

LETTERS TO THE EDITOR

Comments on the Paper by Wickliffe *et al.* (*Radiat. Res.* **159**, 458–464, 2003)

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Wickliffe *et al.* (1) recently published data on mitochondrial DNA (mtDNA) heteroplasmy in laboratory mice exposed to a very low-dose-rate chronic irradiation in the vicinity of the Chernobyl nuclear power plant. Given that the issue of mutation induction by very low-dose-rate irradiation remains controversial (2), it may appear that the results of this publication could provide new insights with regard to the validity of the extrapolation of genetic risk from data on high/medium-dose-rate irradiation. Indeed, according to the authors, the main result of this study suggests that very low-dose-rate chronic irradiation with a cumulative dose of 1.5 Gy does not affect the mutation rate in somatic tissues and that therefore the current linear, no-threshold model of radiation risk (3) should be re-evaluated. It should be stressed, however, that mutation induction at mtDNA in the irradiated animals has never been proven experimentally, which raises serious concerns over the authors' claim.

The authors have analyzed nucleotide variation of mtDNA by sequencing the cytochrome b (*Mtcb*) gene in somatic tissues of inbred mice exposed to the Chernobyl environment. This technique differs drastically from all current *in vivo* approaches for mutation detection in somatic tissues that either employ genetic systems showing relatively high spontaneous and radiation-induced mutation rates (chromosome/chromatid aberrations, micronucleus test) or benefit from the possibility of selection of mutant cells (*Hprt*, *Tk*, *lacZ* assays). In contrast, the mutation rate at *Mtcb* is very low, and mitochondria carrying nucleotide substitutions at this gene cannot be selected for. That is why the procedure of PCR amplification of the bulk mtDNA samples and subsequent cloning of the amplicons used by Wickliffe *et al.* would almost certainly lead to a preferential detection of the most common variants of mtDNA with extremely low chances of detecting any *de novo* mutations. The results of previous studies clearly show that mutation detection at much more unstable tandem repeat DNA loci requires either the amplification of multiple diluted aliquots of DNA [small-pool/single-molecule PCR (4, 5)] or physical selection of rare mutants prior to PCR (6). Since none of these procedures were used by Wickliffe *et al.*, it therefore appears that mtDNA heteroplasmy found in the control and exposed mice should most probably reflect somatic mosaicism arising early in development or even inherited from previous generations.

Even supposing that some of the cytochrome b clones sequenced by Wickliffe *et al.* may have contained "recent" nucleotide substitutions, it still appears highly unlikely that this technique is sensitive enough to detect radiation-induced mutations in somatic tissues. It is reasonable to assume that the relative amount of radiation-induced DNA lesions in the mitochondrial DNA is similar to that in the genomic DNA. The results of numerous publications have shown that exposure to 1 Gy of low-LET X rays or γ rays results in approximately 3,300 initial DNA lesions per mammalian genome (~ 0.5 lesions per 10^6 bp for the diploid genome of 6×10^9 bp), including 40 double-strand breaks, 1000 single-strand breaks, 2000 damaged bases, 150 DNA-protein crosslinks, and 40 bulky lesions (7). Wickliffe *et al.* sequenced 141,600 bp of *Mtcb* copies in mice exposed to a cumulative dose of 1.5 Gy. Given this, the expected number of radiation-induced lesions for this target is about $1.5 \times (140 \times 10^3 \times 0.5 \times 10^{-6}) = 0.1$, and the probability of detecting a single

damaged target is therefore less than 10% (Poisson approximation, $P\{k \geq 1\} = 1 - e^{-0.1} = 0.09$). This implies that the target size analyzed by Wickliffe *et al.* is too small to be affected directly even by a single hit of low-LET radiation, thus providing the most likely explanation for the negative results in their study. The same is also true with the regard to another publication from this group (8). It should be noted that the results of recent studies suggest that the observed radiation-induced increases in mutation rates at some DNA repeat loci cannot be explained by the direct targeting of these relatively short genomic loci (9–11). However, it appears highly unlikely that mutation induction at the protein-coding *Mtcb* gene could be attributed to nontargeted mechanisms.

In conclusion, the negative results obtained by Wickliffe *et al.* are clearly attributed to the very low sensitivity of the technique used in their study and therefore, despite the authors' claim, do not imply that the current model of radiation risk should be even slightly re-evaluated. They also show that the analysis of mutation induction at mtDNA requires more sophisticated approaches for mutation detection.

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Response to the Letter of Y. Dubrova

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In response to Dubrova (1), our intent is to provide a cogent argument for the experimental design in Wickliffe *et al.* (2) and address concerns expressed by Dubrova in his letter.

The central question is “What are the actual, biological consequences of exposure to doses and, more importantly, dose rates of ionizing radiation that cannot be investigated in a controlled laboratory experiment?” (3). This question forms the core of recent efforts to explore the ultra-low-dose component of the current radiation effects models that are used to predict risk. A major weakness of the application of these models to low-dose-rate radiation is the lack of empirical substantiation. Under laboratory conditions, actual continuous, chronic exposure to low-dose-rate radiation is difficult to administer. The only alternative is to find those habitats where this type of exposure exists (3, 4). Our study sites near Chernobyl, Ukraine, represent such habitats. Dose rates, primarily resulting from ¹³⁷Cs γ rays range from a high of 33 mGy/day to background. We have carefully estimated micro- and macrogeographic dose rates in these areas, as well as individual absorbed doses, resulting from internal deposition of radionuclides and external irradiation, in small mammals (rodents and insectivores) collected or housed experimentally in these areas over the last 7 years (5–7). Unfortunately, accurate dosimetry has not been documented in other studies investigating mutagenicity in both animals and humans exposed to Chernobyl radiation. Because of the substantial interindividual variability we have observed in absorbed dose estimates in resident rodent species collected from the same location at the same time, relating “exposure” to Chernobyl radiation, in the absence of accurate dosimetry, with increased genetic mutations in barn swallows is tenuous at best, especially in a species that is migratory and vagile and does not reside in the Chernobyl environment for most of its life (8, 9). In addition, research investigating minisatellite mutation rates in humans is equivocal, and observed increases in mutation rates are more likely a result of the inadequate selection of a suitable reference population than of exposure to Chernobyl radiation (10–14).

To better understand the consequences of living in the Chernobyl environment, we examined somatic mtDNA mutations (site heteroplasmy) after chronic exposure to low-dose-rate radiation at Chernobyl. While we agree that this genetic model has not been adequately validated and is in need of controlled experimentation as suggested by Dubrova (1), we feel the theoretical foundation for examining mtDNA mutations is sound (15). For example, in some mtDNA-associated diseases, mutant mtDNA variants can be tolerated at levels up to 70% of the total mtDNA within cells before individuals exhibit symptoms; this includes diseases associated with cytochrome b gene nucleotide/site mutations (16, 17). This indicates that redundancy in this multiple-copy genetic system can tolerate an increased mutational pressure. In addition, mtDNA, while being monitored by virtually all of the DNA repair machinery save nucleotide excision repair, is repaired less efficiently than nuclear DNA (18). The mtDNA genome resides in an environment that is rich in reactive oxygen species (ROS) because of the electron-transport chain, and the architecture of the mtDNA molecule is relatively simple compared to the highly organized, histone-bound nuclear genome. This suite of attributes likely contributes to the increased substitution rate posited for this genome when compared to the relatively low substitution rate that is typical of most nuclear loci. These characteristics bring up two important points with respect to the concerns voiced by Dubrova (1). (1) Can the results from

the seminal synthesis provided by Frankenberg-Schwager (19) be extended and applied to mitochondrial DNA? Support for this extension remains untested. Therefore, the mathematical extrapolation of the “direct” number of radiation interactions calculated by Dubrova (1) is not justified. Furthermore, the values for each type of interaction, which comprise the number of DNA–protein crosslinks, nucleotide damage, single-strand breaks, double-strand breaks, and bulky lesions, were estimated from several *in vitro* studies exposing simple adherent or suspended cells to high, acute doses of radiation. This difference is important because in our experimental system we exposed a moderately radiosensitive strain of mouse (whole animals) to extremely low doses administered continuously, which represents a different and complex *in vivo* exposure (20, 21). Thus the extension of Frankenberg-Schwager’s (19) estimates to our results is questionable at best. (2) Direct interactions of radioactive energy or induced ROS cannot solely explain the increase in minisatellite mutations (22–24). These studies conclude that some aspect of the DNA repair complement is altered by radioactive exposure, and this is responsible for the significant increase in paternal mutation rate in these repetitive elements. However, this process, termed genomic instability, has not been investigated thoroughly, and the mechanisms by which radiation induces the phenomenon are unknown (25). If reduced DNA repair capacity is responsible, however, this should affect the mutation sensitivity of mtDNA. Of course, this depends on which repair pathway(s) is affected. For example, if base excision repair was diminished, this would theoretically amplify the substitution frequency above that predicted by Frankenberg-Schwager (19), assuming again that we had cause to apply those estimates to mtDNA damage.

Another concern raised by Dubrova (1) was our apparent lack of application of more traditional or standard markers of mutational insult. On the contrary, we have investigated genetic aberrations in several other markers including standard assays for mutagenesis. Several studies have used the peripheral blood micronucleus test, which is a standard assay in radiation research. Chronically exposed native resident rodents, naïve experimentally exposed native rodents, and experimentally exposed laboratory mice (C57BL/6, BALB/c, and Big Blue® [C57BL/6]) mice have been examined for the formation of micronuclei (26–28). Not one of these experiments documented a significant increase in MN formation in exposed mice compared to unexposed mice (26–28). Microsatellite instability, suggested by Ellegren *et al.* (8), has been examined in native resident rodents exposed to Chernobyl radiation (29). A total of 89 meioses were examined, and no *de novo* mutations were evident in the exposed offspring. Thus either barn swallows are uniquely radiosensitive at only a single microsatellite locus or the Chernobyl environment is not really causing genomic instability by disrupting mismatch repair as suggested. Mismatch repair efficiency has not been measured with respect to exposure to Chernobyl radiation.

Finally, we recently examined somatic transgene mutations in Big Blue® *lacI* mice experimentally exposed to a chronic (90 days) cumulative external dose of 3 Gy (¹³⁷Cs γ radiation). It has been demonstrated that an acute dose of 1–3 Gy γ radiation (¹³⁷Cs) induces a significant approximately fourfold increase in the transgene mutant frequency in this same strain (30, 31). No significant differences in the frequency of mutations or specific types of mutations were evident when mice exposed to a chronic dose of Chernobyl radiation were compared to unexposed mice (32). Taken together, and with carefully reconstructed individual doses, our research indicates that any potential mutagenicity resulting from exposure to radiation at our Chernobyl sites is not equivalent to that observed after comparable acute doses. This conclusion is compatible with the lack of mutagenic response evident in our studies of mtDNA somatic mutations (2, 33, 34).

We do not question the mutagenicity of high, acute exposures to ionizing radiation. This research is well documented and supported by several genetic markers, including minisatellites (22–25). The linearity of response over a log range of high, acute doses is unquestioned as well. However, we do question the extension of the empirically derived functions below, and for that matter above, the limits that have been tested experimentally. For our research, we are well above typical background

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levels of radiation exposure rates, but well below the lower limits of the acute doses and dose rates used to generate the dose–response curves that are supported experimentally. Our results indicate that the cumulative effects predicted by these models are inconsistent with the experimental observations of mutagenicity after equivalent cumulative doses administered at disparate rates.

Finally, we feel the discourse expressed herein reflects the best in scientific spirit and serves to refine the focus on truly low-dose radiation research. We commend Dubrova for engendering this spirit and suggest that mutual exchanges of archived genetic material be made to fully explore other potentially relevant markers of mutational insult. It is clear that a single genetic end point cannot unequivocally address the biological significance of chronic, continuous exposure to low levels of ionizing radiation. By applying collaborative efforts, the goals of low-dose radiation research can be met more quickly and comprehensively.

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Comments on the Responses of Utteridge *et al.* (*Radiat. Res.* 159, 277–278, 2003) to Letters about their Paper (*Radiat. Res.* 158, 357–364, 2002)

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The answer of Utteridge *et al.* (1) to three letters (2–4) commenting on the article “Long-term Exposure of $E\mu$ -*Pim1* Transgenic Mice to 898.4 MHz Microwaves does not Increase Lymphoma Incidence” (5) is insufficient and raises new questions.

The attitude of the authors vis-à-vis the issues that have been raised in these comments cannot be accepted. They state: “We are particularly puzzled by the criticism of our study, the results of which are consistent with the remaining body of evidence worldwide, while the study of Repacholi *et al.* remains the anomaly” (p. 277). The reader will not find a single word of criticism in these comments, although criticism would in fact have been more than justified, considering the insufficient presentation of the study. Concerning the body of evidence the authors are alluding to, it must be stressed that the consistency is with respect to the shortcomings inherent in many of them. One of these shortcomings is the fast decline in survival. There is no room here to discuss this in detail. In short, the main problem of long-term animal studies is the necessity to expose at very low levels to avoid interference with heating. At low levels, exposure is expected to raise background rates only moderately (i.e. by a factor of 2 to 4), if at all; otherwise there would be no controversy, because sufficient data for humans would be readily available. Typically the cancer rate in wild-type laboratory animals is low, too low to consistently detect such moderately increased risks. Hence models are used that guarantee an increase in background rates (cancer-prone strains, genetically engineered animals, induction models, implantation of tumor cells). However, there are two conditions for these models concerning sufficiency to study the effects of long-term low-level exposure to EMFs: The rate of spontaneous tumors must not be higher than about 40%, and the decrease in survival must start late and be slow. The first condition is related to power considerations, while the latter is based on the fact that we presently do not know at what stage of carcinogenesis EMFs might exert an effect. Interference at all stages of carcinogenesis is possible, but due to the low level of exposure we must assume either an accumulating mechanism or dose protraction enhancement effects. Hence sufficient time has to be allowed for the exposure to produce an effect. In this light, the comments on the increased background rates and steep decline in survival observed by Utteridge *et al.* (5) in sham-exposed animals is not marginal but refers to a central point of the assay.

The assumption that immobilization stress might have been responsible for the increase in spontaneous rates was well founded by evidence [note that one of the first experiments conducted in this area had demonstrated equivalence between EMF exposure and immobilization (6)]. However, this assumption can be tested by comparison of cage controls with sham-exposed animals. In their response (1), the authors show data on incidences in cage controls that are as high as those reported for sham-exposed animals. While the authors may feel that this strengthens their conclusions, it actually calls the whole experiment into question. First, a spontaneous rate of 80% in cage controls violates the power conditions for the experiment; second, it reveals a fundamental problem that relates to the definition of the end point of the study. The scientific community surely assumed a consistency in definition of end point in the studies of Repacholi *et al.* (7) and the “replication” experiment (5). Repacholi *et al.* (7) submitted mice to pathology if they were found dead or showed abnormal conditions that were considered life-threatening or were causing significant distress to the animals. Hence their end point was related to death or terminal stage of illness and counted if necropsy demonstrated lymphoblastic or non-lymphoblastic lymphoma. Although it would have been better if the earlier study had submitted all mice still alive at termination of the study to necropsy, inclusion of these data would have been prohibited by the definition of the end point, because in this strain

histological signs of malignancy developing in lymphatic tissues might be observed in virtually all animals at this age. This distinction is of utmost importance because the experimental paradigm is stage insensitive; i.e., it cannot differentiate between an effect of an increased likelihood of initiation, an earlier onset of initiation, and an increase in growth rate. Therefore, lumping together data from animals at different stages of illness must be avoided. In this light, extension of the study duration to 24 months also seems problematic. Although the decision to expose for 18 months in the prior experiment (7) was well reasoned, it already exposed far beyond the age for which data on other exposures and spontaneous tumors were available. Further extension of this period must be based on very good arguments, but none have been put forward by the authors.

Although it appears that because of the equality of lymphoma incidence in cage controls and sham-exposed animals, immobilization is not likely to be the cause of the observed increased spontaneous rates, these figures are insufficient considering the discussion in the last paragraph. In their reply, the authors stated correctly that “. . . focusing only on the final mortality incidences at the different exposure levels ignores the natural history of the disease. . . .” (p. 276); however, in this respect the data presented so far are completely insufficient. Survival curves are relevant to assess possible differences in longevity and to consider confounding by concurrent causes of death but are not of direct importance to the study’s objective. The authors should provide graphs of cumulative lymphoma incidence for all groups of animals including cage controls (heterozygote as well as wild-type mice) by time to tumor (or more precisely, by time to tumor-related death or terminal illness). It should be noted, however, that their revised Fig. 1 is still not compatible with their original Fig. 2 and the statement about study onset.

The authors’ statement that by censoring their data at 18 months the apparent discrepancy between their study and that of Repacholi *et al.* (7) vanishes is unsubstantiated. They again neglected the difference between age at necropsy and time after randomization. Even a short look at Fig. 4 of the earlier report would have informed them that Repacholi *et al.* (7) exposed mice close to the age of 20 months and not 18 months. Furthermore, the relevant comparison is not survival but (cumulative) lymphoma incidence.

While the limitations due to logistics are appreciated, simply neglecting the difference between two 30-min exposures a day and a single daily exposure of 1 h is deemed inappropriate, in their response that they “doubt” this “. . . could have affected results significantly” (p. 277). Consider that fractionation of dose is used to enhance the efficiency of radiation therapy. Abundant results from chrono-toxicology that indicate significant time-of-day effects also cannot be neglected.

It is quite understandable that comments were focused on the differences between the studies of Repacholi *et al.* (7) and Utteridge *et al.* (5) because of the apparent discrepancies in the results. However, decisive data to assess differences in outcome were actually lacking, and the authors should therefore be encouraged to present these data in an unambiguous way.

However, this does not mean that information lacking in the original article (5) and not provided in the answer of the authors (1) is of minor importance. Therefore, the points still missing or unclear are listed below:

1. Exactly at what age did exposure of the animals commence?
2. At what time of day were the mice exposed (“morning” is too vague)?
3. What was the range of SAR values at each exposure level during the experiment (including variances introduced by growth of animals)?
4. Despite the well known characterization of the C75BL/6Ntac strain, are there data available that demonstrate consistency of its genetic features?
5. Is there an explanation for the exceptionally high lymphoma rate in the wild-type animals?

In addition to the data on cumulative lymphoma incidence requested above, the authors should compute relative tumor risks with data censored at age 20 months to compare their findings to that of the earlier study.

So far the credibility of the results has not been greatly enhanced by

the statements of the authors, but it is hoped that the perennial discussion will ultimately lead to clarity.

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