OBJECTIVE

The current objective of our research is to investigate the dose-response relationship of the lethal and mutagenic effects of exposure of cells to radon and its decay products. Dose-rate dependence will be studied, as well as the nature of the DNA lesions. The effect of DNA repair on the lethal and mutagenic effects of exposure and on the character of the DNA lesions will be investigated by comparing the response of L5178Y strains which differ in their ability to rejoin X radiation-induced DNA double-strand breaks.

DOE/ER/60658--3

DE91 005591

PROGRESS REPORT: 4/1/1988 - 10/1/1990

Methods

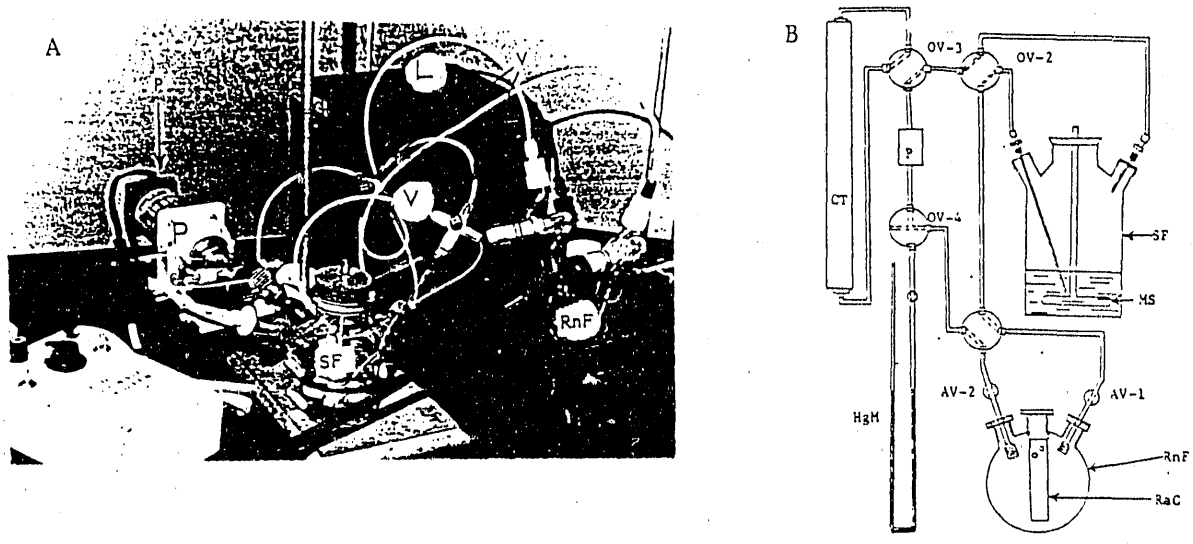
Exposure: For the exposure the cells are suspended in medium previously equilibrated with respect to radon and its decay products. The **dose rate is varied** by the amount of radon and its decay products in solution. The **dose is estimated** by measuring the radioactivity of samples of the medium withdrawn at the beginning and end of the exposure. Gamma radioactivity from Bi-214 is measured using a 1" x 3" NaI crystal and a multichannel analyzer, calibrated with a standard solution of Ra-226. Alpha radioactivity is assayed by scintillation counting, using a Ra-226 solution for calibration and determination of window settings. Medium samples are counted at various times after collection, and the proportion of radon and its decay products in the medium during the exposure is calculated from the decay of the net total alpha activity in each sample according to the Bateman equations (1). The proportion of the various alpha emitters in the medium as determined using an alpha probe constructed at Pacific Northwest Laboratories is in good agreement with the calculated values. The sources of the dose to the suspended cells are radon and its decay products in the medium plus alpha-emitting particles adsorbed to or incorporated into the cells. To determine the contribution of adsorbed or incorporated alpha particles, cells are centrifuged after the exposure, rinsed one time, suspended and assayed by scintillation counting. The background radioactivity is determined by centrifuging a medium sample containing no cells, rinsing the "pellet", suspending it and assaying for radioactivity under the same conditions as used for the cellular pellet. It has been found that the alpha particles adsorbed to or incorporated into the cells varies with the amount of radon and its decay products in the medium and with the time of exposure (2).

The **radon delivery system** allows controlled bubbling of radon into the cell culture medium prior to the addition of serum and cells (Fig. 1). Radium sulfate (37 mCi) is inserted in flask RnF, and the emitted radon is pumped by means of a stream of air through Tefzel tubing and a 4-way valve to the medium in spinner flask SF. The flow rate can be varied from 0 to 144 ml/minute. The plugs and stopcocks are remotely operated and are fitted with radiation-resistant Kalrez O-rings.

The valves and connectors can withstand a pressure of 3000 psi, but the system is operated at a nominal pressure of one atmosphere as monitored with a mercury manometer. At the completion of each radon loading, the residual radon in the system external to flask RnF and spinner flask SF is pumped through an activated charcoal trap. The flask is placed in a 1"-thick lead-shielded box L located in a negative pressure room. Lead bricks (2") are used as additional shielding and reduce radiation levels to acceptable limits. A typical loading of the medium in the spinner flask with radon involves the following sequence of steps. The two Teflon stopcocks on Flask RnF are opened and the radon-enriched air is pumped into the spinner flask and bubbled through 100 ml of medium via a 6" 16G needle. The sterility of the medium is maintained during the bubbling process by sterile filters at the entrance and exit ports of the spinner flask. After two minutes of bubbling at the rate of 100 ml per minute, the pump is stopped, the containment flask closed, and the spinner flask isolated via the 4-way stopcocks. Serum is then added to the radon-loaded medium through a port, and the needles are removed and septa sealed. After loading sufficient flasks for each experiment, the containment flask is isolated with the 4-way valve, and

the residual radon in the system is pumped into the charcoal trap. Cells are added to each spinner flask four or more hours after the loading procedure.

Figure 1. A. Photograph of Radon Generator; B. Diagram of Radon Generator



RaC - Radium containment cylinder; RnF - radon containment flask; AV-1,2 - Ace valves; OV-1,2,3,4 - Omnifit valves; HgM - mercury manometer; CT - charcoal trap; P-pump; SF - spinner flask; MS-magnetic stirrer

Cell Culture: The mouse lymphoma L5178Y (LY) strains which have been used differ in their sensitivity to low-LET radiation. Strain LY-S is deficient in the repair of X radiation-induced DNA double-strand breaks and is radiation sensitive in comparison to strain LY-R (3). Sublines of these strains have been constructed which are either heterozygous (strains LY-S1 and LY-R16) or hemizygous (strain LY-R83) at the thymidine kinase (*tk*) locus (4). We have used these strains to determine radon/radon daughter cytotoxicity, and its mutagenicity at a heterozygous *vs.* a hemizygous locus; to investigate the nature of the lesion size at this locus; and to determine whether or not these cellular responses are affected by the repair capabilities of the exposed cells.

Table 1. PROPERTIES OF L5178Y STRAINS

Strain	Response to Radiation	*tk* Genotype
LY-S1	X radiation sensitive	$tk^{+/-}$
LY-R16	X radiation resistant	$tk^{+/-}$
LY-R83	X radiation resistant	$tk^{+/o}$

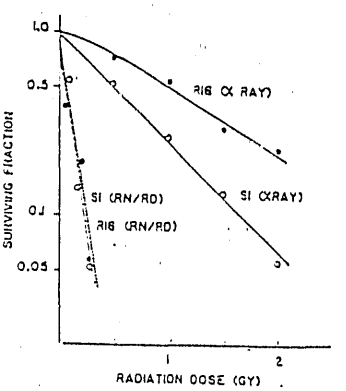
Cytotoxicity is determined by comparing the colony forming ability of control and exposed cells following plating in a soft agar medium (4). The **mutation frequency** at the *tk* locus is measured by incubating the exposed cells for 48 hours to allow expression of mutations, and plating exposed and control cells in selective medium containing trifluorothymidine (4). Only cells lacking thymidine kinase activity can form colonies in this medium. In order to determine the **size of the mutational lesion inactivating the thymidine kinase gene,** independent spontaneous or alpha radiation-induced $TK^{-/-}$ mutants are isolated and expanded in culture. Changes in the restriction fragment electrophoretic patterns of the TK DNA are identified by autoradiography

following hybridization to a labeled TK cDNA probe (5). Extension of the mutational lesion to the neighboring galactokinase (*gk*) gene is assayed by measuring galactokinase activity of homogenates of cells obtained from each independent mutant (5).

Results

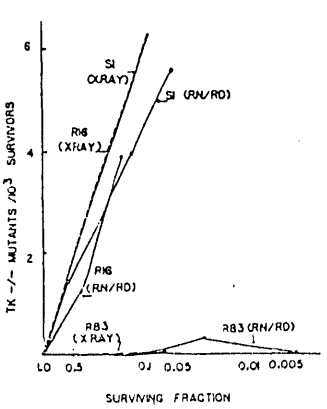
1. Exposure of L5178Y Cells at PNL. The initial exposures of L5178Y cells were carried out at Battelle Pacific Northwest Laboratories. Two L5178Y strains were exposed in each of three experiments at dose rates to the medium of 0.13-0.23 Gy/hr. The dose response curves with regard to colony formation were exponential and similar for strains LY-S1, LY-R16, and LY-R83 (Fig. 2). D_os were 0.09 Gy and D_qs were essentially 0 Gy. Thus, in these experiments, no difference was obtained for the repair-efficient vs. the repair-deficient strains following exposure to radon and its decay products, in contrast to the differences observed following exposure to low LET radiation (Fig.2). (Following exposure to X radiation D_os were 0.67 Gy and 0.90 Gy, and D_qs were 0.05 Gy and 0.5 Gy for strains LY-S1 and LY-R16, respectively.)

Figure 2. Cytotoxicity of Radon/Radon Daughters vs. X radiation: Exposures at PNL



The mutant frequency at the *tk* locus was similar for strains LY-R16 and LY-S1 (1.5-1.75 $TK^{-/-}$ mutants per 10^3 surviving cells at the D_o dose) following exposure to radon and its decay products (Fig. 3). This induced mutant frequency was similar to that obtained after exposure of the cells to equitoxic doses of X radiation (Fig. 3). In contrast to the two heterozygous strains, a very low mutant frequency was obtained in the case of the $TK^{+/o}$ hemizygous strain LY-R83 after exposure to either X radiation or radon and its decay products (Fig. 3). This result indicates that the majority of mutants induced by radon and its decay products (as well as by X radiation) harbor multilocus lesions, since mutants bearing such lesions are poorly recovered in the $TK^{+/o}$ hemizygous strain LY-R83 (4).

Figure 3. Mutagenicity of Radon/Radon Daughters vs. X radiation: Exposures at PNL



2. Exposure of L5178Y Cells at CWRU. A temporary radon generator was assembled using a 3 mCi radium source for radon which was bubbled into the medium as described above. The dose rates ranged from 0.04 to 0.13 Gy/hr to the medium. Exposure of the cells in this radon generator induced much less cell killing than when the cells were exposed at PNL. The D_os were 0.36 and 0.31 Gy for strains LY-R16 and LY-S1, respectively (Fig. 4), The mutant frequency induced by equitoxic doses of radon/radon daughters in strain LY-R16 was similar to that obtained at PNL. The mutant frequency induced in strain LY-S1, however, was less than half of that obtained at PNL (Fig. 5).

Figure 4. Cytotoxicity of Radon/Radon Daughters: Exposures at CWRU vs. PNL

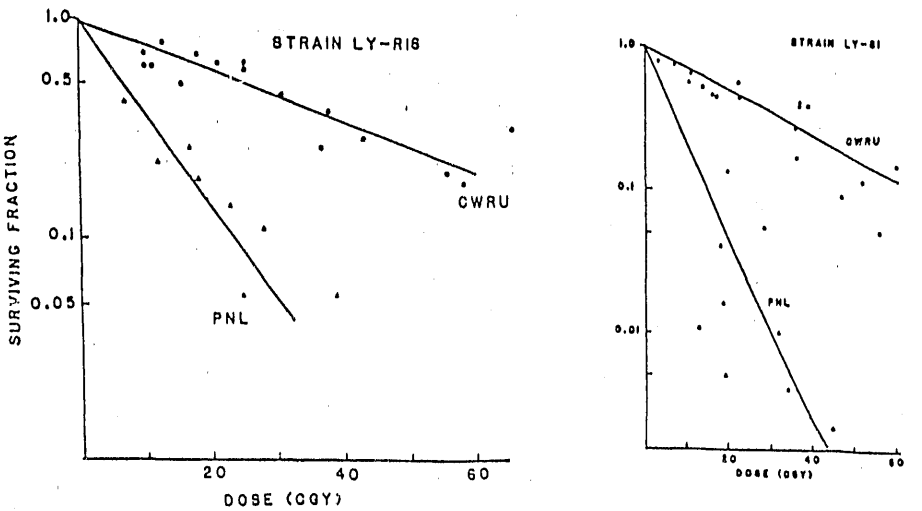
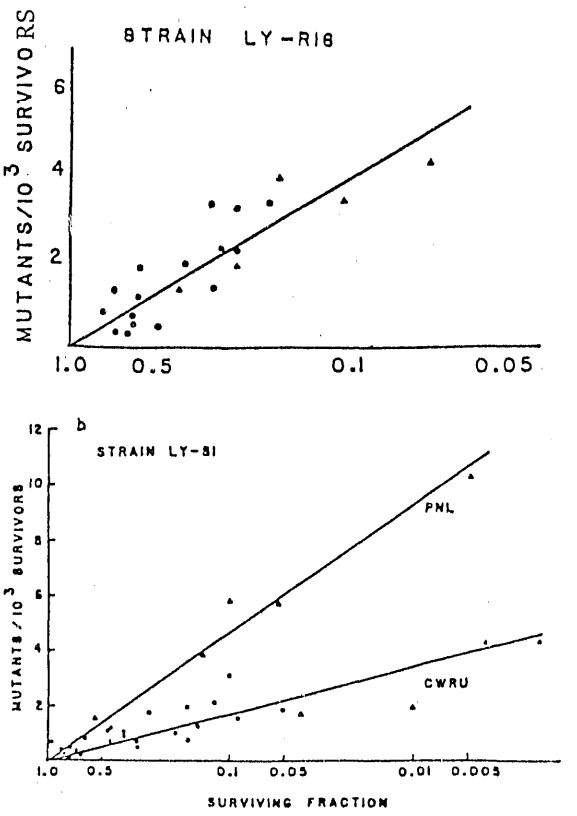
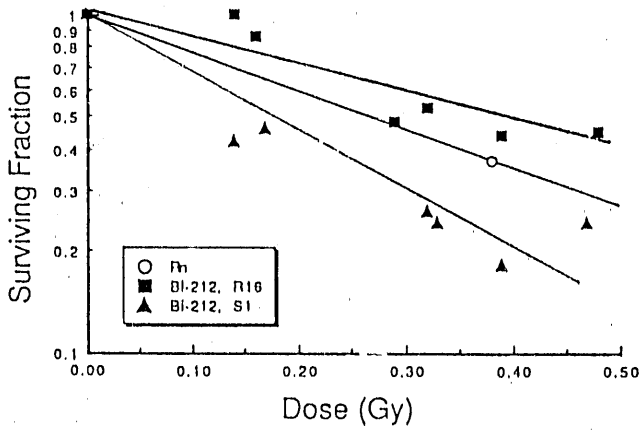


Figure 5. Mutagenicity of Radon/Radon Daughters: Exposures at CWRU vs. PNL



3. Exposure of L5178Y Cells at the University of Chicago. Cells were exposed to alpha radiation from chelated Bi-212 at the University of Chicago. The induced cytotoxicity and mutant frequency were similar to that obtained at CWRU, although the difference in the survival of the radiation-resistant vs. the radiation-sensitive strains was greater after exposure to Bi-212 than to radon and radon daughters (Fig. 6), perhaps because of the relatively higher contribution of low-LET radiation to the dose during Bi-212 exposure.

Figure 6. Cytotoxicity of Alpha Radiation from Bi-212: Exposures at the University of Chicago



4. Investigations of the Mechanism of the Differences in Dose Response upon Exposure at PNL vs. Exposure at CWRU or UC.. We examined several differences in experimental protocols which might explain the differences in dose response at PNL vs. CWRU. We found that holding the cells for four hours following exposure and removal of radon and its decay products (as done at PNL) did not cause a marked difference in dose response (Table 2).

Table 2. Survival as a Function of Holding Time at 25° Following Exposure to Radon and Radon Daughters

Strain	Dose	Surviving Fraction After Various Holding Times (hrs)				
		0	1	2	3	4
LY-S1	0.26	0.56	0.40	0.31	0.50	
	0.37	0.38				0.38
LY-R16	0.14	0.59	0.77	0.50	0.68	

Since dose rates were much lower at CWRU than at PNL, the longer exposure time at 37° resulted in cells accumulating in the G2 period (Fig 7) To determine if differences in cell cycle distribution could be responsible for the differences in dose-response, we compared exposures at 37° with exposures at 25°. Little cell cycle progression occurs at the lower temperature (Fig. 7) No difference in dose response was obtained at the two temperatures, however (Fig. 8).

Figure 7: Cell Cycle Distribution at Various Times During Exposure of L5178Y Cells to Radon/Radon Daughters at 25° and 37° at CWRU

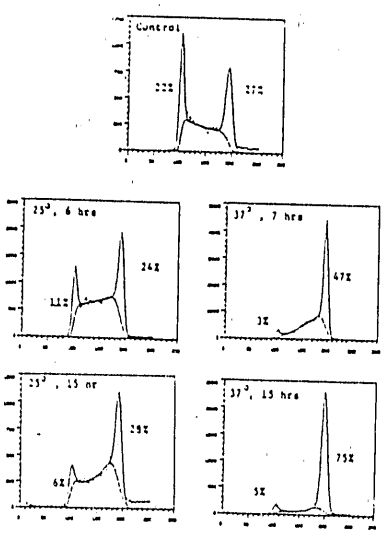
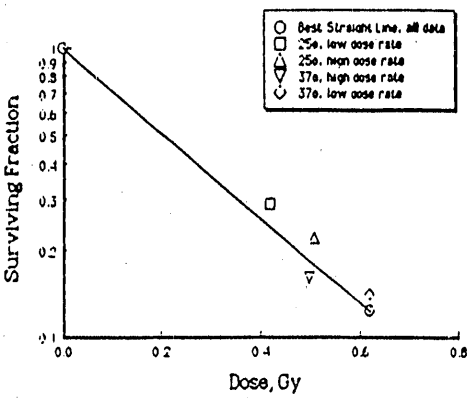


Figure 8. Cytotoxicity of Radon/Radon Daughters: The Effect of Dose Rate and of Temperature



The similarity of the dose response obtained at CWRU with that obtained upon exposure to chelated Bi-212 at UC (Fig. 6) suggested that a possible difference between exposure at PNL and CWRU could be the level of attachment of radon decay products to the cells. This was found to be the case, with attachment at PNL amounting to ten times that observed at CWRU (Table 3). In order to determine the contribution of the attached alpha radioactivity to the total dose, it will be necessary to determine the kinetics of attachment and the dependence of attachment on the concentration of radon daughters in the medium (see below).

Exposure System	Alpha cpm / cell
CWRU	0.023
PNL	0.21

After the exposure the cells were centrifuged, transferred to a new centrifuge tube, washed with medium, resuspended in fresh medium, and the suspension was assayed for cells and alpha particles per ml.

5. Interlaboratory Comparison of the Response of CHO cells to Alpha Radiation. The response of the CHO cell line, C-18, was compared upon exposure to radon and its decay products at PNL and CWRU and to chelated Bi-212 at UC. For determination of the dose, the steady-state concentrations of radon and its daughters and of Bi-212 in the various media were determined, and the dose contribution of the radon daughters attached to or incorporated into the cells was calculated (2). When these factors were taken into account, the dose response of the CHO cells to alpha radiation was found to be very similar at all three institutions (Fig 9, Table 4, from ref. 2). Thus, in contrast to the results obtained with L5178Y cells, the dose-response curves obtained for CHO cells were very similar at CWRU and PNL, once the dosimetry was carefully determined. The contribution to the dose of alpha-emitting particles attached to the cells may be much lower for CHO cells than for L5178Y cells because of the low cytoplasm/nucleus ratio in the L5178Y cells. The determination of the contribution to the dose of radon daughters attached to L5178Y cells is one of our high priority objectives in the near future (see research proposal).

Response of CHO Cells to Alpha Radiation: Comparison of Results Obtained at Three Different Laboratories

Figure 9. Dose Response Curves: CHO Cells

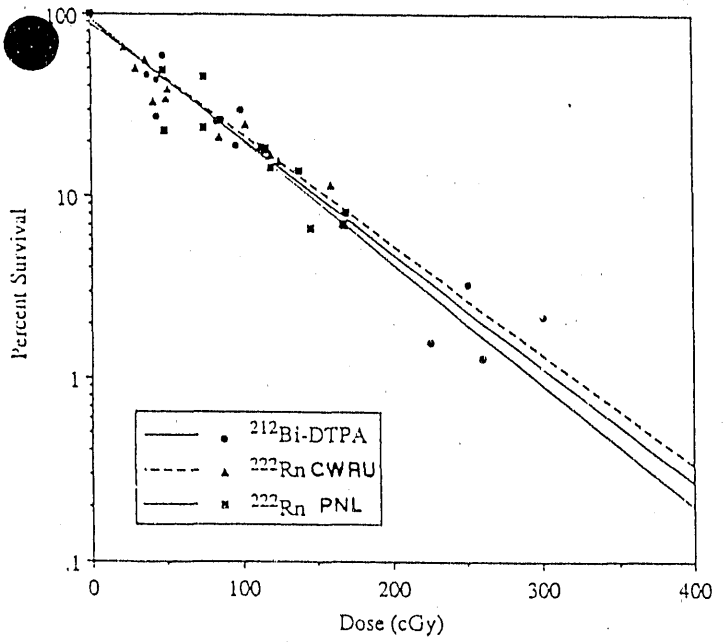


Table 4: Dose Response Parameters: CHO Cells

RADIATION SOURCE	N	D_Q	n
^{212}Bi	4	0.66 +/- 0.20	1.2 +/- 0.25
^{212}Bi-DTPA	4	0.60 +/- 0.12	0.9 +/- 0.11
^{222}Rn CWRU	4	0.63 +/- 0.03	0.9 +/- 0.07
^{222}Rn PNL	4	0.61 +/- 0.10	0.9 +/- 0.12

1 Results for D_0 (in Gy) and n are mean and s.e.m. of N number of experiments.

6. Analysis of Alterations of TK DNA in $TK^{-/-}$ Mutants. Strain LY-R16 (but not LY-S1) possesses a restriction fragment length polymorphism upon digestion with the restriction enzyme NCO1 that enables the differentiation of the two *tk* alleles (5). We have found that the majority of $TK^{-/-}$ mutants induced by exposure to radon and its decay products have lost the entire active *tk* allele similar to $TK^{-/-}$ mutants induced by X radiation and to spontaneous $TK^{-/-}$ mutants (Table 5). Interestingly two of the radon/radon daughter-induced mutants lost the **inactive** rather than the active *tk* allele; a possible explanation for such an event could be inactivation (but not loss) of the active *tk* allele, followed by a conversion of the inactive allele to the original inactive allele. However, since a similar loss was observed in a spontaneous mutant, it is uncertain whether this unusual event was induced by alpha radiation, or whether the two independent mutants picked from the radon-exposed cultures were actually spontaneous mutants (which were present in a ratio of approximately 1:5 in these two experiments).

Table 5. LOSS OF THE ACTIVE *tk* ALLELE IN $TK^{-/-}$ MUTANTS OF STRAIN LY-R16

Origin of $TK^{-/-}$ Mutants	Mutants Lacking *tk* Allele			
	tk^+ Allele (6.3 kb band)		tk^- Allele (4.7 kb band)	
	Fraction	%	Fraction	%
Spontaneous	29/36	80	1/36	3
High Dose-Rate X Radiation	21/22	95	0/22	0
Radon & Radon Daughters	21/25	84	2/25	8

7. The Extent of the Lesion Inactivating the *tk* Gene. The extension of the lesion inactivating the tk gene to the neighboring *gk* gene (within 200 kb of the *tk* gene) was assessed by determining galactokinase activity in homogenates of the $TK^{-/-}$ mutants. For the repair-efficient strain LY-R16, the percentage of $TK^{-/-}$ mutants with lesions extending to the *gk* gene was much greater following exposure to radon than to high dose-rate X radiation (Table 6). However for the repair-deficient strain LY-S1, the percentage of mutants with lesions extending to the *gk* gene was somewhat less following exposure to radon/radon daughters than to X radiation (Table 6). These results indicate that DNA lesions resulting in long deletions are repaired in strain LY-R16 following exposure to X radiation but that this repair is less efficient with regard to lesions induced by radon/radon daughters. Neither type of lesion appears to be repaired well in strain LY-S1.

Table 6. $TK^{-/-}$ MUTANTS WITH DEFICIENT GALACTOKINASE ACTIVITY

Origin of $TK^{-/-}$ Mutants	% $TK^{-/-}$ Mutants with GK Activity Less than 80% of Parental Strains			
	LY-R16		LY-S1	
	Fraction	%	Fraction	%
Spontaneous	15/25	(60)	12/23	(52)
High Dose-Rate X Radiation	3/25	(12)	20/36	(55)
Radon & Radon Daughters	12/27	(44)	9/23	(39)

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