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## Effect of $\gamma$ Radiation on Membrane Fluidity of MOLT-4 Nuclei

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MCCLAIN, D. E., TRYPUS, C. A., AND MAY, L., Effect of  $\gamma$  Radiation on Membrane Fluidity of MOLT-4 Nuclei. *Radiat. Res.* 123, 263-267 (1990).

These experiments measured the effect of  $\gamma$  radiation on the nuclear envelope using doxyl-fatty acid spin-label probes. Nuclei were isolated from cultured MOLT-4 cells, a radiation-sensitive human T-cell lymphocyte. Membrane fluidity was measured from the electron paramagnetic resonance spectra of the probes. MOLT-4 cells were grown under standard conditions, and suspensions were exposed to <sup>60</sup>Co  $\gamma$  radiation at room temperature. The spectra of 5-doxylstearic acid in the nuclei were those of a strongly immobilized label. A difference in the membrane fluidity was detected in a series of experiments comparing labeled irradiated and nonirradiated nuclei. The change in fluidity was measured by comparing the changes in the order parameter, *S*, of the spin label in irradiated nuclei with those in control nuclei. The change in the *S* ratio is dependent on radiation dose, increasing with doses up to 15 Gy. The maximum change of the order parameter with time after irradiation occurs 16-20 h after radiation exposure. These observations are correlated with changes in cell viabilities. © 1990 Academic Press, Inc.

### INTRODUCTION

The nuclear envelope consists of a double-layer membrane perforated by pore complexes and lined on its nucleoplasmic surface by a protein scaffold consisting primarily of the nuclear lamins A, B, and C (1, 2). The envelope serves as a semipermeable barrier between the cytoplasmic and the nucleoplasmic spaces, regulating transport of messenger RNA and other macromolecular traffic through the pore complex. Although the function of the lamins is not yet understood, they appear to serve a structural role, supporting the nuclear membrane (3) and providing attachment sites for chromatin (4, 5). Alteration of the nuclear envelope by radiation could severely affect the metabolic and reproductive viability of the cell.

Many studies indicate that the nuclear envelope is sensitive to radiation. The first morphological change observed in the irradiated lymphocyte is a dilation and invagination

of the nuclear envelope (6). Sato *et al.* (7) detected radiation-induced changes in the negative charge on nuclear membranes by measuring the electrophoretic mobility of isolated nuclei. They showed that there is a good correlation between the mobility of intact nuclei and the surviving fraction of three cell lines.

Damage to the nuclear envelope is related to the survival of the cell. Cole *et al.*, in a series of experiments beginning in the 1970's, showed that limiting radiation exposure to the region of the nuclear envelope in intact cells can lead to division delay and cell death (8-10). Schneiderman and Hofer (11) found that cells containing DNA labeled with <sup>125</sup>IUdR during S phase did not experience enhanced division delay, while cells with DNA labeled during late-S/G<sub>2</sub> phase did. Chromatin is bound to the nuclear envelope during G<sub>2</sub> phase but not during S phase. Therefore, ionizations that occur proximal to the nuclear envelope are more harmful to the cell than those that do not.

The mechanism by which radiation damages the envelope remains unclear, and the specific sites of damage are unknown. In an effort to understand more about the effects of radiation on the nuclear envelope, we used a fatty acid spin-label probe to measure radiation-induced changes in nuclear membrane fluidity. Our results show that  $\gamma$  irradiation results in increased nuclear membrane fluidity in MOLT-4 cells *in vivo*. Nuclei purified from the irradiated cells exhibit changes in the electron paramagnetic resonance (EPR) spectra of the spin-label probe 5-doxylstearic acid that are related to radiation dose and time after irradiation. This damage is correlated with cell viability.

### MATERIALS AND METHODS

#### Cell culture

MOLT-4 is a human leukemic T-lymphocyte cell line originally isolated from a patient with acute lymphoblastic leukemia and previously characterized as radiosensitive (12) with a *D*<sub>0</sub> value of 0.49 Gy for X rays (13). Cells (ATCC CRL 1582) were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, NY), penicillin (100 units/ml, GIBCO), and streptomycin (100  $\mu$ g/ml, GIBCO) in loosely capped plastic culture flasks at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. Subculturing the cells every 3-4 days maintained them in log-phase growth. Doubling time of the cells was 24-30 h.

#### Irradiation

Irradiation was performed at room temperature, using the Armed Forces Radiobiology Research Institute's Theratron <sup>60</sup>Co source at a dose

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rate of 0.85 Gy/min. Cells were irradiated as a suspension in growth medium at a density of  $0.5\text{--}1.0 \times 10^6$  cells/ml.

#### Nuclei Purification and Spin-Labeling

Cells were removed from medium by centrifugation and washed twice with nuclei buffer [NB: 10 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4]. The washed pellet was resuspended in hypotonic Hepes buffer (10 mM Hepes, 2 mM MgCl<sub>2</sub>, 1 mM PMSF) to a concentration of  $1\text{--}3 \times 10^7$  cells/ml and allowed to swell for 12 min on ice. Cells were then homogenized by seven strokes in a Dounce homogenizer ("A" pestle). The homogenate was centrifuged (750g at 4°C for 7 min) to pellet the nuclear fraction. The pellet was washed twice with NB before labeling with the spin-label probe. Nuclei were labeled by adding 5-doxy stearic acid (Molecular Probes, Inc., Eugene, OR;  $10 \mu\text{l}$  of  $2.3 \times 10^{-3}$  M solution in methanol) to 1 ml of the nuclei suspension ( $0.5\text{--}1.0 \times 10^8$  nuclei). Methanol used to dissolve the spin label had no effect on the EPR spectra of either control or irradiated nuclei. The suspension was incubated for 10 min on ice, then centrifuged (13,000 rpm in an Eppendorf microcentrifuge for 7 s at 4°C) to pellet the nuclei, which were then washed once with NB and stored on ice until analyzed. The single wash reduces handling steps yet is sufficient to remove all detectable levels of free spin label.

Our experience indicated that the MOLT-4 nuclei tend to aggregate during purification unless all steps are done gently, expeditiously, and on ice at all times, using buffers containing PMSF.

#### Electron Paramagnetic Resonance

Immediately before obtaining a spectrum, the nuclei were resuspended into 50  $\mu\text{l}$  of NB. The suspension was aspirated into a 50- $\mu\text{l}$  disposable micropipet that was then sealed and inserted into an EPR quartz tube (two-thirds filled with ordinary household oil) located in the EPR cavity. All EPR spectra were obtained at room temperature with the operation parameters fixed for any given experiment. Typical settings on the spectrometer were: field set, 3380 G; scan range, 100 G; scan time, 8 min; time constant, 0.128 s; modulation amplitude, 2 G; and microwave power, 16 mW. The order parameter,  $S$ , of the strongly immobilized probe was calculated from these spectra using the equation:  $S = (T_{\parallel} - T_{\perp}) / (3(T_{zz} - a')/2)$ , where  $2T_{\parallel}$  is the outer hyperfine splitting,  $2T_{\perp}$  is the inner hyperfine splitting,  $a'$  is  $(T_{\parallel} + 2T_{\perp})/3$ , and the tensor  $T_{zz} = 32$  G [14, Fig. 1].

#### Viability Measurements

Cell viability was assayed by trypan blue vital dye exclusion by adding one part of 0.1% trypan blue in deionized water to three parts of the medium containing the cells. After a 5-min equilibration period, cells were counted under an optical microscope. Cells taking up the dye were scored as nonviable and expressed as a fraction of the total number of cells counted.

#### Biochemical Determinations

The plasma membrane marker enzyme 5'-nucleotidase was determined by colorimetry at 660 nm by monitoring the production of inorganic phosphate resulting from the hydrolysis of adenosine monophosphate (Sigma Diagnostics Procedure No. 675) [15].

Protein was determined using the Bio-Rad standard protein assay.

## RESULTS

The nuclear isolation procedure used in these experiments produces nuclei devoid of visible cellular debris (as judged by light microscopy). Because of concern that significant contamination of the nuclei preparations by other cel-

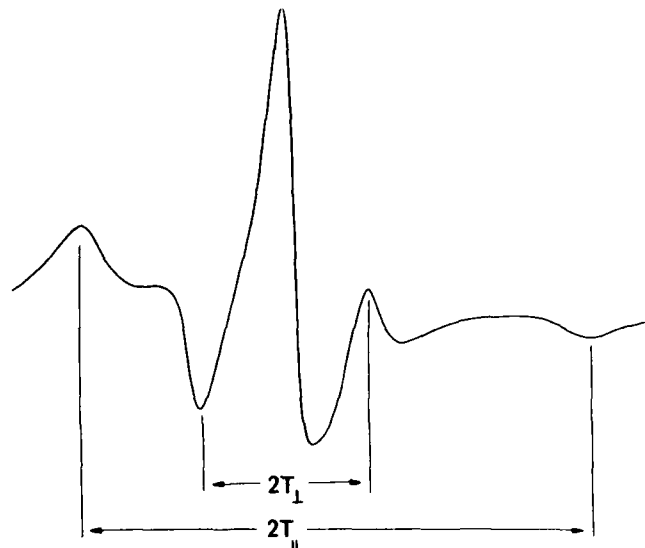


FIG. 1. EPR spectrum of nuclei incubated with 5-doxy stearic acid. The spectrum is typical of that of a membrane-bound spin-probe.  $S = (T_{\parallel} - T_{\perp}) / (3(T_{zz} - a')/2)$ , where  $2T_{\parallel}$  is the outer hyperfine splitting,  $2T_{\perp}$  is the inner hyperfine splitting,  $a'$  is  $(T_{\parallel} + 2T_{\perp})/3$ , and the tensor  $T_{zz} = 32$  G [14]. An increase in  $S$  indicates an increase in the fluidity of the membrane.

lular membranes could affect the interpretation of our data, we analyzed the purity of our nuclei preparations by assaying for the plasma membrane marker, 5'-nucleotidase. Five percent of the activity present in the whole cell remains in the purified nuclei preparation. In addition, nuclei preparations from irradiated cells do not demonstrate any difference in contamination compared to controls. Two other isolation techniques tested, using sucrose gradients, result in somewhat less contamination of the nuclei, but produce a greater tendency for the nuclei to aggregate despite the use of different buffers designed to inhibit this phenomenon. All isolation procedures tested, however, produced identical EPR spectra.

Figure 1 shows the first-derivative EPR spectra obtained with the spin-label probe, 5-doxy stearic acid, in nuclei from nonirradiated MOLT-4 cells. The probe incorporates into the lipid portion of the nuclear membrane and produces a spectrum typical of a membrane-immobilized spin-label probe. The order parameter,  $S$ , is an empirical indicator of the spin-label mobility in the membrane. An increase in  $S$  implies a more fluid nuclear membrane lipid environment. In these experiments  $S$  was remarkably consistent in control cells from preparation to preparation. Sixty independent measurements of nuclei from nonirradiated cells produced a value of  $1.001 \pm 0.002$  (mean  $\pm$  SEM). Greater variability was observed in nuclei from irradiated cells (Figs. 2 and 3).

The data in Fig. 2 demonstrate that fluidity of the nuclear membrane increases with time after irradiation. No significant change is observed until 15 h postirradiation; after that

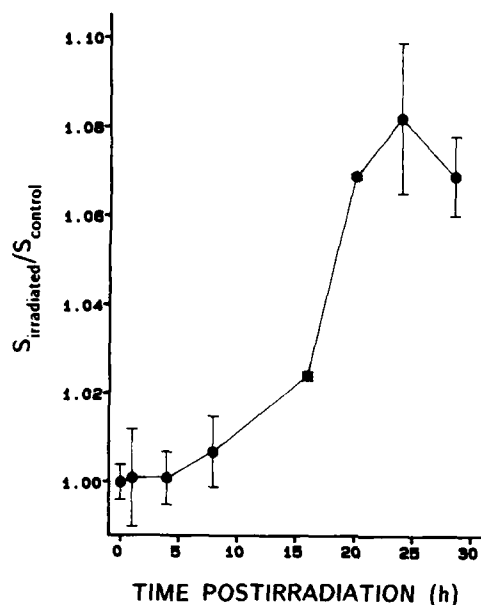


FIG. 2. Ratio of  $S_{\text{irradiated}}/S_{\text{control}}$  varies with time post irradiation. MOLT-4 cells were exposed to 7.5 Gy  $^{60}\text{Co}$   $\gamma$  radiation and their nuclei extracted and labeled. Values represent the means  $\pm$  SEM from nine determinations of  $S$  (three independent experiments) from control and irradiated nuclei, respectively.

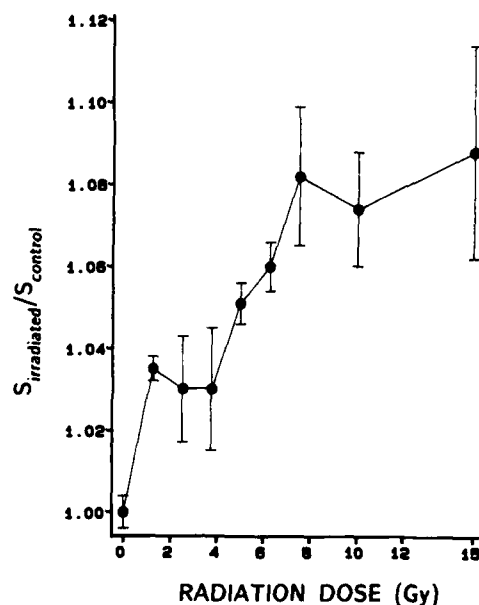


FIG. 3. Ratio of  $S_{\text{irradiated}}/S_{\text{control}}$  varies with radiation dose. MOLT-4 cells were exposed to various doses of  $^{60}\text{Co}$   $\gamma$  radiation and their nuclei extracted and labeled 24 h postirradiation. Values represent the means  $\pm$  SEM from nine determinations of  $S$  (three independent experiments) from control and irradiated nuclei, respectively.

the membrane fluidity increases to the maximum observed at 20–24 h.

Radiation dose also influences membrane fluidity (Fig. 3). Exposures as low as 1.25 Gy produce a significant increase in the membrane fluidity compared with controls. Increases in nuclear membrane fluidity are roughly proportional to dose up to 7.5 Gy, with larger doses producing smaller increases.

Measurements of the viability of irradiated cells indicate that the number of cells scored as viable by their ability to exclude trypan blue decreases with both radiation dose and time after irradiation. Figure 4 shows that radiation doses greater than 5 Gy reduce the number of viable cells measured 24 h postirradiation to a minimum. Figure 5 shows that 5–7 h after exposure to 7.5 Gy, viability decreases steadily until a minimum is observed at 24–30 h postirradiation. Comparison of these viability curves with the increase in membrane fluidity (Figs. 2 and 3) indicates an inverse relationship between viability and fluidity.

## DISCUSSION

Although many studies have provided indirect evidence that the nuclear envelope is sensitive to radiation, few studies have identified specific alterations in the envelope that occur as the result of radiation exposure. Sato *et al.* (7) reported that radiation induces a net loss of negative surface charge on nuclei from three cell lines. This change was

shown to correlate with the ability of the cells to survive radiation exposure.

Our experiments are the first to measure a change in the membrane fluidity of the nuclear envelope of nuclei iso-

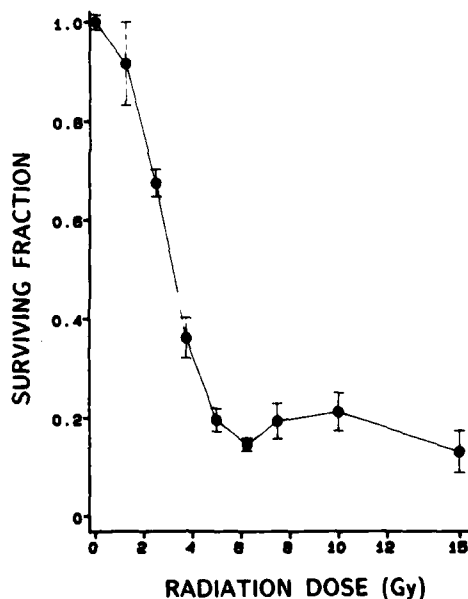


FIG. 4. Viability of MOLT-4 cells assayed by trypan blue exclusion after exposure to various radiation doses. Viability is expressed as the mean  $\pm$  SEM (three determinations) of the normalized fraction of irradiated cells/control cells that exclude trypan blue.

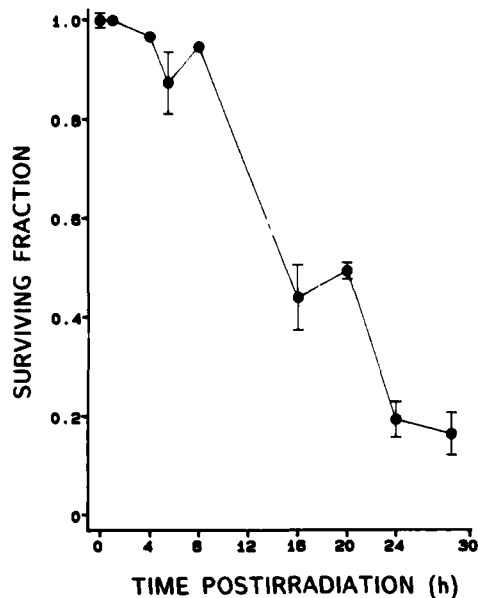


FIG. 5. Viability of MOLT-4 cells measured by trypan blue exclusion at various times after radiation exposure. Viability is expressed as the mean  $\pm$  SEM (three determinations) of the normalized fraction of irradiated cells/control cells that exclude trypan blue.

lated from irradiated cells compared with that from non-irradiated cells. The change in fluidity increases with radiation dose and time after irradiation, and is related to the viability of the cells. The radiation doses required to produce the change in nuclear membrane fluidity and the time course of the changes observed are similar to those reported by Sato *et al.* Our qualitative observation that nuclei from irradiated cells tend to aggregate in relation to radiation dose also suggests that exposure to radiation produces changes in the nuclear surface. The protease inhibitor PMSF and cold temperatures greatly inhibit this effect, suggesting that protease substrates that are not exposed in nuclear envelopes of nonirradiated cells are exposed by radiation.

Fluidity changes in the plasma membrane have been shown to be associated with lipid peroxidation (16, 17) and alterations in the properties of membrane proteins (18–20). The role of either in radiation injury to the cell is still unclear [for reviews see (21, 22)]. Also, the radiation doses used to produce detectable radiolytic effects on the molecular components of membranes are often in the 10- to 1000-Gy range, far higher than the doses required to alter fluidity in our experiments. This may imply that low levels of lipid peroxidation or protein damage induce fluidity changes in the nuclear envelope or that the irradiated nuclear membrane is altered by another mechanism.

The changes in nuclear membrane fluidity we observed may arise from either alteration of the lipids themselves or alterations to sulfhydryl groups or other amino acid resi-

dues, leading to conformational changes in membrane proteins that influence membrane lipid structure. Any such fluidity changes could affect the viability of the cell. For example, a radiation effect that changes the lipid environment of pore complex proteins or alters the pore proteins themselves could disrupt the bidirectional, transmembrane traffic of proteins and mRNA. Radiation-induced alteration of membrane lipids could affect the binding of nuclear membrane-bound proteins such as lamin B, which is thought to mediate the attachment of chromatin to the nuclear envelope through lamins A and C (23).

Further study of radiation effects on nuclear membrane fluidity could determine the primary site of radiation damage. A more detailed characterization of the changes in lipid fluidity in our system might use lipid spin probes with the doxyl group located at different positions along the hydrocarbon chain, which would allow measurement of membrane fluidity at various depths within the lipid bilayer. The effect of radiation on membrane proteins could be studied by measuring changes in the mobility of probes bound to those proteins.

Much evidence suggests that the nuclear envelope is a target of radiation in the cell, the impairment of which affects cell viability. We have demonstrated a radiation-induced change in nuclear membrane lipid fluidity not previously reported. The specific membrane alterations producing this change remain unknown. Whether this is a primary effect of radiation or a secondary expression of damage awaits further study.

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