

2-15-1991

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### Recommended Citation

Seed, T. M. and Niiro, G. K. (1991) "Alpha-Particle Irradiation-Induced Change in Bronchopulmonary Macrophage Morphology, In Vitro," *Scanning Microscopy*. Vol. 5 : No. 2 , Article 25.

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## ALPHA-PARTICLE IRRADIATION-INDUCED CHANGE IN BRONCHOPULMONARY MACROPHAGE MORPHOLOGY, *IN VITRO*

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(Received for publication May 16, 1990, and in revised form February 15, 1991)

### Abstract

Bronchopulmonary macrophages, isolated from canine lungs by saline lavage and grown in tissue culture for short periods, were acutely irradiated with a range of doses of either Americium-241 alpha particles (0.03-48 Gy) or 250 keV x-rays (0.5-24 Gy). Following a 24-hour reincubation and "expression" period, cells were examined for radiation-induced changes in overall viability, as well as in cell morphology and ultrastructure. Results indicated that neither quality of radiation had much effect on cell viability over dose ranges examined, but substantial changes in cell volume, surface topography, and cytoplasmic features were noted, especially in the alpha-particle-irradiated specimens. Results support the concept that the limiting plasma membrane of the targeted macrophage is a sensitive subcellular target for ionizing radiation, especially high-linear-energy-transfer heavy particles.

**Key words:** Alpha radiation, heavy particles, high-LET, macrophages, bronchopulmonary macrophages, ultrastructure, electron microscopy.

### Introduction

The nucleus of the eucaryotic cell, with its vital genomic elements, is the principal subcellular organelle responsible for reproductive cell death following ionizing radiation exposure. With other modes of cell death, e.g., interphase cell death or apoptotic cell death (programmed cell death), other subcellular organelles seem to be selectively targeted and damaged and, at least in part, responsible for cell death. One of the more sensitive of these subcellular targets is the plasma membrane. Various investigators have demonstrated apparent causal relationships between plasma membrane damage following irradiation and resulting cell death [1, 4, 5, 7, 13-15, 17-19]. The more convincing of these studies have made use of non-dividing, terminally differentiated but functionally competent cells. Use of the latter cell types allows for a more effective separation between radiation-mediated nuclear processes which elicits reproductive failure and, in turn, cell death from radiation-induced plasma membrane alterations that cascade into the loss of selected but critical cell functions and, finally, into loss of cell viability [1, 2, 4, 5, 7, 17-19]. By eliminating the confounding variables associated with cell replication and related repair events in the study of radiation-induced cell lethality, critical non-nuclear processes (e.g., plasma membrane-associated ion transport imbalances) are causally highlighted. In terms of the latter, we asked the question whether or not selected types of subcellular damage such as induced plasmalemmal changes might be augmented and more tightly correlated to other cellular impairments following exposure to densely ionizing heavy particles rather than to sparsely ionizing electron or photon-type radiations.

We, therefore, examined the differential effects of high-linear-energy-transfer (high-LET) alpha particles versus low-LET x-rays on the viability and surface morphology of cultured bronchopulmonary macrophages (i.e., vital, functionally mature, and non-dividing lung cells).

This study was part of our ongoing effort to characterize and model radiation dose-dependent pathologic responses of selected pulmonary cell types following exposure to radon gas (i.e., a naturally occurring, gaseous,

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alpha-emitted radionuclide of significant environmental and toxicological concern).

## Materials and Methods

### Collection of Bronchopulmonary Macrophages

Bronchopulmonary macrophages were collected from normal adult beagles under sterile conditions by the saline lung lavage technique originally described by Muggenburg and Mauderly [8]. In brief, endotracheal tubes were inserted into the trachea of dogs under anesthesia. Both left and right principal bronchi and lungs were flooded, rinsed with 1 L of physiological saline, and subsequently drained. The saline wash was collected and centrifuged (1000 rpm for 10 minutes at 4°C). The pelleted cells were resuspended in saline and recentrifuged. The latter process was repeated two additional times. The final washed cell pellet was maintained on ice until the time of cell culturing.

### Cell Culturing of Bronchopulmonary Macrophages

Cells from the lung lavages were resuspended at  $10^6$  cells/ml in a modified McCoy's 5A based nutrient media, originally described by Greenberg et al. [3], but modified by excluding the  $10^{-6}$  M hydrocortisone additive [12]. Four milliliter aliquots of the cell suspension were plated out in specially fabricated 54-mm glass-ringed Petri dishes with ultrathin ( $1.5 \mu\text{m}$ ) mylar plastic growth support surfaces. The latter provided an alpha particle-transparent window for the subsequent irradiation of the adherent macrophage population. The culture plates were incubated in 37°C in 5%  $\text{CO}_2$ , >95% humidity, for 72 hours, after which the culture plates were washed 1x in ice-cold Hanks balanced salts solution containing 10% horse serum (HBSS+HS) in order to remove the non-adherent cells. More than 95% of the mylar-adherent cells were macrophages, as defined by morphologic and cytochemical (non-specific esterase) criteria. Following washing, 5 ml of fresh, ice-cold HBSS+HS was added to the plates. The plates were then transported to the radiation facilities and irradiated according to methods described below.

### Macrophage Cell Irradiation

Cell cultures were irradiated at room temperature ( $\sim 20^\circ\text{C}$ ) with either 4.2 MeV  $^{241}\text{Am}$  alpha particles, to total cumulative doses ranging from 3 to 4800 cGy or with 250 keV x-rays to total doses ranging from 50 to 2400 cGy. Non-irradiated cultures served as zero dose controls. For both types of radiations, cultures (cells) were irradiated perpendicular to the collimated sources. Dose rates of 50 cGy/min and 84 cGy/min were used for the x-ray and  $^{241}\text{Am}$  particle irradiations, respectively. The alpha-particle fluence for the  $^{241}\text{Am}$  irradiation was 613 alpha particles per  $\text{mm}^2$  per cGy. Design, operation, and dosimetry of the alpha source has been described in detail elsewhere [10].

Immediately following irradiation, the cultures were drained of the HBSS+HS bathing solution and re-fed with complete tissue culture media. The cultures

were returned to the  $\text{CO}_2$  incubator and reincubated for an additional 24 hours to allow for the expression of morphological damage.

### Specimen Preparation

Immediately following the expression period, the cultures were processed for both cell viability testing and a combination of microscopic examinations. For cell viability testing, adherent cells were removed from the mylar growth surface with a rubber policeman, collected, and washed once in ice-cold HBSS+HS, and resuspended to  $10^6$  cells per ml. One-tenth milliliter aliquots of each cell suspension were mixed directly on a microscope slide with 0.1 ml volumes of 0.4% trypan blue-saline and cover-slipped. Following a 15 minute incubation at room temperature, the slides were examined at 40x by light microscopy. The number of cells excluding the dye per 100 cells counted was used as the numerical estimate for cell viability.

For microscopic analyses, we used light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For LM, the cultures were quickly rinsed in saline, fixed for 5 minutes with methanol, and stained with Giemsa reagent (1 ml stain : 50 ml  $\text{H}_2\text{O}$ , for 45 minutes). The stained specimens were mounted either directly or indirectly (first on small plastic support disks) on microscope slides.

For both types of electron microscopy, duplicate cultures were partially drained of culture media and fixed with 5 ml of freshly prepared 1.25% glutaraldehyde in Sorenson's phosphate buffer (pH 7.1). After 5-10 minutes, the fixative was replaced with fresh fixative, and the cultures were allowed to sit overnight at 5-8°C. The fixed cultures were then rinsed thoroughly in Sorenson's buffer. The fixed cell specimens on the thin mylar growth support were mounted with Super Glue<sup>®</sup> on 13-mm circular plastic culture disks specially designed for EM processing (Thermanox slips; Miles Scientific).

For TEM, the specimens were processed by conventional methods, previously described [11, 12]. In brief, the specimens were postfixed for 45 minutes in 1% osmium tetroxide and dehydrated in ascending concentrations of ethanol (i.e., 5-minute baths in 30, 50, 70 and 95% ethanol, and 1 hour in 100% ethanol), followed by a final dehydrating step for 1 hour in 100% propylene oxide. Specimens were infiltrated overnight with a 50:50 mix of propylene oxide and Epon 812, and finally placed in pure Epon for 8-12 hours. Infiltrated specimens were placed face-down on Epon-filled embedding capsules. Following curing (60°C for 48 hours under vacuum), the supporting plastics were snapped off the surface of the specimen. Specimen blocks were trimmed and ultrathin sections were cut, mounted, stained by standard EM procedures [11]. Prepared thin sections were examined with a Siemens 101 electron microscope operating at 80 keV.

For SEM analysis, specimens were post-fixed in osmium and dehydrated in ascending concentrations of acetone following the process described above for the ethanol dehydration used for TEM specimens. Follow-

ing dehydration in 100% acetone, the specimens were transferred to a critical point drying bomb and dried with liquid CO<sub>2</sub> used as the intermediate fluid. Once dried, the specimens were mounted on aluminum studs and sputter-coated with a light coating of gold (7-14 nm) in order to prevent charging. The specimens were examined with either a Cambridge Mark IV or a JEOL 840a scanning electron microscope.

#### Analysis

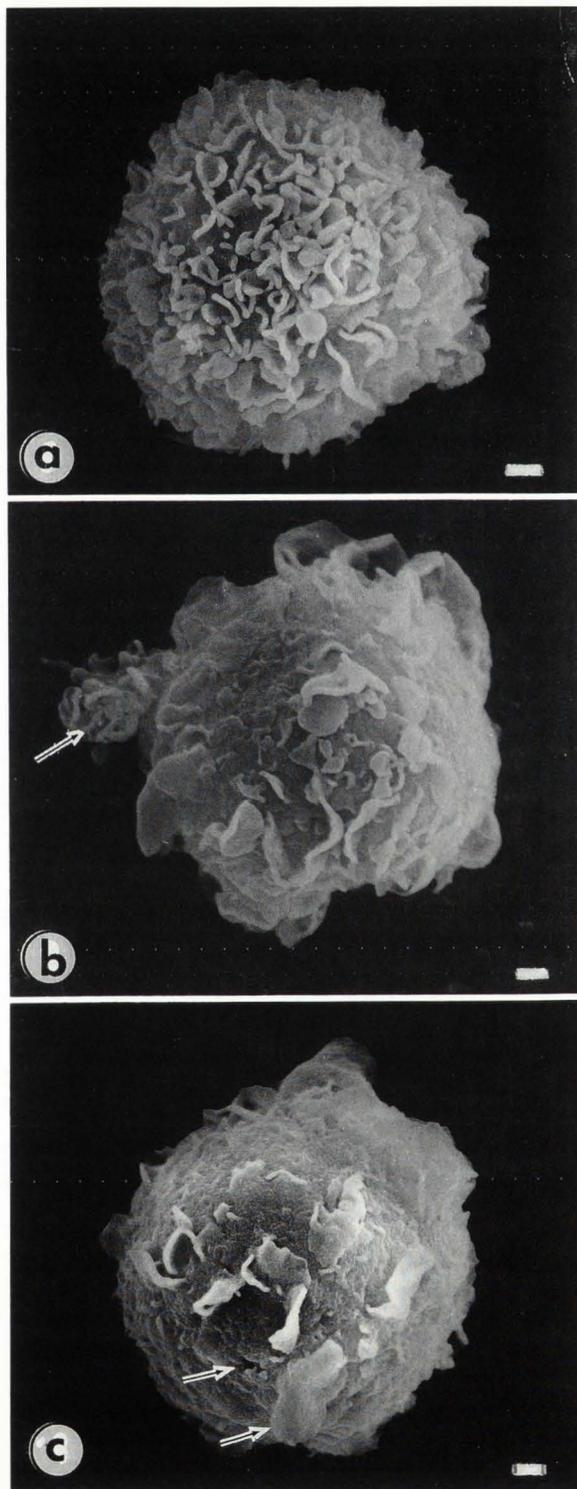
For topography analysis by SEM, both control and irradiated specimens were initially surveyed and the major morphologic variants characterized from four experiments in which the full range of  $\alpha$ -particle and x-ray doses were applied. The total number of specific morphologic variants within the total number of microscopic fields (100-225 fields;  $\sim 5000 \mu\text{m}^2$  per field) were counted at each radiation dose level and equated as a percentage of the total cell population per field assessed. From the latter estimates, average values (i.e., percentage of morphologic subtype) were calculated for each cell type at each radiation dose level (see Fig. 1 legend for description of morphologic subtypes). For analysis, the data were proportionally weighted according to the number of microscopic fields examined for each specimen. Statistical significance of the various changes were determined by using the Student's T test.

#### Results

##### Radiation-Induced Modification of Cell Surface Topography

Cultured bronchopulmonary macrophages exposed to increasing doses of 4.2 MeV alpha-particle radiation (3-4800 cGy) exhibited a marked series of topographic changes, as illustrated in Fig. 1 (a-c). The topographically dense, full-volumed cells with abundant surface ridges, ruffles, and microvilli were the dominant cell type in non-irradiated specimens, as well as in specimens irradiated at the lowest dose tested, i.e., 3 cGy (Fig. 2a). With moderate increments in the alpha dose, the percentage of these topographically dense cells

dropped; i.e., from  $\sim 77\%$  ( $\pm 6.7$ ,  $\pm 3.8$  S.E.) in both the non-irradiated and lightly irradiated (3 cGy) populations; to  $\sim 49\%$  ( $\pm 7.3$ ,  $\pm 7.5$  S.E.) at the moderately low doses of 25 and 50 cGy; to  $\sim 23\%$  ( $\pm 4.3$  S.E.) at 4800 cGy, the highest dose tested (Fig. 2). The latter



**Figure 1 (a-c).** Representative SEM micrographs showing the dominant macrophage types, relative to cell surface topography, within either control or irradiated populations. **Fig. 1a** shows the dominant, topographically dense macrophage-type within non-irradiated, control preparations, as well as within low-dose samples. **Fig. 1b** shows the surface features of dominant, topographically sparse cells with reduced surface density of microvilli, ridges and ruffles, characteristically found in increasing numbers at intermediate to high radiation doses. **Fig. 1c** illustrates a prominent subtype of the topographically sparse cell population that is found in increasing numbers at intermediate to high radiation doses, namely relatively smooth-surfaced, small-volumed cells with markedly reduced surface densities of microprojections. Line markers =  $1 \mu\text{m}$ .

changes were significant, with estimated P values  $\leq 0.038$ . In a reciprocal fashion, the percentage of topographically sparse cells within the population increased as the radiation dose increased, i.e., from  $\sim 18\%$  ( $\pm 7.7$  S.E.) in the controls; to  $\sim 33-46\%$  ( $\pm 7.5, \pm 7.9$  S.E.) at 50 and 150 cGy; to  $\sim 59\%$  ( $\pm 3.3$  S.E.) at 4800 cGy (Fig. 2a). At radiation doses  $\geq 25$  cGy, sizeable numbers ( $\sim 12-26\%$ ) of small-volumed cells, denuded of their normal complement of surface projections, were consistently seen (Figs. 1c and 2a). The latter increase with radiation dose in the number of small-volumed, topographically sparse cells proved to be highly significant ( $P \leq 0.036$ ), except for the 600 cGy dose level ( $P = 0.149$ ).

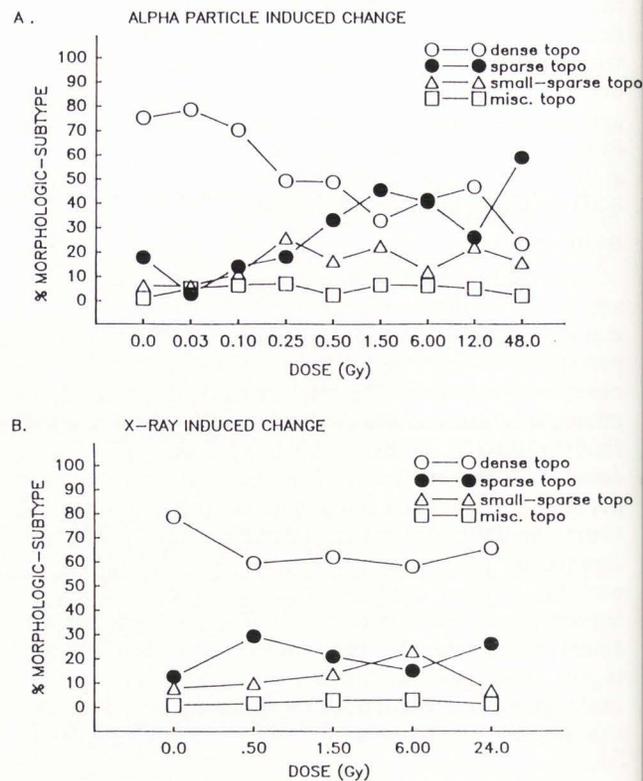
Knowing the alpha particle flux rate of 613 per  $\text{mm}^2$  per cGy and the average cross-sectional area of  $107 \mu\text{m}^2$  for the mylar-adherent macrophages, we estimated the number of alpha-particle hits per macrophage irradiated with the relatively low doses of 25 and 50 cGy to be 1.6 and 3.3, respectively. At the high doses of 2400 and 4800 cGy, the number of hits was 157 and 315, respectively.

In contrast, macrophage cultures irradiated with equivalent doses of x-rays showed less pronounced changes in the numbers of these topographic variants, especially at the higher doses (Fig 2b). Moderately low doses of x-rays (50 cGy) reduced the percentage of topographically dense cells from  $\sim 79\%$  ( $\pm 6.6$  S.E.) to  $\sim 59\%$  ( $\pm 4.9$  S.E.), while increasing the percentage of topographically sparse cells, i.e., from  $\sim 13\%$  ( $\pm 1.6$  S.E.) to  $\sim 29\%$  ( $\pm 6.0$  S.E.) (Fig. 2b). The latter decline in topographically dense cells approached statistical significance with an estimated P value of 0.053, while the increase in sparse cells was significant with an estimated P value of 0.042. However, the magnitude of these changes remained relatively constant despite further increases in radiation dose (Fig 2b).

#### Ultrastructure of Induced Cytoplasmic and Plasmalemmal Changes

As shown by TEM, in cross-section, slender surface ruffles and microvilli that normally extended outward from the surface of the macrophage, tended to be folded over, often forming circular figures, and were often either partially or totally enveloped in near-surface cytoplasm (Fig. 3a). Residual bodies were commonly seen in associated with these enveloped surface microprojections. Such images suggested a process by which damaged and collapsed microprojections were removed by their inward movement, cytoplasmic envelopment, and subsequent degradation within lysosomal vacuoles (residual bodies).

In contrast, large surface blebs were seen with increased frequency at the higher radiation doses. The thin limiting plasma membranes in blebbed regions were often whorled, fragmented, and commonly found exterior to the cell proper (Fig. 3b). Opposite to the apparent inward movement (interiorization) of damaged plasma membrane, this blebbing process suggested that a complementary, but opposite and outward process



**Figure 2 (a & b).** Change in proportion of the morphologic variants within the irradiated macrophage population with increasing doses. Fig. 2a illustrates the alpha particle-induced change. Fig. 2b shows the x-ray-induced change. Abbreviations: dense topo, topographically dense cells; sparse topo, topographically sparse cells; small sparse, small topographically sparse cells; misc, cells with other topographical features.

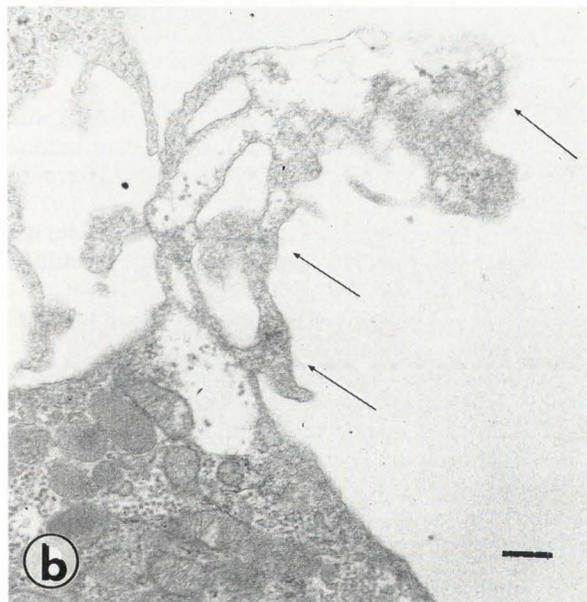
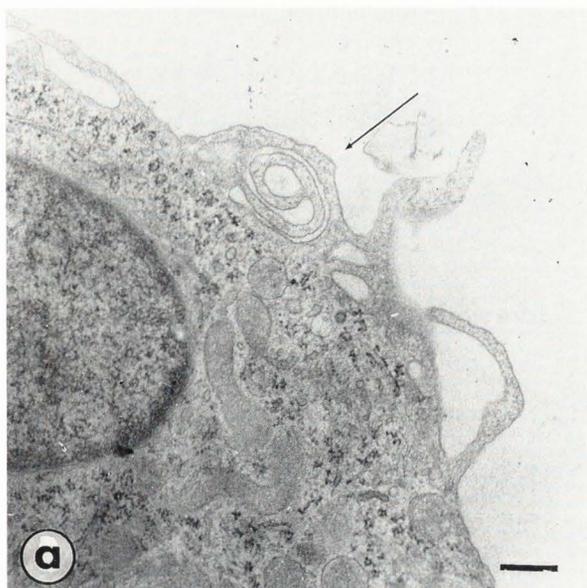
(exteriorization), exists for the cell to remove damaged membrane. These two complementary processes are shown schematically in Fig. 4.

#### Effect of Radiation Exposure of Cell Viability

Macrophage viability, as assessed by trypan blue dye exclusion, was maintained at a 90% or better level despite the wide range doses and the two qualities of radiations applied (Fig. 5).

#### Discussion

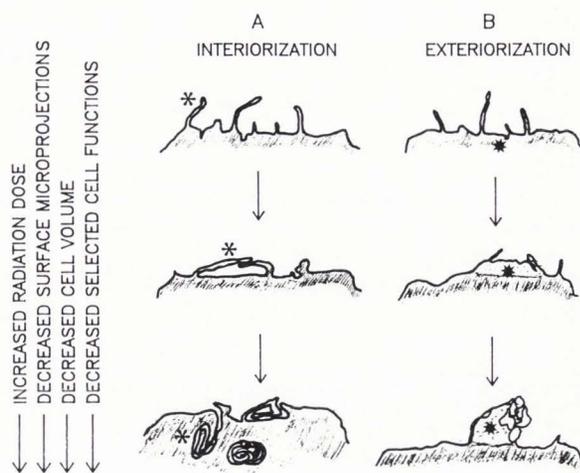
Mature terminally differentiated macrophages, regardless of origin, are generally considered highly radioresistant. However, one must define the term radioresistant in terms of the nature of the biological end point being assessed. Clearly, in terms of overall cell viability as measured by trypan dye exclusion, bronchopulmonary macrophages are indeed highly radioresistant, as shown here in this study by more than 90% cell viability, even following irradiation with high-LET alpha particles to doses as high as 4800 cGy. Only at higher doses is



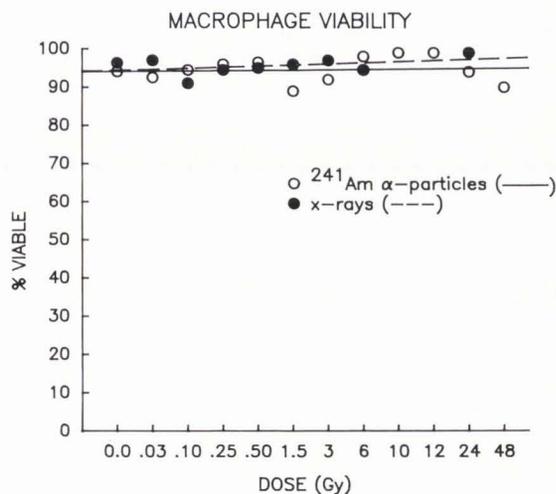
**Figure 3 (a & b).** Cross-sectional features of representative macrophages exposed to high doses of alpha particles showing prominent plasmalemmal changes. **Fig. 3a** illustrates the induced infolding of cell surface microprojections following irradiation. **Fig. 3b** shows the induced surface blebbing and associated loss of surface microprojections following irradiation. Line markers = 0.3  $\mu\text{m}$ .

there substantial reduction in viability, e.g., 50% reduction in viability of rodent alveolar macrophages irradiated *in vitro* with 11,500 cGy of x-rays as observed by McLennan [7]. However, at lower viability-sparing radiation doses delivered *in vitro*, a number of principal

MECHANISMS OF RADIATION-ELICITED LOSS OF CELL SURFACE MICROPROJECTIONS



**Figure 4.** A schematic illustrating the hypothesized two complementary but distinct processes involved in the loss of radiation-damaged surface microprojections.



**Figure 5.** Effect of alpha and x-ray radiation exposures on overall macrophage cell viability.

cellular functions of macrophages, including phagocytic capacity and related oxidative and lysosomal enzyme processes clearly are modified, not only quantitatively but qualitatively as well [2, 7]. Radiation doses in the range of 28 Gy have been reported to significantly suppress phagocytic capacity of primary alveolar macrophages shortly following irradiation [7], whereas at lower dose levels (8-20 Gy) and extended postexposure sampling times, both primary macrophages as well as

established macrophage cell lines (e.g., J774.1 cells) respond with modestly increased phagocytic capacity, along with related increased cell "activation" processes (e.g., increased lysosomal enzyme and ectoenzyme content, hydrogen peroxide production, oxidative metabolism, increased cell spreading, substrate adherence, and cell volume, etc.) [2,9,16]. At still lower exposure levels (e.g., <6 Gy), irradiated macrophages and principal cellular functions seemingly remain quiescent [9].

The above observations led us to assume that the relatively low-to-moderate irradiation doses that so effectively elicit topographic alterations of the surface of the macrophage have little influence on overall viability or on suppression of a primary cell function, namely, phagocytosis. Subsequent studies indeed support the latter assumption (as reported here, as well as unpublished observations). Nevertheless, other vital cellular functions are compromised by relatively low-dose, alpha-particle irradiation. One such function involves the production by the mature macrophage of a hormone (macrophage colony stimulating factor, M-CSF), whose dual function is to both maintain viability and stimulate clonal proliferation of small progenitorial macrophage (monocyte) subpopulations within tissues [6]. Recently, we have shown that low doses (3-50 cGy) of  $^{241}\text{Am}$  alpha particle radiation elicit a substantial reduction in the capacity of the macrophage to elaborate M-CSF (Seed et al. unpublished observations). The estimated  $D_{25}$  value (dose required to elicit a 25% level of suppression) is ~32 cGy, which equates to an alpha particle flux density in the cell growth volume of  $2.0 \times 10^4 \text{ mm}^{-2}$ . The estimated microflux at this  $D_{25}$  level of suppression is two alpha particle "hits" per cell, with each particle depositing ~16 cGy of absorbed dose. Similar microflux estimates for the number of alpha particle "hits" to the nucleus of macrophages falls below 1 (0.8) at the  $D_{25}$  dose level. The latter tends to indicate that the critical cellular target being affected by alpha particle irradiation is something other than the nucleus.

Although the noted radiation-induced changes in macrophage cell surface topography might not directly affect phagocytic function or overall viability, they may indeed perturb hematopoietin production--a key cellular function that ultimately serves to regulate the size and viability of the macrophage population at large. One can envision how substantial loss of cell surface area following irradiation might well affect both the pattern and overall efficiency of cytoplasmic and plasma membrane transport of newly synthesized hormone (e.g., via reduction in plasmalemmal translocation sites).

Our observations and microdose estimates are consistent with the concept that the plasma membrane is a sensitive subcellular target for relatively low doses of high-LET alpha particles. The latter concept is certainly not novel but is broadly supported by the work of other investigators using a battery of plasma membrane end points and a variety of both reproductively active and inactive cell types [1, 4, 5, 7, 13-15, 17-19].

## Acknowledgments

We wish to thank Dr. Robert Schlenker for kindly allowing us to use the  $^{241}\text{Am}$  irradiator and Mr. Billie Oltman for assisting in these irradiations. Further, we thank Mrs. Fran Coose and Mrs. June Lear for their secretarial help, and Mrs. Susan Barr for her editorial assistance. This work was supported by the U.S. Department of Energy, Office of Health and Environmental Research, under Contract No. W-31-109-ENG-38.

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#### Discussion with Reviewers

**C. Ts'ao:** The changes (i.e., surface topographical changes of irradiated cells) are presented in precise quantitative terms, can you clarify the way they are quantified? If Fig. 1a is rated "dense-topo", 1b "sparse-topo", and 1c "small sparse-topo", what about cells with surface changes between a, b, and c? Where was the line drawn?

**Authors:** The identification of specific morphologic variants was based on the percentage of cell surface covered with microprojections: e.g., the dense-topo cells had a minimum surface cover of >50%, with an average cover of >75%; sparse-topo cells had a maximum cover of 50%, with an average cover of about 25%. For the most part, separating and counting the various variants did not present a problem; however, in those cases where cells presented with topographic relief close to the cut off cover value of 50%, the counter decided (in a relatively subjective fashion) to which class the cell belonged.

**C. Ts'ao:** Since this is largely a morphologic study, the investigators should concentrate their discussion on morphologic changes and compare their (results) with those reported by others.

**Authors:** We accept this comment and provide the read-

er with a short discussion (below) of related observations made by other investigators, relative to the observations made here. However, the reader must understand that such comparisons might well be superficial due to major differences in experimental designs and protocols used in these various experiments. Further, we know of no strictly comparable high-LET radiation-morphologic response studies having been assessed and reported. In the study by McLennan et al. [8], very high doses (115 Gy) low-LET x-rays were shown to elicit marked cell surface alterations of primary alveolar macrophages within a 24 hour post-exposure period. The dominant change noted was the shift from the normal topographic pattern of high surface density of short ridged and ruffled micro-projections to an abnormal pattern of surface blebs of varying sizes, clear zones (microprojection-free cell surface areas), surface pits, and invaginations. This radiation-induced shift in surface topography was similar to what we observed here with primary canine macrophages exposed to relatively low doses of high-LET alpha particles. Other investigators have observed similar effects (e.g., surface blebbing and pit formation) using a broad dose range of low-LET photon irradiation, with a variety of cell types having varying mitotic and differentiative potentials [1, 17-19]. The magnitude of these induced cell surface responses are constrained radiologically (radiation dose, dose-rate, quality of radiation), temporally, and by the irradiated cell's inherent capacity to process and repair damaged plasma membrane. The more common of the radiation-induced surface responses (i.e., blebbing and pitting) might well represent manifestations of the cell's damage processing and repair capacities. Our observations here, as well as those of others [4, 5], are consistent with this concept; i.e., that radiation-damaged plasma membrane is corrected by two opposing processes, namely exteriorizing and jettisoning damaged membrane, versus interiorizing and degrading damaged membrane.

**J.R. Maisin:** How do you explain that the decrease of percentage of topographically dense cells and the increase in sparse cells following a dose of 50 cGy of alpha particles, or of x-rays, remains more or less constant despite further increase in radiation dose?

**Authors:** The major component of the "low dose" (<50 cGy) effect is the loss of high surface density of microvilli and small ruffles and ridges. The large surface projections, e.g., large ruffles, ridges, and surface blebs, appear to be quite resistant to relatively high radiation doses, especially to low-LET x-rays and, to a lesser extent, alpha particles. The simplest explanation is that cytoskeleton network supporting the various species of expressed surface topographies are different and, in turn, have different thresholds for radiation damage.

**J.R. Maisin:** Is it possible to correlate the ultrastructure of induced cytoplasmic and plasmalemmal changes as shown by TEM with the cell surface topography as shown by SEM?

**Authors:** Yes, but only indirectly through evaluating static SEM and TEM images of the same structures. This was not done, however, in this study.

**J.R. Maisin:** It would be of interest to know if the substantial reduction of the capacity of the macrophage to elaborate M-CSF after low doses of Americium-241 is increased with increasing doses of radiation?

**Authors:** The magnitude of suppression continues to increase only up to moderate levels of alpha radiation (about 100 cGy) doses, after which the response wanes.

**Z. Somosy:** What is the possible molecular mechanism(s) of micro-morphological changes (with) alpha irradiation?

**Authors:** One possible explanation is that the densely ionizing alpha particles disrupts the subplasmalemmal cytoskeleton network and, in turn, basic movement and expression of the various species of cell surface micro-projections.

**D.B. Warheit:** Are *in vitro* radiation exposures relevant for assessing radiation effects in exposed animals or humans?

**Authors:** The answer to this is an "unqualified" yes -- and, unfortunately, a "no" as well. It all depends on the end point in question. In terms of the macrophage population studied here, there is no reason to doubt that similar effects noted *in vitro* wouldn't be noted *in vivo*, although this needs to be conclusively demonstrated.

**D.B. Warheit:** Why didn't the authors assess macrophage functional parameters such as phagocytosis and chemotaxis?

**Authors:** Although not reported here, phagocytic capacity was assessed and found not to be significantly altered by relatively low to moderate doses of irradiation (low-LET photon irradiation). Chemotaxis was not studied, although we agree wholeheartedly that this end point probably should be examined in future studies.

**D.B. Warheit:** Why did the authors use horse serum in their HBSS?

**Authors:** We use horse serum as a basic serum supplement in all of our canine cell cultures. Canine tissue cells seem to grow better when horse serum is included in the basal media.

**D.B. Warheit:** Why didn't the authors use trypan blue solutions on the adherent cells to assess viability instead of using a rubber policeman to remove the cells from the mylar surface?

**Authors:** The dye-exclusion assays (as well as the phagocytosis assays) could have been performed equally as well *in situ* with the same results obtained. However, because the latter assay method would have required separate cultures to be set up exclusively for this assay (with restrictive culture and radiation conditions), we chose the more convenient route of assessing viability following cell dislodgement from the mylar surface.

**D.B. Warheit:** Why were the samples processed for SEM dehydrated in acetone instead of ethanol?

**Authors:** Acetone was used for the simple reason that it saved steps (amyl acetate) in the preparation of the samples for critical point drying and without compromising the quality of final SEM preparations.