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Mutation Research 568 (2004) 111–120

Fundamental and Molecular Mechanisms of Mutagenesis

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Review

Genotoxicity in the eyes of bystander cells

Tom K. Hei[a,b,*], Rudranath Persaud[a], Hongning Zhou[a], Masao Suzuki[c]

[a] *Center for Radiological Research, College of Physicians and Surgeons, Columbia University, Vanderbilt Clinic 11-205, 630 West 168th Street, New York, NY 10032, USA*
[b] *Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, 60 Haven Avenue, B-1, New York, NY 10032, USA*
[c] *International Space Radiation Laboratory, National Institute of Radiological Sciences, Chiba, Japan*

Received 19 April 2004; received in revised form 2 July 2004; accepted 2 July 2004
Available online 1 October 2004

Abstract

The controversial use of a linear, no threshold extrapolation model for low dose risk assessment has become even more so in light of the recent reports on the bystander phenomenon. The answer to the question as to which of the two phenomena, bystander versus adaptive response, is more important has practical implication in terms of low dose radiation risk assessment. In this review, genotoxicity is used as an endpoint to introduce the two phenomena, provide some insight into the mechanisms of bystander effect and to bridge the two low dose phenomena which operate in opposite directions: the bystander effect tends to exaggerate the effect at low doses, by communicating damage from hit to non-hit cells whereas the adaptive response confers resistance to a subsequent challenging dose by an initial low priming dose.
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Keywords: Bystander phenomena; Genotoxic effects; Adaptive response

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* Corresponding author. Tel.: +212 305 8462; fax: +212 305 3229.
E-mail address: tkh1@columbia.edu (T.K. Hei).

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doi:10.1016/j.mrfmmm.2004.07.015

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1. Introduction

Ever since X-rays were shown to induce mutation in *Drosophila* more than 70 years ago, the prevailing dogma has been that the genotoxic effects of ionizing radiation such as mutations and carcinogenesis are due mostly to direct damage to the nucleus. As such, generations of students in radiation biology have been taught that such heritable biological effects are the consequence of a direct radiation–nuclear interaction. Although there has been circumstantial evidence to suggest that this simple statement is not strictly true as early as the 1940s, for example, Kotval and Gray had shown that α-particles which passed close, but not through, the chromatid thread had a significant probability of producing chromatid and isochromatid breaks or chromatid exchanges [1], the modern day definition of a bystander effect derived mainly from the work based on micro-dosimetric principles conducted more than a decade ago [2].

It was shown that, following a low dose of α-particles from a plutonium source, a larger proportion of cells showed biological damage than were estimated to have been hit by an α-particle; specifically 30% of the cells showed an increase in sister chromatid exchanges even though less than 1% were calculated to have undergone a nuclear traversal [2]. It is reasonable to assume that the non-hit cells in the vicinity of a hit one contributed to the induction of biological damage, or a bystander effect. However, the number of cells hit was estimated by a calculation, based on the fluence of α-particles and the cross-sectional area of the cell nucleus. The conclusion was thus of a statistical nature since it was not possible to know on an individual basis which cells were hit and which were not.

To demonstrate the induction of a radiation induced bystander effect unequivocally, studies have been carried out using a single particle microbeam where a defined proportion of cells in a confluent monolayer are irradiated with a preset number of α-particles through either the nucleus or cytoplasm. The Columbia University microbeam has become an invaluable tool in defining the bystander effect both phenomenologically as well as mechanistically.

2. The Columbia University microbeam

The design and layout of the Columbia University single particle microbeam has been described previously [3]. Briefly, each cell attached in a monolayer to a thin polypropylene base of a cell culture dish is identified and located by using an image analysis system, and its coordinates are stored in a computer. The cell dish is then moved under computer control such that the centroid of each cell nucleus (or a region of the cytoplasm remote from the nucleus, according to the plan of the particular experiment) is in turn positioned over a highly collimated shuttered beam of α-particles generated by a 4 MeV van de Graaff accelerator.

Each cell is exposed to a predetermined exact number of α-particles and a detector positioned above the cell signals to close the accelerator shutter when the desired number of particles (e.g., one) is recorded, after which the next cell is moved over the beam. Con-

tinuous developments in hardware and software have increased the microbeam throughput so that individual cells can be irradiated, one at a time, in about 1 s; this permits sufficient cells to be exposed for mutation and oncogenic transformation studies. Using this device, the bystander effect for α-particle irradiation has been demonstrated for a variety of biological endpoints [4–8]. In this review, genotoxicity will be used as an endpoint to establish the bystander phenomenon; to probe the possible mechanism involved; to ascertain the modifying effects of adaptive response; and to evaluate the implication for low dose radiation risk assessment.

3. Genotoxicity as a bystander endpoint

3.1. The mutagenic assay

Since individual cells are irradiated one at a time in the microbeam studies, a sensitive mutagenic assay system is essential. The human hamster hybrid A_L cells, developed by Waldren et al. [9], contain a full complement of hamster chromosomes but only one human chromosome (chromosome 11) fulfill this requirement. Chromosome 11 contains the *CD59* gene (formerly known as the *M1C1* gene) at 11p13.5 that encodes for the CD59 cell surface antigen (also known as the S1 antigen). By the use of the E7.1 monoclonal antibody, mutations can be scored in the human chromosome with high specificity and quantification since mutations ranging in size from a single base pair to chromosomal mutations involving loss of the entire chromosome 11 (140 Mb) are detectable [10–13]. The *CD59* gene is 27 kb in size and has four exons. PCR primers are available to determine the mutant spectrum involving the *CD59* gene. In addition to the *CD59* gene itself, there are several other marker genes identified on both the short and long arms of chromosome 11. By using probes for these genes, one can delineate the presence or absence of particular DNA segments in order to define the spectrum of the mutants generated as described [10,11,13].

3.2. Defining radiation induced bystander mutagenesis

The relatively high mutagenic sensitivity of the A_L assay and the speed of the Columbia University microbeam have made it possible to assess the bystander mutagenic potential of α-particles. Using the microbeam and an image analysis system, Zhou et al. irradiated 20 % of randomly selected A_L cells with a lethal dose of 20 α-particles each. Under the experimental conditions, approximately 70% of the cells were in direct contact with an irradiated cell. Since all directly hit cells were reproductively dead, the mutant fraction obtained from the cell population should be similar to background mutant yield (averaged 64 ± 15 per 10^5 progeny among the cells used in these experiments) assuming that there was no interaction between the hit and non-hit cells. As shown in Fig. 1, the measured mutant fraction when 20% of the cells were irradiated with 20 α-particles each was 196 ± 34 per 10^5 progeny, a value three times higher than the expected background value [4]. The results suggest that unirradiated cells acquire the mutations indirectly. In other words, irradiated cells clearly induce a bystander mutagenic response in neighboring cells that were not directly traversed by α-particles.

However, bronchial epithelial cells exposed to environmental radon in homes rarely have more than one particle traversal at any one time. The next critical question to ask is whether this bystander effect can be demonstrated at low doses of α-particle, a dose as

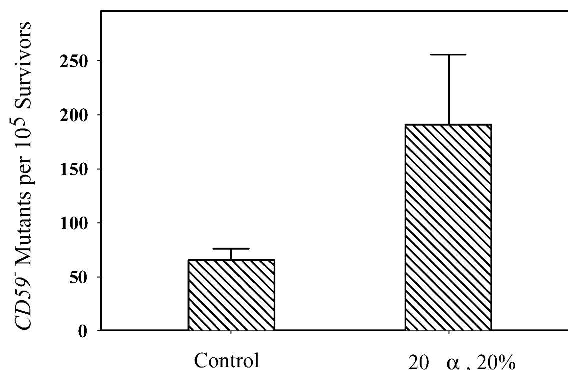

Fig. 1. Mutant fraction at the CD59 locus in neighboring, non-irradiated human hamster hybrid (A_L) cells in which 20% of the population was randomly irradiated with a lethal dose of 20 α-particles each. Background mutant fraction from control populations was shown for comparison. Data are averaged from eight independent experiments. Bars represent ±S.D. (replot using data from reference 4).

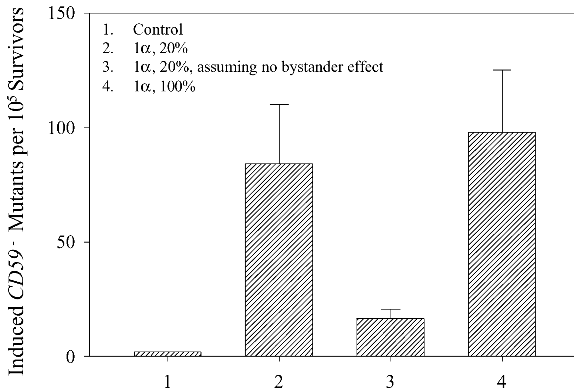

Fig. 2. The bystander effect for mutation at the CD59 locus in the A_L cells when 20% of the cells received a single nuclear traversal by α-particles. This mutant fraction is not different from the incidence when every cell in the population received a single α-particle. Data are from three to seven independent experiments. Bars represent ±S.D.

low as a single traversal per cell. Bystander mutagenesis in A_L cells induced by a single α-particle through the nucleus is shown in Fig. 2. Consistent with our previous finding, traversal of the nucleus with a single α-particle was only slightly cytotoxic to A_L cells resulting in a surviving fraction of ∼0.79 ± 0.05 [14]. Furthermore, the yield of *CD59*⁻ mutants induced in populations of A_L cells in which 100% of the cells had received exactly one α-particle through the nucleus was not significantly different from the mutant fraction obtained when only 20% of the cells were hit with a single α-particle [15].

To ascertain if this bystander mutagenic effect can be demonstrated with a lower density of irradiated cells, the experiments were repeated with different proportion of hit cells. The yield of *CD59*⁻ mutants induced in populations of A_L cells in which 5, 10 or 100% of the cells had received exactly one α-particle through the nucleus has been examined [15]. The mutant fraction when 5% of the cells had been irradiated was 58% of that when all of the cells were irradiated (induced mutant fractions were 57 and 98, respectively). It is of interest to note that there was no difference in the yield of mutants when the fraction of irradiated cells increased from 10 to 100%. This could be a reflection that the fraction of non-irradiated cells in the population that were in direct contact with, and affected by, an irradiated cell had reached a plateau at 10% and suggests that cell density is important in bystander mutagenesis.

3.3. Is the bystander mutagenic effect an artifact of the A_L cells

To demonstrate that the bystander genotoxic effect observed thus far with the A_L cells is not an artifact of the human hamster hybrid cells and the findings are relevant to human lung cancer incidence as a result of exposure to environmental radon, it is necessary to show that primary human bronchial epithelial cells exhibit a similar bystander genotoxic response as well. Since mutagenesis studies in primary epithelial cells are difficult to conduct due to the limited life span of the cultures, we use another highly sensitive genotoxic endpoint, that of G_2 phase premature chromosome condensation technique, to measure chromatid breaks in bystander normal human bronchial epithelial (NHBE) cells. In addition to gene mutations, chromosomal aberrations are an important class of DNA damage induced by α-particles. Using the Calyculin A-induced G_2 phase premature chromosome condensation (G_2PCC) assay, we showed previously that bystander genotoxicity could also be demonstrated in A_L cells [15]. Commercially available NHBE cells were plated on microbeam dishes and irradiated as described above. The incidence of chromatid-type breaks induced in these cells where a single α-particle was delivered to the nuclei in either 20 or 100% of the cultures was determined. As shown in Fig. 3, in a population in which every NHBE cell was irradiated, 90% of the cells contained three or more chromatid breaks (Fig. 3B). Assuming no interaction between the irradiated and non-irradiated cells, 78% of the cells were expected to contain no breaks when 10% of the cells were hit with one particle through the nucleus (Fig. 3C). In actuality, only 38% of the cells in this population showed no chromatid breaks (Fig. 3D). Furthermore, the profile of chromatid breaks was very different from that in which 100% of the cells in the population were hit. The proportion of NHBE cells with multiple chromatid breaks that was so prevalent in directly hit cells were much smaller in the bystander population. These data clearly illustrated that the bystander genotoxic response can also be demonstrated in NHBE cells and is not an artifact pertaining to the A_L cells.

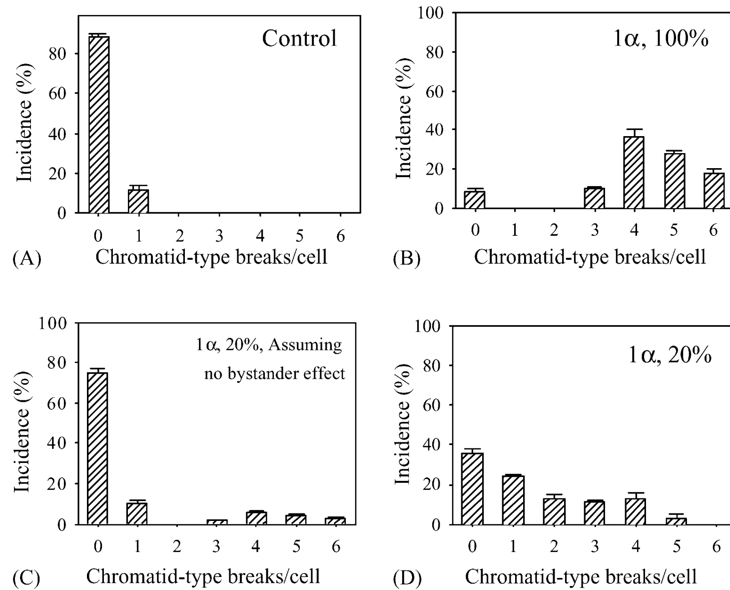

Fig. 3. Induction of chromatid-type breaks per cell from populations of commercially available primary human bronchial epithelial (NHBE) cells in which 0, 10 or 100% of cells were traversed by exactly one α-particle through the nuclei. Chromatid-type breaks include chromatid breaks and acentric fragments, and were scored from a minimum of 50 G_2 phase premature chromosome condensation samples per experiment as described [45]. The estimated chromatid-type breaks per cell assuming no interaction between irradiated and non-irradiated cells were calculated as described [4,15].

4. Mechanism of bystander mutagenesis

4.1. *Effects of oxygen scavengers on bystander mutagenesis*

There is evidence that reactive oxygen species (ROS) released into tissue culture medium may mediate the bystander effect [16,17]. ROS 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 hydroxyl radicals, and it can protect mammalian cells against the toxic and genotoxic effects of a variety of agents such as ionizing radiation, asbestos fibers, and arsenic in which oxyradicals are known to mediate their biological effects [18–20]. There is evidence that in cells pretreated with 0.2% DMSO 24 h before irradiation and maintained in it throughout the expression period, the bystander mutation frequency was like that in cells without DMSO treatment [4]. Similarly, treatment with 8% DMSO 10 min before and 10 min after irradiation, which reduced the mutagenic response due to cytoplasmic irradiation [21], did not affect the bystander mutation fraction in the present experiments [4]. DMSO treatment by itself was non-toxic and non-mutagenic to A_L cells under the experimental conditions used in the study.

4.2. *Effects of N-acetyl-cysteine on the bystander effect*

N-acetyl-cysteine (NAC), the *N*-acetyl derivative of the amino acid cysteine, is a sulfhydryl group donor that is able to cross plasma membrane and maintains intracellular glutathione levels by providing cysteine for its biosynthesis in the cells [22]. Clinically, NAC has both antioxidant and anti-inflammatory effects. There is evidence that A_L cells pretreated with NAC (10 mM) 24 h before irradiation and maintained in culture throughout the expression period had essentially no effect on bystander mutagenesis when 20% of the cells were randomly irradiated with a single α-particle [23]. NAC

treatment by itself was non-toxic and non-mutagenic to A_L cells under the experimental conditions. These data indicate that free radicals generated from irradiation have limited effects on the induction of bystander mutagenesis.

4.3. *Effects of non-specific inhibitor of gap-junctional communication on the bystander effect*

Since cell density dependence of the bystander effect, as described above, implies cell-to-cell contact in the process, the relationship between gap junctional activity and α-particle induced bystander mutagenicity was investigated in two ways: (1) the use of octanol to inhibit gap junction-mediated intercellular communication [24] and (2) using genetically engineered cells that lack gap junctions. In the first set of studies, A_L cells were treated with a non-toxic, and largely non-mutagenic dose of octanol (1 mM) beginning 2 h before and up to 3 days after irradiation. Octanol reduced the yield of induced $CD59^{-}$ mutants from 92 ± 35 to 16 ± 3 per 10^5 survivors [15]. Treatment of octanol alone resulted in a low but detectable mutant fraction of $\sim 10 \pm 4$. Although this result indicates a role of gap junctions in the bystander mutagenic response, octanol is a non-specific inhibitor of gap junctions, and can have wide ranging effects on other cellular structures and functions including membrane fluidity [25]. Therefore, to investigate more specifically the role of gap junction mediated cell-to-cell communication with α-particle-induced bystander mutagenicity, it is necessary to use cells in which gap junctional activity was suppressed by a dominant negative connexin construct.

A_L cells genetically deficient in connexin 43 shows no gap junctional communication and no bystander genotoxic responses.

Connexin 43 is the principal protein component of gap junctions [26] and there is good evidence that connexin itself (assembled in a lipid bilayer) is sufficient and necessary for the generation of gap junction channels [27,28]. Using the standard scrape-loading test as a measure of gap junctional activity [26], it was found that the migration of Lucifer yellow was completely blocked in A_L cells carrying the dominant negative connexin 43 vector [15].

In contrast, the dye was found to migrate many cell layers in distance among wild type A_L cells as well as cells carrying a connexin 43 overexpressing construct. Significantly, A_L cells showed a higher bystander mutagenic yield than that of vector control [15]. In contrast, there was little, if any, bystander effect among cells carrying the dominant negative vector. These data clearly show that the connexin 43 vector is working well in the transfected cells and that gap junction intercellular communication is critical in mediating the bystander mutagenic process.

4.4. *Medium effect in bystander genotoxicity*

Previous studies have shown that medium from irradiated culture, upon transferal to non-irradiated cells, can induce increased biological effects in the latter. Mothersill and Seymour first demonstrated a highly significant reduction in cloning efficiency in both non-irradiated normal as well as malignant epithelial cell lines that had received medium from ^{60}Co-gamma-ray irradiated cultures [29]. These results suggested that irradiated cells secreted a cytotoxic factor into the culture medium, which was capable of killing non-irradiated cells. Furthermore, transferring medium from low LET-irradiated cultures to un-irradiated cells lead to increased levels of various bystander effects, such as cell killing [30,31], neoplastic transformation [32] and genomic instability [33].

To ascertain whether irradiated medium, with or without accompanied cell cultures, can induce bystander genotoxic endpoints in the A_L cells, custom-designed double mylar dishes were used. One side (with or without cells) was irradiated with α-particles using a broad beam from the track segment mode of a 4 MeV van de Graaff accelerator [34]. Since α-particles can only traverse a very limited distance, cells plated on the other side of a medium-filled mylar dish would not be irradiated by the α-particles. There is evidence that non-irradiated target cells attached to the top mylar layer had a much higher number of chromatid-type aberrations when there was a bottom layer of cells in the medium filled chambers than just medium alone [35]. In fact, very few chromatin fragments were induced in the non-hit bystander cells in the top layer when only medium were irradiated. This increase in

Table 1
Characteristics of the bystander effect

Multiple pathways likely
P53 gene function is NOT essential for the process
In confluent cultures, gap junction mediated cell-to-cell communication is essential
In sparse cultures, secreted signaling molecules, particularly, reactive oxygen and reactive nitrogen species are involved
It is not clear if ROS are only the initiating signaling event which triggers other down stream, more stable secondary signaling pathways

the bystander chromatid breaks showed a time dependent factor since the incidence increased with increasing incubation period [35]. Furthermore, when transferring the medium from these cell-irradiated dishes to fresh A_L cultures, chromatid-type aberrations were produced in the never irradiated cells. Using the same experimental set up, Zhou et al. found no induction of *CD59*$^-$ mutations but an increased in cytotoxicity conducted under similar experimental conditions [34]. These results suggest that certain, yet to be identified, modulating factors, secreted from the irradiated cells on the bottom mylar layer into the medium, induce some non-repairable chromosomal changes, resulting in an increased incidence of chromatid breaks but no mutagenesis due to increased in cell lethality in the non-irradiated, bystander cells.

The mechanism of radiation induced genotoxicity is not clear and is likely to be complex and involves multiple pathways. Table 1 lists the possible processes involved. It is likely that a multiple signaling cascade involving both an initiating event and downstream signaling steps are necessary to mediate the bystander process. Since gap junctional communication plays an important role in mediating the bystander signaling, Table 2 lists the many possible signaling molecules involved in the process. Studies are currently underway in many laboratories in identifying the true nature of the signaling molecules.

Table 2
Possible nature of the bystander signaling molecules

Size <1000 Da that can pass through gap junctions
Reactive oxygen species
Reactive nitrogen species
Long lived organic radicals
Protein hydroperoxides
The cytokine TGF β

5. Interaction between bystander mutagenesis and adaptive response

5.1. Adaptive response

Adaptive response is the phenomenon whereby exposure to a low priming dose of a DNA-damaging agent exerts a protective effect such that subsequent high dose exposures are much less damaging. A variety of DNA-damaging insults can elicit adaptive responses in organisms as diverse as bacteria, yeast, animals, and plants, making them more resistant to killing and mutation. Radio-adaptive response is the phenomenon by which cells, pre-exposed to a low dose of ionizing radiation, acquire a resistance to a subsequent higher dose of radiation. An X-ray priming dose as low as 0.01 Gy is sufficient for the adaptive response to occur [36]. The high LET radiations at low doses are very inefficient at inducing adaptation [37], most likely because a single traversal of a particle already does too much damage to activate the adaptive cascade.

The first of the regularly reproducible experiments to show an adaptive response to low doses of radiation were reported as the reduction of induced chromosome aberrations in human lymphocytes [38]. Subsequent adaptive response studies showed the reduction of micronuclei and sister chromatid exchanges in Chinese hamster V79 cells [39], the reduction of mutation frequency in human lymphocytes [40], the reduction and altered spectrum of mutants in human-hamster hybrid A_L cells [41], and the reduction in micronuclei formation in human lymphocytes [42]. Moreover, mutagenic adaptation shares common features with clastogenic adaptation, such as priming dose level, time between priming and challenging treatments, degree of induced protective effect (40–75%), transitory response, and inhibition by 3-aminobenzamide. De novo synthesis of transcripts and proteins is required for the expression of the adaptive response, since it is abolished by the presence of actinomycin D or cycloheximide during the period between the priming and challenging doses [37,43].

5.2. Effect of low LET priming dose on bystander mutagenesis

To define the interaction between adaptive response and the bystander effect, experiments were conducted

to determine whether low dose, low LET radiation can suppress bystander mutagenesis induced by high LET radiation. A_L cells irradiated with either a 0.02 or a 0.1 Gy dose of X-rays resulted in a low but significant induction of mutations at the CD59 locus [8]. Consistent with previously published data, irradiation of 10% of a confluent cell population with a single α-particle each through the nuclei resulted in a bystander mutant yield that was roughly four times higher than expected assuming that there was no interaction between hit and non-hit cells [4]. Pretreatment of cells with a low (0.02 or 0.1 Gy) dose of X-rays significantly reduced this bystander mutagenesis by 62 and 58%, respectively [8]. An increase in the priming dose decreased the inhibitory effect such that pretreatment with 0.5 Gy of X-rays reduced the bystander mutant yield by only 12%, and the difference was no longer statistically significant. A similar mutagenic response was found if 10% of the cells were given a near lethal dose of 20 α-particles each delivered directly to the nuclei. These results imply that in the presence of low-dose radiation stress, bystander mutagenesis is suppressed by the adaptive response, though the mechanism(s) is unclear.

6. Summary

Accurate risk assessment of human exposure to ionizing radiations traditionally has been compromised, in that reliable data are available only for relatively high doses. Cancer risk estimates over the doses ranging from 0.05 to 2.5 Sv are available from the epidemiological study of the A-bomb survivors [44]. Risks at lower doses can only be inferred by an extrapolation from the high dose risks. The question is, what is the risk of radiation exposures above the level of natural background but below the lowest dose for which risks are known from the A-bomb survivors? Both ICRP and NCRP recommend a linear no-threshold extrapolation, but this has generated a great deal of controversy and is a much debated issue, since it involves issues of major societal and economic concern. As a consequence, a considerable amount of research effort has been directed at the mechanisms of mutagenesis/carcinogenesis in the hope that this would shed some light on the shape of the dose-response relationship, and therefore on the validity of the linear extrapolation.

The bystander effect contributes to this debate by implying that the biological effects of low doses, where not all cells are traversed by a charged particle, are amplified by the transfer of factors to unirradiated neighbors. If phenomena demonstrated in vitro are applicable in vivo then the bystander effect implies that a linear extrapolation of risks from high to low doses may underestimate rather than overestimate low dose risks.

The adaptive response, on the other hand, implies that prior exposure to a low dose of a DNA damaging agent renders cells resistant to a subsequent exposure. These two phenomena have opposite effects for cellular radiobiological endpoints: deleterious in the case of the bystander effect as damage is communicated from hit cells to their neighbors, and beneficial in the case of adaptive response as a small priming dose confers resistance to subsequent dose. The radiobiological responses at low doses are likely to be a complex interplay among many factors including direct damage, the bystander effect, the adaptive response and the genetic background of the individuals involved. The identification of causally linked genes involved in these processes should provide important clues to the radiobiological consequences of their interactions.

Acknowledgements

The authors would like to thank Ms. Sarah Baker for critical reading of the manuscript. Work supported by funding from the National Institute of Health Grants CA 49062, ES 12888, NIH Resource Center Grant RR 11623, Environmental Center Grant ES 10349 and from the US Department of Energy DEFG-ER63441.

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