

# Changing Perspectives on the Genetic Doubling Dose of Ionizing Radiation for Humans, Mice, and *Drosophila*

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This presentation is devoted to comparing the frequency of radiation-induced mutations in humans, mice, and *Drosophila*. However, I reserve for a later presentation at this Symposium (Neel, 1999) a discussion of two recent challenges to the validity of the estimates I will be presenting (Dubrova et al., '96; Gardner et al., '90).

## MEASUREMENT OF GENETIC DAMAGE

Early on in the various studies of humans and experimental organisms that were directed at the genetic effects of radiation, the issue arose as to the most appropriate manner in which to present these effects, both to the scientific community and to a concerned public. One school of thought favored presenting findings simply in terms of risk of mutation per gene per generation per unit of radiation [in the early days, per roentgen (rad) or per roentgen equivalent man (rem), now per Gray (Gy) (100 rad = 1 Gy) or per Sievert (Sv) (100 rem = 1 Sv)]. A second school argued that inasmuch as (spontaneous) mutation was a fact of life, presenting "added" risk with no references to "customary" risk (from spontaneous events) failed to provide the necessary perspective, and that a more useful estimate was the "doubling dose." This is defined as the exposure to ionizing radiation (or other mutagen) that will produce the same frequency of mutation per generation as occurs spontaneously. It may be expressed either as the dose per gamete or the dose per zygote. Because radiation-induced rates may differ in the male and female, the zygotic doubling dose is not simply twice the spermatogonial doubling dose. In the studies in Japan on the children of atomic bomb survivors, because we studied the results of the radiation of a total population, the estimate we have derived is the zygotic doubling dose. Although there have been, and remain, uncertainties concerning the total impact of spontaneous mutation on any animal population, most geneticists have an (intuitive?) feeling for this impact, and what doubling the impact might imply for a population. The "doubling dose" treatment has become standard, especially for across-species comparisons, and is pursued in this paper. As we shall see, simple though the concept is, the derivation of a figure that will include the impact of all aspects of the complex mutational process has proved very difficult. In particular, because it depends on a ratio, of induced to spontaneous rate,

each component of which has biological variability as well as an error of estimation, the error in the estimate of the doubling dose is apt to be large but indeterminate. The estimate of the doubling dose can also be influenced by such factors as stage of germ cell at irradiation, type of mutation scored, sex of animal, or population structure (humans) or precise research design (experimental organisms), and in across-species comparisons, one must employ similar indicators as far as possible (Muller, '59). In the decade following the atomic bombings, the uncertainties were such that no less a presence than Haldane ('55) could suggest that for humans, the doubling dose might be 0.03–0.05 Gy of acutely delivered ionizing radiation, suggesting that background radiation from natural sources might make a substantial contribution to so-called spontaneous mutations.

There are two especially troublesome issues in the comparisons to be made. The first is the differing radiation exposures across species at which doubling doses are derived. For instance, most of the *Drosophila* data were derived at acute exposures of approximately  $\geq 30$  Gy, whereas the mouse data were usually derived at exposures of 3–6 Gy. The average *combined* (mother and father) gonadal exposure of the atomic bomb-exposed parents of the children examined in Japan is currently estimated at 0.4- to 0.5-Sv equivalents, but with continuing uncertainty about the magnitude of the (highly effective) neutron component in the ionizing radiation. Because of the special interest in the human doubling dose, we shall, wherever possible in comparisons, use the "low-dose" data from experimental organisms. The second issue arises from the well-documented greater sensitivity of mature and maturing sperm than spermatogonia to ionizing radiation. As the human data are predominantly based on the irradiation of gonial-stage cells (or immature oocytes), we will also resort, wherever possible to similar data from experimental organisms.

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## HUMAN DATA

The principal corpus of data concerning the genetic effects of radiation on humans is derived from follow-up studies in Japan, under the aegis of the Atomic Bomb Casualty Commission–Radiation Effects Research Foundation. This has been the work of many investigators, both Japanese and American. I mention especially my long-time association in these studies with W.J. Schull. As of now, the reproductive performance of atomic bomb survivors living in Hiroshima and Nagasaki is complete. Most of the studies to be mentioned have been based on a cohort (or subset thereof) consisting of 31,150 children born to parents, one or both of whom were within 2,000 m of the hypocenter at the time of the bombing (ATB) and a matched comparison cohort of 41,066 children born to survivors beyond this distance, or to parents now living in either city but not there ATB. Over the years, data have been collected regarding these children ( $F_1$ ) concerning untoward pregnancy outcomes (major congenital malformation and/or stillbirth and/or neonatal death); sex of child; malignant tumors with onset before age 20; death of liveborn infants through an average life expectancy of 26.2 years, exclusive of death from malignancy; growth and development of liveborn infants; cytogenetic abnormalities; and mutations altering the electrophoretic behavior or function of a selected battery of erythrocyte and blood plasma proteins. In short, over the years, as wide a variety of endpoints with a genetic component have been studied as possible, in an effort to obtain a balanced view of the genetic effects of radiation. The parents have been assigned gonadal radiation exposures based on a formula developed by the Radiation Effects Research Foundation (RERF). Since very few of the children incorporated into this study were conceived within the first few months after the bombings, this is essentially a study of the effects of the radiation of immature germ cells. In a series of analyses that involved fitting a linear radiation dose-response regression model to the occurrence of the various indicators of radiation damage, the model including up to six concomitant variables depending on the indicators in question, there was no statistically significant effect of combined parental exposure on any of these indicators, but the net regression was slightly positive (summarized in Neel et al., '90; Neel and Schull, '91).

If we assume that despite the absence of a statistically significant regression of any of the indicators on parental exposure, the derived regressions are nonetheless the best current indicators of the genetic effects of radiation on humans, these regressions can be employed to generate a doubling dose. Because all the regressions evaluate the effects of essentially nonoverlapping indicators (i.e., are independent of each other) and are based on the same cohorts or subsets thereof, it is legitimate for the appropriate traits to combine these additively (Table 1). The sum of the individual Table 1 regressions for the qualitative traits was 0.00375 per Sv equivalent, with a considerable but difficult-to-

**TABLE 1. Summary of the regression of the various indicators on parental radiation exposure and the impact of spontaneous mutation on the indicator**

Trait	Regression per combined parental Sv	Contribution of spontaneous mutation
UPO	+0.00264 ± 0.00277	
$F_1$ mortality	+0.00076 ± 0.00154	0.0033–0.0053
Protein mutations	–0.00001 ± 0.00001	
Sex-chromosome aneuploids	+0.00044 ± 0.00069	
$F_1$ cancer	–0.00008 ± 0.00028	0.00002–0.00005
	0.00375	0.00632–0.00835

From Neel et al. ('90) in *American Journal of Human Genetics*, 46:1053–1072, published by the University of Chicago. Reprinted by permission of the University of Chicago Press. © 1998 by The American Society of Human Genetics. All rights reserved.

define error. This regression should apply to the mutational component in our indicators; we estimated that each generation of newborns, something like 6.3–8.4 infants per 1,000, would ultimately exhibit one of the qualitative indicators under study because of spontaneous mutation in the parental generation. The estimate of the doubling dose—in this case, a zygotic doubling dose—falls between 0.0063/0.00375-Sv equivalents and 0.0084/0.00375 Sv equivalents, or 1.7–2.2 Sv equivalents, with a poorly defined, but certainly large, error term. I want to emphasize our awareness of the sources of error in this estimate (Neel et al., '90) but, at the same time, also emphasize our belief that the assumptions that entered into this estimate were on the conservative side. The lower boundary to this estimate, at the 95% probability level, is about 1 Sv equivalent (Neel et al., '90) but, in the absence of a statistically significant difference between the children of the exposed and the control children with respect to any of the indicators, an upper bound to the estimate cannot be computed. Thus, it cannot be excluded, on a statistical basis, that the true zygotic doubling dose for humans is as much as 4- to 5-Sv equivalents. This degree of uncertainty is unfortunate but inherent in the situation. Furthermore, the somewhat lower socioeconomic status of parents exposed to the atomic bombs in the first decade after the bombings may have inflated the apparent genetic effect of the parental exposures (Kato et al., '66), as would any underestimate of the neutron exposures, with both factors biasing our estimate downward.

## DOMESTIC MOUSE DATA

Although my assignment for this symposium was to summarize the studies on the children of atomic bomb survivors, recent developments regarding the parallel studies on mice should be brought into the picture as well. While the human data have been accumulating, the mouse has served as the principal human paradigm for the genetic effects of radiation. The doubling dose

estimate developed above was substantially higher than most of the extrapolations from the experiments with mice undertaken by various national and international bodies. When this discrepancy became apparent, Susan Lewis and I (Neel and Lewis, '90) undertook to review the totality of the mouse data. Unfortunately, for reasons we have discussed in some detail in that earlier review (most notably, the immaturity of the mouse fetus at birth and the intra-litter competition effect both before and after birth), although effects of paternal radiation on the frequency of congenital defects, stillbirths, and early survival were demonstrated in the offspring of radiated males, these data really cannot be compared directly with the human data.

The most appropriate data for comparison with the human data would seem to be the result of the various specific locus-phenotype test systems, although I am the first to admit the reservations to be attached to this comparison. By far the most influential of the specific locus systems has been the 7-locus test system of Russell ('51). So many important insights have issued from this system—e.g., the recovery of induced mutations from radiated female mice only in the first several post-radiation litters, the lesser genetic effectiveness of chronic as contrasted with acute radiation—that the system warrants special scrutiny. In his first report, Russell ('51) reported an average induced rate of  $2.6 \times 10^{-7}$  per locus per 0.01 Gy for spermatogonia, and a spontaneous rate of  $7.6 \times 10^{-6}$  per locus, concluding, after a review of the male *Drosophila* data then available (based on radiation of a mixture of male germ cell stages), that the mouse per locus rate was “considerably higher than that found in *Drosophila*.” (This statement was not in the context of a doubling dose.) On the basis of these and further data (Table 2), the estimate of the murine gametic doubling dose for this system was later set at 0.44 Gy. The only other comparable mouse-specific multiple locus data of this type are those of Lyon and Morris ('66, '69) who, on the basis of six different mouse loci, observed an induced rate after acute relatively high-dose radiation of  $7.6 \times 10^{-8}$ /locus/0.01 Gy, approximately one-third of the rate in the Russell experiments. Unfortunately, in the relatively small series of Lyon and Morris, no mutations were observed in the controls, so that no doubling dose can be computed.

The results from eight different attempts to develop data from which a radiation doubling dose for mice could be calculated, based on more-or-less specific locus (or specific phenotype) approaches, are shown in Table 2. Note the wide range in the various estimates, to which we found it impossible to attach errors in the usual statistical sense. Not shown there (because the data do not lend themselves to the calculation of a doubling dose) are the important results reported by Roderick ('83), who estimated for mice a per locus recessive lethal mutation rate in postspermatogonial cells per locus from ionizing radiation of only  $0.35 \times 10^{-8}$  per 0.01 Gy, whereas for the Russell 7-locus

**TABLE 2. Summary of the gametic doubling doses for acute, “high-dose” radiation of spermatogonia yielded by the various specific-locus/specific-phenotype systems developed in the laboratory mouse\***

System	Doubling dose (Gy)	Origin of treated males
1. Russell 7-locus	0.44	101 × C3H
2. Dominant visibles	0.16	Various
3. Dominant cataract	1.57	101/E1 × C3H/E1
4. Skeletal malformations	0.26	101
5. Histocompatibility loci	>2.60	C57BL/6JN
6. Recessive lethals (3 studies)	0.51	DBA
	0.80	1.77 C3H/HeH × 101/H
	4.00	CBA, C3H
7. Loci encoding for proteins	0.11	Various
8. Recessive visibles	3.89	C3H/HeH × 101/H
	Av. 1.35	

\*References to the sources of the data and the doubling dose calculations will be found in Neel and Lewis ('90). From Neel and Lewis ('90) in Annual Review of Genetics, 24:327–362. Reprinted by permission of Annual Reviews. Copyright © 1990 by the Annual Reviews.

system, the corresponding rate for all post-spermatogonial mutations was  $45.32 \times 10^{-8}$  per 0.01 Gy with approximately 80% of these mutations homozygous lethal. As Roderick pointed out, this was about a 100-fold difference, although the error term to be attached to his estimate was large but difficult to calculate.

The simple average of all the estimates in Table 2, unweighted because we could think of no good way to weight the individual studies, was a male gametic doubling dose of 1.35 Gy, with an indeterminate error. There are several reasons to approach this estimate with caution. First, the data from many of the systems used in Table 2 are absolutely minimal for the generation of a doubling dose. The Oak Ridge data should dominate the estimate, forcing us to look at them with great care. Second, in his very first papers, Russell recognized that the assumption that the loci he studied were representative of the genome was key. There are now data for the mouse indicating a 7-fold range in the rate per locus with which spontaneous mutation results in phenotypic effects (Green et al., '65; Schlager and Dickie, '67). In Russell's data, radiation produced 18 times more mutations at the *s* locus than at the *a* locus, surely a signal to extrapolate with caution (reviewed in Searle, '74). Furthermore, in a somatic cell mutagenization experiment in our laboratory using the TK6 line of human lymphocytoid cells and employing ethylnitrosourea as mutagen, the protein products of 263 loci were scored for the occurrence of mutants resulting in electrophoretic variants, employing a two-dimensional polyacrylamide gel system (Hanash et al., '88). Ten of the 263 loci whose protein products were being scored



were known to be associated with genetic polymorphisms. The induced mutation rate at these 10 loci was 3.6 times greater than at the monomorphic loci, an observation with a probability of  $<0.004$ . The relevance of all these observations to the possible bias in the Russell system was that to set his system up, Russell drew on loci characterized by genetic variation. There had to be at least two alleles known for each of the loci in his system, and it helped in creating the optimum phenotype for scoring if there were even more alleles available to choose among. This use of loci for which variants were readily available introduced a bias toward higher mutability.

Third, the mouse doubling-dose estimates of Table 1 are male based. The demonstration (Russell, '65) that although the offspring of radiated female mice exhibited about the same amount of genetic damage as the offspring of radiated males in the first few litters after treatment, there was no apparent damage in the later litters of these females, created a dilemma for risk setting. Was the human female similar to the mouse female in this respect? To be conservative, in extrapolating to the human situation, the mouse male-derived risks have usually been applied to both sexes. The zygotic doubling dose implied by these data would thus become 2.7 Gy but, because of the lack of induced mutations in the late litters of females, this is almost certainly an underestimate of the mouse zygotic doubling dose. By contrast, in the Japanese data, radiated females sustained about half the total population dose on which the doubling dose estimate is based.

The fourth reason that the murine-based estimate of 1.35 Gy may be conservative is the apparent failure in the past to include either the observed clustered mutations or the mosaic mutations encountered in the studies of the Russells in the doubling dose estimates derived from their data by various groups. L.B. Russell ('64) described some 40 specific locus mutations, which in the course of the experiment at Oak Ridge, occurred in the offspring of both irradiated and control mice as clusters of two or more. Specifically, "21 had one irradiated parent and 19 came from a contemporary control population of slightly smaller size." It is unclear how many of these occurred in the basic 7-locus series, which provided the mutation rates quoted above. More recently, Russell and Russell ('96) have also described a series of some 37 mosaic mutants that appeared in the  $F_1$  of both radiated and control mice, none of which has apparently been incorporated into the doubling dose calculations of the past that used the Russell data. In a brief abstract, Selby ('96) recently suggested that because of the failure to incorporate clusters into the calculations, "the size of the doubling dose has been underestimated by at least a factor of three." No similar estimate is yet available for the effect of noninclusion of the mosaic mutants, but it could be a factor of two. These clusters, apparently reflecting a relatively high mutation rate in the "perigametic—very early zygote" interval (Muller, '59), are well documented in humans

and *Drosophila* and have been, by purpose or default, included in past doubling dose estimates for these species (reviewed in Woodruff and Thompson, '92). The *Drosophila* data, however, suggest that only some 40% of all spontaneous mutations occur as clusters, so that while their omission from a calculation of the doubling dose would have biased the estimate downward, it would not be by a factor of three.

As a population geneticist working with a nonexperimental organism, I do not enjoy the luxury of manipulating my material as does the experimentalist, a luxury that can, however, lead to oversimplification of a complex issue. From the population standpoint, there are both theoretical and practical reasons why cluster and mosaic mutations must be properly incorporated into the doubling-dose issue. First, when Mother Nature goes to work on a newly fertilized egg carrying a mutant gene not present in either parent, she (or, more technically, the process of natural selection) does not ask exactly when and how that mutation originated. She scores the totality of all the newly arisen mutations represented in the zygote, which is what we have, in effect, attempted to emulate in the study in Japan. She does not stop to ask whether the mutation occurred as a member of a cluster. Second, in the past, clusters have certainly been included in the studies on radiation-induced sex-linked lethals in *Drosophila*. Meaningful comparisons between the large body of *Drosophila* data and the mouse data are impossible without including the clusters in the mouse data. The same is true, in principle, for comparisons involving the human data, in which the small sibship size makes cluster detection more difficult than for mice and *Drosophila*. Third, although the frequency of clusters may not be altered by radiation under the special circumstances of the design of the Russell study, with the radiation usually delivered at 12 weeks of age, in the human experience, such as the exposures from the atomic bombs or the Chernobyl disaster, exposure to both sexes is at all ages and all stages of gametogenesis or fetal development, including the period particularly susceptible to the occurrence of what will become "clustered mutations." Until the full body of the Russell data is laid out in an appropriate fashion, it is impossible to estimate the magnitude of the correction to the doubling dose estimate derived from the specific locus data, and we shall for now continue to employ the male gametic doubling dose estimate of 1.35-Sv equivalents developed by Neel and Lewis ('90) and derive the zygotic doubling dose by simply doubling the male estimate, knowing these estimates will almost surely be revised upward in the future.

#### ***DROSOPHILA* DATA**

In a parallel report (Neel '98) I have included a considered discussion of the genetic effects of ionizing radiation on *Drosophila*. The general impression among radiobiologists is that the mouse is "much" more sensitive to the genetic effects of radiation than *Drosophila*.

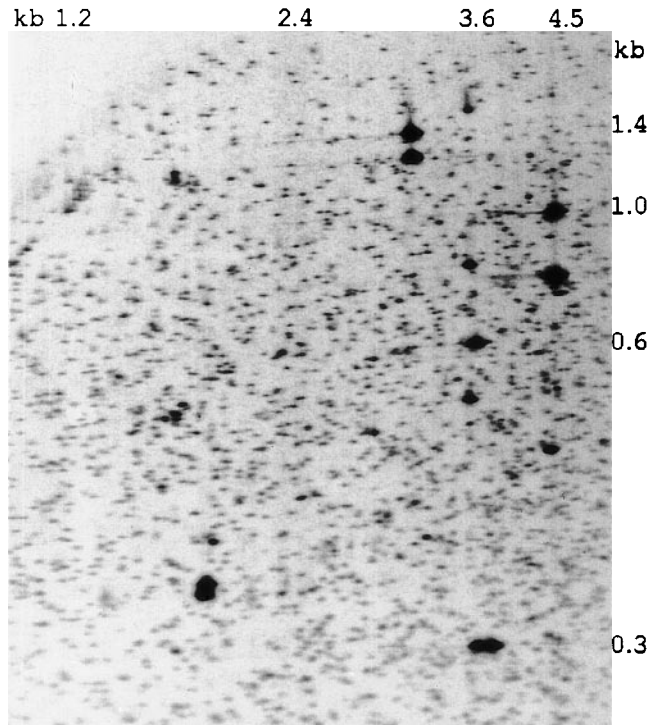
This stems largely from an early paper by W.L. Russell ('56) who, comparing his results with results of a *Drosophila* specific locus system developed by Alexander ('54), suggested the mouse exceeded *Drosophila* in sensitivity to induced mutation by a factor of 15. When, however, all the *Drosophila* data are surveyed, I develop a zygotic doubling dose for immature *Drosophila* germ cells of 10.0 Gy, resulting in a "sensitivity ratio" with respect to the mouse of 3–4, *Drosophila* still appearing to be somewhat less sensitive.

### PERSPECTIVE

The estimates that have been presented of the zygotic doubling dose of acute ionizing radiation for humans, mice, and *Drosophila melanogaster* are approximately 2.0-Sv equivalents, 2.7 Gy, and 10.0 Gy, respectively. Many assumptions have entered into these estimates. The errors are large, and there are reasons to suspect that the estimates for humans and mice are underestimates. Those who disagree are invited to tender their own estimates. The current genetic guidelines for human exposures to ionizing radiation would seem suitably conservative.

For some time, it has been apparent that despite the enormous differences in duration of life, number of germline cell divisions, mean body temperature, and reproductive strategies, the spontaneous mutation rates in *Drosophila*, mouse, and humans are "surprisingly" similar (reviewed in Neel, '83; see also Drost and Lee, '95). Now it appears that the amount of ionizing radiation necessary to double that spontaneous rate may also agree within a factor of approximately four or even less. These comparisons are still imprecise and soft but, if in general correct, will certainly call for many discussions in the future concerning radiation biology and evolutionary strategy. Why do such diverse organisms have such similar genetic doubling doses? Have higher eukaryotes "adjusted" to respond per generation to a mutagenic insult in proportion to their spontaneous mutation rate per generation, or is this to some extent a matter of gene or nuclear target size (Abrahamson et al., '73), but recall how much of the human genome is thought to be "junk" DNA? The *Drosophila* spontaneous rate per unit time would, of course, be much greater than the mouse rate. In turn, this is much greater than the human rate, but how and why, then, the "adjustment" (if this is not coincidence) that results in the same relative genetic response to ionizing radiation per generation?

It seems desirable to ask: Where do we go from here; how can we reduce the uncertainty in these doubling dose estimates? Figure 1 represents a two-dimensional display of an enzymatic digest of human genomic DNA in which the fragments have been isotopically labeled (Asakawa et al., '94, '95; Kuick et al., '95). About 2,000 DNA fragments can be visualized, varying in size from 1.0 to 5.0 kb in the first dimension and from 0.3 to 2.0 kb in the second dimension. Depending on gel conditions and the enzymes employed, a number of such prepara-



**Fig. 1.** Computer-processed image of a two-dimensional gel of enzyme-digested genomic DNA obtained from one of the lymphocytoid cell lines established at the Radiation Effects Research Foundation. See text for further description.

tions exhibiting largely different DNA fragments can be prepared from a single individual. To enter a genetic analysis, a battery of these fragments must exhibit positional and quantitative stability, which we have shown to be the case. The variation to be observed is of two types, quantitative or qualitative. Two types of genetic event create this variation, namely, insertions/deletions/inversions of the DNA and nucleotide substitutions altering the cutpoints of the enzymes employed for the DNA digestion. With respect to quantitative variation, if spots that are the product of two homologous DNA fragments are to be distinguished with the requisite accuracy from spots that are the product of only one fragment (the hallmark of a null mutation), the coefficient of variation (CV) of spot intensity should be approximately  $\leq 0.12$ . Some 500 of the spots in a single gel meet these standards.

In an examination of preparations based on three Japanese father–mother–child trios, some 10% of those 500 spots were found to exhibit variation that segregated within families according to Mendelian principles. We suspect that, with an expanded series, some 20% of spots will exhibit genetic variation. A mutation should consist in a variant present in a child not present in either parent. Given the technique employed, mutations resulting in quantitative variation should be detected substantially more frequently than mutations resulting in qualitative variation. Thus, the

technique should be especially efficient for the detection of deletions, the predominant type of radiation damage to DNA. We have already established that a variant fragment among the spots under consideration can be recovered and cloned and the DNA sequenced. Thus, although the identity of most of these spots is unknown, in time their sequences can be established and a match-up with data in the various Gene Banks should establish the identity of the gene or other type of DNA with which each is associated. Our program has already established sufficient sequence data for a match-up procedure for some 20 spots.

Two genetic pilot studies using this technique are now under way. The first involves a search at RERF for mutant DNA fragments in the offspring of male mice exposed to 5 Gy of ionizing radiation, under the direction of Dr. J. Asakawa. The second is a collaborative study between RERF and our laboratory, employing Epstein-Barr virus (EBV)-immortalized cell lines of father-mother-child trios, for approximately one-half of which trios one or both parents were exposed to the atomic bomb, the other half of the trios serving as controls. These studies should determine the efficiency of the approach and facilitate a decision concerning the feasibility of a full-scale study. Although we are convinced that this technique has much to offer, we also recognize that a major effort is required if this approach is to contribute materially to the understanding of the genetic effects of radiation on humans and mice.

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