles was significantly higher than expected from target theory calculations of the number of cells that had actually received an alpha particle (8, 9, 38). There is recent evidence that this bystander effect may also be extended to include mutation in CHO cells (39). In addition, medium from cultures of cells irradiated with γ rays can kill unirradiated cells (10) and cells in contact with cells internally irradiated by short-range ^{3}H-β particles have a reduced clonal survival (40). Using a precision charged particle microbeam, we recently reported that irradiation of cellular cytoplasm with either a single or an exact number of alpha particles results in mutation in the nucleus while causing little toxicity, and that free radicals mediate the process (14). The study provided a clue that cytoplasmic targets may contribute to the bystander phenomenon. To extend these observations, we present in the present study clear evidence, not based on target theory Poisson calculations, that mutations are induced in cells not traversed by an alpha particle. We further show that mutagenesis depends on cell–cell communication and that the spectrum of mutations induced is unlike that found spontaneously.

Using the nuclear cross sectional area of 108 μm^2 measured for A_L cells, we calculated that a dose of ≈12 cGy of 90 keV/μm alpha particles from track segment irradiation where attached cells are exposed to a board beam of monoenergetic particles would be required to deliver an average of one particle traversal per nucleus based on random, Poisson distribution (19). Our direct measurement showed that ≈20% of the irradiated cells were killed by a single alpha particle traversal through the nuclei, and >99% of the cells grown on the microwell dishes were killed by 20 alpha particles through the nucleus. These data are consistent with our previous findings (19).

Interaction between irradiated and non-irradiated cells has been of interest to biologists and geneticists for decades. Lorimore *et al.* (41) reported recently that alpha particle irradiation induced chromosome instability in the descendants of unirradiated stem cells and suggested that instability could be attributed to interactions between the irradiated and non-irradiated cells. There is evidence that culture medium exposed to alpha particles can produce a SCE-inducing factor(s) and that exposure of unirradiated cells to factor(s) present in the residual medium can induced the production of SCE in these cells (38). These findings are reminiscent of the earlier report of Stone *et al.* that demonstrated that irradiation of bacterial culture broth by UV light for a period of 3 hr significantly enhanced the mutational phenotype of penicillin-resistance in *Staphylococcus aureus* (42). However, in the present study using the microbeam, there was <3 μl of medium present per microbeam dish during irradiation. It is, therefore, unlikely that medium would play a significant role in mediating the bystander mutagenic effect. Because DMSO is highly effective in scavenging hydroxyl radicals, our data would seem to rule out the role of this radical species, although other long-lived radicals not scavenged by DMSO could be involved (31).

It is of interest to note that the bystander mutagenic effect induced among unirradiated A_L cells in which either 10 or 20% of the cell population were irradiated with 20 alpha particles each are not much different (Fig. 2). This finding is consistent with our previous report on mutagenicity induced by cytoplasmic irradiation with alpha particles (14). The decrease in bystander mutant yield could reflect that the production of mediators of mutation was saturated because the number of unirradiated cells in direct contact with an irradiated cell between the 10 and 20% population was not much different (data not shown).

Our present finding with lindane is consistent with that of Azzam *et al.* (13), who reported that expression levels of p53, p21, CDC2, Cyclin B1, and RAD51 were significantly modulated in confluent, density-inhibited human diploid cell populations exposed to doses in which only a small fraction of nuclei were expected to be traversed by an alpha particle track. The extent of modulation of p53, p21 was found to be significantly reduced in the presence of lindane, which suggested that cell–cell communication was involved in the bystander effect (13). Extracellular communication from one cell to another over extracellular space triggers various kinds of intracellular signal transduction processes in the receiving cell. Modulation of the intracellular physiology of the target cell can affect the up- or down-regulation of intercellular communication, which is essential in tissue homeostasis (43). However, the nature of the signaling molecule(s) involved in the communication between alpha particle-traversed and -non-traversed cells remains to be established. It is likely that multiple pathways are involved in mediating the bystander effect. Our present finding with DMSO is consistent with data obtained in our preliminary dilution experiment in which cells irradiated with 20 alpha particles are mixed with a fixed proportion of control cultures (80 and 90%) to achieve either 10 or 20% irradiated population. No enhancement in bystander mutagenic effect was detected in these mixing studies, suggesting that cell–cell contact was required and that labile mediator(s) appeared unlikely to be involved in the response. Our studies provide clear proof that irradiated cells may induce bystander mutagenic response in neighboring cells not directly traversed by alpha particles and suggest that signal transduction pathway other than hydroxyl radical-mediated oxidative stress may play a critical role in mediating the bystander phenomenon.

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