

SUBCHRONIC EXPOSURE OF BALB/C AND C57BL/6 STRAINS OF *MUS MUSCULUS*
TO THE RADIOACTIVE ENVIRONMENT OF THE CHORNOBYL, UKRAINE
EXCLUSION ZONEBRENDA E. RODGERS,*† RONALD K. CHESSER,‡§ JEFFREY K. WICKLIFFE,† CARLETON J. PHILLIPS,† and
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Abstract—Environmental contamination resulting from the Chernobyl, Ukraine, disaster offers a unique opportunity to examine the in vivo biological effects of chronic, low-dose exposure to radiation. Laboratory studies of acute exposure to ionizing radiation have been used to estimate risk and potential human health effects by the extrapolation of laboratory data to situations of low-dose environmental radiation exposure. Few studies, however, have explored the biological consequences of low-dose exposure via in situ environmental radiation in a sentinel species. In the present study, laboratory strains of *Mus musculus* (BALB/c and 57BL/6) were placed in environmental enclosures in the Red Forest region of the Chernobyl exclusion zone. Blood samples were obtained every 10 d, and the micronucleus (MN) test was employed to assess the potential for cytogenetic damage from exposure to Chernobyl radiation. Radionuclide uptake was monitored throughout the study, and dose was estimated for each individual as well as for their offspring. Total dose for the mice experimentally exposed to this environment averaged 1162 mGy for BALB/c (30 d) and 1629 mGy for C57BL/6 (40 d). A higher MN frequency for both strains was observed at day 10, although this change was only statistically significant in the C57BL/6 mice ($\chi^2_3 = 13.41$, $p = 0.003$). Subsequent samples from C57BL/6 resulted in values at or less than the initial frequencies. In BALB/c mice, an increase in MN was also evident at day 30 ($\chi^2_3 = 10.38$, $p = 0.006$). The experimental design employed here allows for the incorporation of traditional laboratory strains, as well as transgenic strains of *Mus*, as sentinels of environmental radiation contamination.

Keywords—Chernobyl Micronucleus test BALB/c C57BL/6 Chernobyl

INTRODUCTION

The effects of acute exposure to ionizing radiation are well documented in inbred strains of the mammalian model *Mus musculus* [1–3]. However, biological effects of chronic, low-dose exposure to radiation in these animals are less well known. Contamination resulting from the Chernobyl, Ukraine, disaster offers a unique opportunity to examine the in vivo biological effects of chronic, low-dose exposure to environmental radiation. Previous studies have reported that native rodent species (*Clethrionomys glareolus*, *Apodemus* sp., *Microtus* sp.) resident in the most radioactive regions near Chernobyl do not demonstrate statistically significant increases in their levels of mitochondrial DNA heteroplasmy, micronuclei (MN) formation, or chromosomal aberrations [4–6] when compared to the same species collected in regions of low contamination. Comparative data sets on strains of laboratory mice would be valuable in the application of these results to human health risks and the establishment of regulatory statutes. The present study was undertaken to assess the effect of exposure to environmental radiation contamination in the Chernobyl exclusion zone on two radiosensitive strains of *M. musculus*, BALB/c and C57BL/6 [1,3,7].

Attempts have been made to accurately estimate risk and to monitor levels of environmental radiation contamination by using wildlife species as sentinels [6,8–13]. Although wildlife studies have contributed to our understanding of the ecological

risk posed by the levels of radiation contamination in the Chernobyl region, their utility in assessing human health risk is minimized by several factors. Of great importance is that a considerable amount of variation exists in the sensitivity of organisms to radiation. In humans, the LD50 is approximately 3.5 to 5.0 Sv (1 Sv \approx 1 Gy [14]), whereas one of the most common rodent species in the exclusion zone, *C. glareolus* (bank vole), has an LD50/30 (i.e., acutely administered dose that would be lethal to 50% of the population in 30 d) of more than twice that (11 Gy) [8]. Other voles common to the Chernobyl exclusion zone include species of *Microtus*, which have an LD50/30 of more than 10 Gy [15]. Therefore, native species, although valuable in documenting radionuclide accumulation and distribution of ^{90}Sr and ^{137}Cs in the exclusion zone [12,16,17], may not be the most appropriate indicator for the risk of human exposure to the Chernobyl environment. Few studies have explored the biological consequences of low-dose, continual environmental radiation exposure in the *Mus* model system, even though the BALB/c (LD50/30 = 5.5 Gy) and C57BL/6 (LD50/30 = 6.0 Gy) strains of *M. musculus* demonstrate sensitivity to radiation [1,3,7] more similar to that of humans.

Herein, we report the results of a study in which two laboratory strains of *M. musculus* were placed in environmental enclosures in the Red Forest region of the Chernobyl exclusion zone. Radionuclide uptake was monitored throughout the study period. Accumulated dose was calculated for each sample period, and the micronucleus test was employed to assess the

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Fig. 1. Environmental enclosures placed in the Red Forest region of the Chernobyl, Ukraine, exclusion zone.

potential for cytogenetic damage from exposure to low-dose radiation. Micronuclei are cytoplasmic nuclear bodies that are formed by incomplete expulsion of complete chromosomes or chromosomal fragments from the nuclei of daughter cells during cytokinesis. Because MN are due to chromosomal aberrations or spindle-fiber anomalies, elevated levels of MN in the bone marrow or peripheral blood are accepted as a measure of genotoxic damage resulting from exposure of stem cells to aneugenic or clastogenic agents, such as ionizing radiation [18–23]. Micronuclei can be visualized in mouse polychromatic erythrocytes as early as 4 d and as late as 7 to 8 d postexposure to a mutagen. Subchronic exposure and periodic sampling of peripheral blood allowed for assessment of MN induction in the exposed mice relative to radionuclide uptake and accumulated dose.

MATERIALS AND METHODS

Study site

Enclosures to house laboratory strains of *M. musculus* were placed in the Red Forest approximately 1.5 km west/southwest of Reactor 4 of the Chernobyl Nuclear Power Plant (UTM:36 295545 U5697040). This experimental site was chosen because, in previous studies, rodents collected at this locality in the Red Forest consistently demonstrated the highest concentrations of ^{137}Cs and ^{90}Sr [12].

In May 1999, which was 13 years after the meltdown of Reactor 4, 11 enclosures, measuring $1 \times 0.67 \times 0.67$ m, were constructed from untreated cedar and galvanized wire mesh and placed in a 30×20 -m area of the Red Forest. Hinged tops were fastened with bolts and wing nuts to allow access to the animals during periodic sampling. The enclosures were placed on a site excavated to a depth of approximately 30 cm. After an enclosure was in place, the soil and vegetation that had been removed from the placement site were returned to the enclosure. The soil was seeded with alfalfa and watered to encourage growth of cover inside the enclosure before introduction of the study animals. Each enclosure was supplied with a water bottle and a nest box (Fig. 1).

Sampling

Animal husbandry and handling conditions were in accordance with the Texas Tech University Animal Care and Use Committee (Lubbock, TX, USA) guidelines and approved pro-

tol. In July 1999, laboratory strains of *M. musculus* (C57BL/6: $n = 7$, $\delta = 4$, $\text{♀} = 3$; BALB/c: $n = 8$, $\delta = 1$, $\text{♀} = 7$) were purchased from a research breeding colony in the city of Chernobyl, Ukraine. Ideally, we would have used an equal number of males and females from each strain; however, unlike in the United States, the availability of inbred strains is limited in Ukraine. These animals were sexed, weighed, measured, and assigned a voucher number. Before introduction to the Red Forest, a unique toe clip was given to each animal for further identification. From the toe clip, a blood smear was prepared and used to establish the initial MN frequency for each individual. The toe was preserved in lysis buffer [24] and used for subsequent DNA analyses.

Specific activity of ^{137}Cs in mice was measured by whole-body counts using a 7.62-cm NaI crystal (Canberra Industries, Meridian, CT, USA) enclosed in a lead shield. Each mouse was counted for 3 to 10 min. Although longer reading times may have provided more accurate estimates of initial internal radioactivity, efforts were made to reduce stress to the animal by limiting the time of confinement in a 4×6 -cm cylindrical scintillation jar for the count. Background assessments were made after every fifth count. The counting methods, calibration, and standardization are detailed by Chesser et al. [12]. All specific activity values for ^{137}Cs reported herein are for whole-body and wet weight. Specific activity values for ^{90}Sr are for wet weight of bone. Data were recorded and analyzed using a Libretto laptop computer (Toshiba American Information Systems, Tokyo, Japan) and Gamma Vision software package (EG&G Ortec, Oak Ridge, TN, USA).

Before introduction into the enclosures, one male and two female mice were placed into separate cages in the laboratory and monitored for overall health and compatibility for 24 h. On July 14, 1999, seven adult C57BL/6 mice were introduced into enclosures in the Red Forest. Eight adult BALB/c mice were introduced 10 d later. Uncontaminated food and water were supplied ad libitum. Mice were recaptured and returned to the laboratory for sampling every 10 d. On the evening before sampling, Sherman live traps (H.B. Sherman, Tallahassee, FL, USA) were set in the enclosures. Mice not caught in the traps were removed while in their nest boxes or by excavating their burrow systems. All animals were returned in live traps containing food and bedding to the lab facility at Chernobyl City, where they were then transferred to cages. Animals were monitored for body mass, whole-body counts were measured, and a blood sample was taken. Animals were returned to the enclosures in the Red Forest on the evening of their day of their capture.

Offspring were collected in two of the five enclosures ($n = 11$) during the course of the study. Newborns were removed from the enclosure at the time of discovery, returned to the lab, and euthanized as previously described by Baker et al. [5]. Whole-body counts and weights were recorded, blood smears were made, and voucher numbers were assigned. One-half of each neonate was cryopreserved, and one-half was preserved in lysis buffer [24]. On termination of the study (30 d for BALB/c, 40 d for C57BL/6), all animals were anesthetized and euthanized. Bone and muscle samples were taken for radiostrontium and radiocesium analysis, respectively. Voucher specimens and vital tissues are housed at the Museum of Texas Tech University.

Accumulated dose calculations

The activities of ^{90}Sr from bone and soil samples and of ^{137}Cs from muscle samples were determined according to the

Table 1. Activity and ^{137}Cs concentrations in Becquerels per gram (Bq g^{-1}) wet weight for BALB/c *Mus musculus* mothers and their offspring born in environmental enclosures in the Red Forest region of the Chernobyl, Ukraine, exclusion zone^a

Mother TK no.	Mother specific activity of ^{137}Cs (Bq g^{-1})		Mother dose (mGy/d)	Newborn TK no.	Newborn specific activity of ^{137}Cs (Bq g^{-1})	Newborn dose (mGy/d)
	Before exposure	After 30-d exposure				
81597 BALB/c Exposed 30 d	3.47	73.77	0.054	82520	69.01	0.05
				82521	67.68	0.049
				82522	70.74	0.051
				82523	64.97	0.047
				82524	71.8	0.052
81593 BALB/c Exposed 30 d	2.06	41.59	0.031	82525	38.55	0.028
				82526	67.01	0.049
				82527	64.24	0.047
				82528	64.82	0.047
				82529	58.91	0.043
		82530	63.21	0.046		

^a Dose rates in milliGrays per day (mGy/d) were calculated for concentrations of ^{137}Cs only. The TK numbers refer to catalogue numbers for The Museum, Texas Tech University (Lubbock, TX, USA).

methods described by Chesser et al. [12]. Specific activities and concentrations in soil samples from the enclosures were $1,296 \text{ Bq g}^{-1}$ (1.71×10^{12} unstable atoms/g) for ^{90}Sr and $3,317 \text{ Bq g}^{-1}$ (4.54×10^{12} unstable atoms/g) for ^{137}Cs . Procedures necessary to determine uptake and accumulated dose rates in the enclosures are described in detail by Chesser et al. [16] and include animals from this study. The primary interest for the present study was to calculate the dose caused by the uptake of radionuclides for the period after the mice were introduced into the enclosures and to relate that dose to MN induction.

Micronucleus assay

Blood smears were returned to the laboratory at Texas Tech University and stored, stained, and scored as previously described [6]. Slides were randomized and coded for blind analysis by a single investigator, who analyzed 5,000 polychromatic erythrocytes per individual per sampling period.

RESULTS

Study Animals

All mice survived the study period and appeared to be in good general health at the termination of the study. With the exception of pregnant females, no weight fluctuations were observed in the mice during their captivity. A total of 11 young

were born in the enclosures. Radionuclide concentrations of mothers and their young are reported in Table 1. The gestation period of *M. musculus* is 21 d, so neonates collected after August 4, 1999 (C57BL/6), and August 11, 1999 (BALB/c), likely were conceived in the enclosures ($n = 11$). In addition to the 11 progeny born in the enclosures, 4 of the 10 female mice were pregnant at the termination of the study.

Dose accumulation

Accumulated dose estimates for ^{137}Cs and ^{90}Sr from internal and external sources are presented in Table 2 and Figure 2, a and b. Variation in the sample size is due to an inability to recapture an animal without subjecting it to undue stress or excessive disturbance to the enclosure.

Internal dose from ^{137}Cs

The number of unstable atoms of ^{137}Cs is expected to approach a constant (i.e., asymptotic) value with time. Therefore, the asymptotic value for the number of unstable atoms of ^{137}Cs per gram of tissue was calculated according to the method described by Chesser et al. [16]. The uptake rate, representing the daily loss rate by physical decay and biological processes, was also calculated for each strain. Using calculated uptake rates and dose accumulation, internal dose rates could be es-

Table 2. Micronucleus (MN) frequencies and accumulated dose estimates from internal and external ^{90}Sr and ^{137}Cs in BALB/c and C57BL/6 strains of *Mus musculus* exposed to radiation in the Red Forest near the Chernobyl Nuclear Power Plant, Ukraine^a

Day	Strain	Mean % MN	Internal dose ^{90}Sr (mGy)	Internal dose ^{137}Cs (mGy)	External dose (mGy)	Total dose (mGy)
0	BALB/c ($n = 8$)	0.24 (± 0.25)	—	—	—	—
	C57BL/6 ($n = 8$)	0.29 (± 0.15)	—	—	—	—
10	BALB/c ($n = 8$)	0.29 (± 0.21)	3.51	29.29	333.3	363.11
	C57BL/6 ($n = 7$)	0.39 (± 0.16)	7.23	25.29	333.3	365.82
20	BALB/c ($n = 8$)	0.23 (± 0.17)	13.92	74.68	666.6	755.2
	C57BL/6 ($n = 6$)	0.29 (± 0.15)	28.64	71.82	666.6	767.05
30	BALB/c ($n = 8$)	0.32 (± 0.17)	30.99	131.26	999.9	1,162.16
	C57BL/6 ($n = 7$)	0.27 (± 0.15)	63.79	126.23	999.9	1,189.92
40	C57BL/6 ($n = 7$)	0.24 (± 0.16)	112.28	183.57	1,333.2	1,629.05

^a Dose estimates in milliGrays (mGy) were calculated according to the methods described by Chesser et al. [12,16]. Standard error is shown in parentheses.

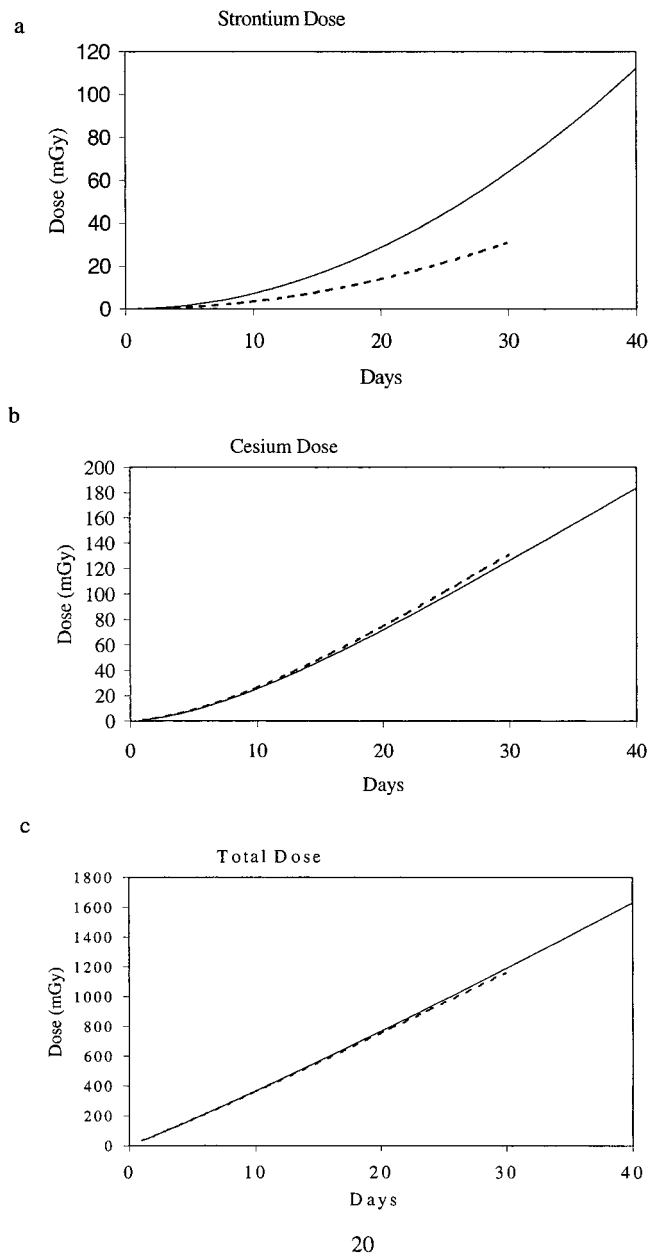


Fig. 2. Dose estimates for BALB/c and C57BL/6 strains of *Mus musculus* during exposure to the Red Forest environment near Chernobyl, Ukraine, from (a) ^{90}Sr , (b) ^{137}Cs , and (c) combined internal and external sources of radiation. The solid line represents the expected dose accumulation; the dashed line represents BALB/c and C57BL/6 dose accumulation.

estimated for the two strains of mice [16]. The accumulated dose from intramuscular ^{137}Cs for the study period averaged 131.26 mGy in BALB/c and 183.57 mGy in C57BL/6 mice.

Internal dose from ^{90}Sr

Concentration values for ^{90}Sr were determined using the specific activity measured in Bq g^{-1} (i.e., the number of nuclear disintegrations per second per gram of tissue) [16]. Activities for the two strains of mice were 1,826 Bq g^{-1} for C57BL/6 and 742 Bq g^{-1} for BALB/c. Using the methods described by Chesser et al. [16], the uptake rates measured in number of unstable atoms per gram were determined. The uptake rates were then used to calculate the accumulated internal dose from

^{90}Sr for a given time period. Approximately 30.99 mGy of ^{90}Sr in BALB/c and 112.28 mGy of ^{90}Sr in C57BL/6 mice were incorporated during captivity in the enclosure.

Internal dose rates for ^{137}Cs and ^{90}Sr are presented in Table 2. Doses from internally deposited ^{137}Cs and ^{90}Sr during the enclosure study totaled 162.26 for BALB/c and 295.85 mGy for C57BL/6 mice.

External dose

External dose rates, calculated from ^{137}Cs and ^{90}Sr in the soil [16], are presented in Table 2. The accumulated external doses during the study period from ^{137}Cs and ^{90}Sr were estimated at 999.90 and 1,333.20 mGy for 30 and 40 d, respectively.

Total dose

Total dose and the contributions from ^{137}Cs and ^{90}Sr are presented in Table 2 and in Figure 2c. The total dose resulting from internal and external sources of radiation was 1,162.16 mGy for BALB/c and 1,629.05 mGy for C57BL/6 mice during exposure to the Red Forest environment.

Micronucleus frequencies

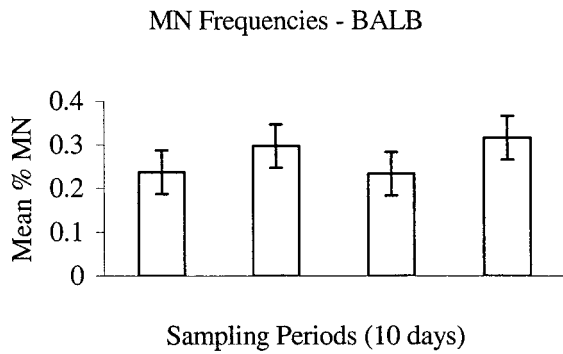
The initial frequency of micronucleated polychromatic erythrocytes averaged 0.24% ($\pm 0.15\%$, SE) for BALB/c and 0.29% ($\pm 0.25\%$, SE) for C57BL/6 mice. Frequencies for day 10 samples averaged 0.29% ($\pm 0.21\%$, SE) for BALB/c mice and 0.39% ($\pm 0.16\%$, SE) for C57BL/6 mice. Although a higher MN frequency for both strains was observed in the day 10 sample, pairwise *t* test showed no statistically significant fluctuation from initial frequencies (*t* test, $p > 0.05$) (Fig. 3). However, subsequent analysis using a nonparametric test, χ^2 , showed MN in C57BL/6 mice to be significantly elevated at day 10 ($\chi^2_3 = 13.41$, $p = 0.003$). The change in MN from the initial to the day 10 value in BALB/c mice was not statistically significant ($\chi^2_1 = 1.81$, $p = 0.178$). However, for BALB/c mice, a statistically significant change in MN frequencies was observed across time periods ($\chi^2_2 = 10.38$, $p = 0.006$) due primarily to fluctuations between each sampling period. Mean MN frequencies for each sampling period and corresponding specific activity levels of ^{137}Cs are presented in Table 2.

DISCUSSION

In a previous study, we did not observe any increase in MN frequencies in a population of voles living in the Red Forest region near Chernobyl [6]. One possible explanation for our observations in these resident voles was that intense selective pressure from living in a contaminated environment [25] had resulted in a population of radioresistant voles. An alternative explanation might be that initiation of enhanced repair mechanisms may have occurred in response to continual exposure to an environmental contaminant. To further address this question, we exposed naive voles to the Chernobyl environment [17]. No increase in MN formation was observed in the naive voles from subchronic exposure to Chernobyl radiation. *Clethrionomys glareolus* had been reported previously to demonstrate increased MN frequencies in response to much smaller doses of radiation [26]. Even so, we began to question whether *C. glareolus* was an appropriate sentinel species for evaluation of low-level radiation contamination. The results of these investigations [6,16,17] led us to design the study reported here.

We chose the BALB/c and C57BL/6 strains because of their known sensitivity to acute doses of radiation [1,3,7]. Our null

a



b

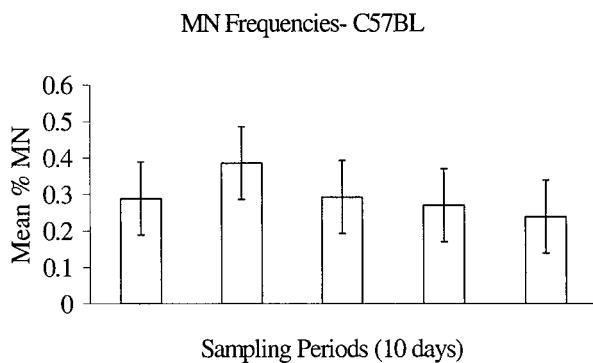


Fig. 3. Mean percentage micronucleated (MN) polychromatic erythrocytes for (a) BALB/c and (b) C57BL/6 strains of *Mus musculus* placed in environmental enclosures in the Red Forest of the Chernobyl, Ukraine, exclusion zone and sampled every 10 d.

hypothesis was that these mice would exhibit increased chromosome breaks from exposure to radiation in the Red Forest. The genetic response of laboratory strains of *M. musculus* exposed to the Chernobyl environment was similar to that of native rodent species of the Red Forest that are the product of multiple generations of exposure. Such comparisons are important when results from laboratory animals and those from resident sentinel species are being used in environmental risk assessments.

We did not observe a strong genotoxic response, but we did observe a significant increase in MN in C57BL/6 mice after 10 d and in BALB/c mice after 30 d. The statistical significance of the observed trends in MN induction in *Mus* is suggestive of a greater sensitivity to subchronic, low-dose radiation than we had observed previously in native rodent species [6,17]. Therefore, the BALB/c and C57BL/6 strains of *Mus* appear to be a more appropriate sentinel when examining short-term response to low-dose environmental radiation contamination. The apparently ephemeral MN induction, followed by a return to initial frequencies, observed in the C57BL/6 strain may be indicative of induced repair in those mice. Further studies are required to determine if this is the case. Also of concern are the long-term consequences of the chromosome breaks, represented by MN. Studies employing chromosome painting are currently underway in our laboratory to determine whether the MN observed represent un-

stable chromosome breaks (i.e., of little consequence to long-term health) or stable rearrangements (i.e., indicative of damage at the progenitor cell level).

The two strains of mice examined in our study incorporated radionuclides at almost identical rates during their captivity. Observed differences in the uptake of ^{90}Sr between the two strains of *Mus* likely were caused by two factors. First, difficulties existed with the initial assumptions of the model in estimating asymptotic concentrations and, therefore, uptake rates for ^{90}Sr over a period as short as 30 to 40 d [16]. More than 750 d of exposure may be necessary for ^{90}Sr levels to reach equilibrium in introduced mice [16]. Therefore, error may be introduced when estimating uptake values over short time frames, as in the enclosure studies. Alternatively, the two strains may have some currently unrecognizable difference in their assimilation efficiencies of ^{90}Sr . Regardless, the experimental design employed here allows for the use of other traditional laboratory strains, as well as transgenic strains of *Mus*, as sentinels for environmental contamination, including radioactivity.

The dose rates presented here and estimated by Chesser et al. [16] are far in excess of the International Atomic Energy Agency recommendation of 1 mGy d^{-1} as the upper limit for terrestrial vertebrates [27]. If the impact of radiation dose is cumulative, as is widely held, then detrimental effects from such doses would be expected, because a mouse exposed to the Red Forest environment would receive a total dose of more than $14,016 \text{ mGy year}^{-1}$. Although this annual dose exceeds the LD50/30 ($\sim 5.5\text{--}6.0 \text{ Gy}$) if administered acutely, similar doses have been documented for free-ranging rodent species in the region with no observed increase in mortality [12]. Furthermore, the scientific literature indicates reproductive inhibition would be expected at these doses [28]. Instead, *Mus* successfully bred and reproduced in this environment. Free-ranging resident species of the Red Forest, as well as naive *C. glareolus* relocated from reference sites, have also been reproductively successful while exposed to similar or even greater doses of radiation [17].

Historically, the effects of radiation have been investigated either in laboratory studies with inbred strains of *Mus* or in environmental field studies with native mammal species. Laboratory studies using fractionated doses attempt to document effects from other-than-acute exposures, but large doses are still administered. However, a paradox exists in the use of data generated from laboratory studies to estimate risk for exposure and remediation of environmental contamination. So-called low-dose laboratory studies differ from chronic field exposures in several ways. Low-dose laboratory reports typically use doses higher than are possible during a lifetime of exposure to any contaminated site in the world [29–31]. Radiation doses are often fractionated in laboratory studies as well, thereby differing from acute doses, but such doses are not continual, as in a radionuclide-contaminated environment. Additionally, animals in radionuclide-polluted environments are receiving doses from both internal and external sources. The radionuclides present and their associated energies (alpha, beta, gamma) can vary greatly between contaminated environments. Therefore, if laboratory studies are to be used to estimate the risk in natural environments of elevated radiation, or in environments contaminated from anthropogenic sources, efforts should be made to investigate environmentally relevant levels of radiation, and those doses should be administered in a fash-

ion that closely mimics those experienced by organisms living in contaminated environments.

Our results document that these two strains of *Mus* can survive and reproduce in environmental enclosures in the most radioactive area adjacent to Reactor 4 of the Chernobyl Nuclear Power Plant. Although mammal species vary in sensitivity, mammals are the most radiosensitive group of organisms [31,32], and mammalian model systems are the most suitable tests to evaluate the potential for human health effects from exposure to ionizing radiation. Because data generated from studies of *Mus* are commonly used to estimate multiple risk factors in humans, to predict potential disease states, or to assess toxicity of various compounds, we wanted to assess the utility of using inbred strains of *Mus* as sentinels in situations where environmental contamination is a concern. We combined laboratory and field approaches by exposing two radiosensitive strains of *Mus* to the environmental radiation contamination resulting from the Chernobyl meltdown. The experimental design employed could also be used to explore the effects of exposure to other contaminated environments. Experiments of this design have the value of providing a reference point for better understanding relationships between laboratory and field studies when examining the effects of mutagens on the benchmark mammalian model, *M. musculus*.

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