Chapter 6

Laboratory Determination of Heritable Mutation Rates

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Chapter 6

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INTRODUCTION

The genetic information of all organisms is encoded in DNA (with the exception of some viruses that depend on RNA), and mutations have been found in all species that have been examined. The biological similarity of DNA structure and function in all species has allowed scientists to study mutation rates and mechanisms of mutagenesis in several species for over half a century. In addition to these efforts in basic research, in recent years the biotechnology industry has provided impetus, including financial incentives, for sophisticated inquiry into mutations and mutagenesis in bacteria and yeast. The industry's interest is in using mutagenesis to benefit from some of the particularly useful characteristics of certain microorganisms, such as the ability of some bacteria to digest oil, and the fermentation capabilities of various yeasts.

Another reason for studying mutations in lower organisms is to identify agents that cause mutations in their DNA, agents that might act similarly on human DNA. For instance, concern about human risks from the genetic effects of ionizing radiation led to the funding of extensive mouse studies, which have provided quantitative data on factors that influence the yield of various types of heritable mutations. In the case of chemicals, however, concern about cancer, not about heritable mutations, has been the primary motivation for developing short-term laboratory test systems to detect mutations. In the last decade, as more and more evidence incriminates somatic mutation as an early event in the development of cancer, the properties of mutagenicity and carcinogenicity have been conjoined, making identification of mutagens an important part of efforts to identify cancer-causing agents. Many of the tests for mutagens measure a chemical's capacity to alter the DNA of bacteria or yeast (145). While such a short-term test in a lower organism may distinguish between agents that can and cannot change DNA, it provides no information for making quantitative estimates of mutagenic potency in humans nor does it show that the chemical can necessarily reach animal or human gonads in an active form to cause heritable mutations (108). Whole animal tests specifically designed to detect germ-cell or heritable mutations provide some information of that type.

RODENT TESTS FOR HERITABLE MUTATIONS

At least 15 tests and variations on them are available for the detection and measurement of heritable mutations induced in mammalian germ cells. Lower organisms, notably Drosophila, are the subjects of similar tests, but most of the information about mutation rates comes from mice. The three most widely used rodent tests are described below (58,108).

The Dominant Lethal Test

The dominant lethal test detects mutations that cause the death of rodent embryos. The usual procedure in this test is to treat male mice with a suspected mutagen and then mate them with untreated females. If the treatment causes a mutation in a male germ cell that is lethal at some stage

in the development of the embryo (after fertilization), the lethal events are detectable by dissecting female uteri a few days after implantation of embryos (implantation occurs about nine days after fertilization). If there are more dead embryos than expected (compared with matings of unexposed mice) and/or more implantation sites than expected in the uterus where there is no longer an embryo, the effect is assumed to be the result of a mutation in the male germ cell. Chromosomal studies in mice have shown that almost all "dominant lethal" events are associated with major chromosome abnormalities.

Human analogs of the postimplantation fetal losses counted in dominant lethal tests are spontaneous abortions (miscarriages). Fifty to 60 percent of spontaneous abortions are associated with numerical and structural chromosome abnormalities, the same basic types of abnormalities detected by dominant lethal tests (12). These similarities between mice and men, and the general pragmatic acceptance of higher animals as predictors for human risk, underlie the idea that agents that are positive in a mouse dominant lethal test may also cause spontaneous abortions or chromosome abnormalities in liveborn infants.

The Heritable Translocation Test

The heritable translocation test detects breakage and rejoining of fragments from different chromosomes. When a translocation involves no loss of genetic information (a "balanced translocation"), often it has no adverse effect on the functioning of the carrier individual. The unusual chromosomes produced by the breaking and rejoining are present in all cells, and they can pair with each other and divide and function like normal chromosomes. However, such translocations cause reproductive problems because the germ cells may have incomplete sets of genetic material and may lead to unbalanced chromosome abnormalities in offspring, often resulting in death during embryonic or fetal development. Heritable translocations are thus detectable in mice because they result in reduced litter size in the second generation after exposure to the suspect agent, or in sterility in the first generation. In addition, cytogenetic studies can detect abnormal chromosomes and can be used to study the association between reduced fertility and chromosome abnormalities.

Specific Locus Tests

Since 1927, when Herman Muller first demonstrated that radiation causes mutations in fruit flies (8 1), there has been concern about possible genetic effects of radiation in human beings. Following World War II, in anticipation of widespread use of nuclear power, the Atomic Energy Commission (AEC) contracted for research on the mutagenic effects of radiation in mice to provide an experimental underpinning for estimating effects in human beings, Specific locus tests that detect mutations in a few specific genes in mice were developed for that purpose. This work and use of specific locus tests to measure chemically-induced mutations have provided the animal data most useful for estimating human risk.

Through research supported by the AEC, William L. Russell (109) bred a strain of "tester" mice that differs from wild-type mice in 7 morphological features—coat colors, patterns of pigmentation, eye color, and ear shape—that are recessive traits. Each of the features is thought to be controlled by a different genetic locus. Tester mice are homozygous for the recessive alleles of these genes, while wild-type mice are homozygous for the dominant alleles. When the tester strain is mated to a strain of wild-type mice, the offspring should all be heterozygous at the seven test loci, and therefore they should appear wild-type because the the wild-type alleles are associated with the dominant phenotype. However, if a germinal mutation occurs in a gene for one of the seven loci in the wild-type strain, causing the dominant allele not to be expressed, the offspring from that mutation will have a phenotype characteristic of the tester strain.

In a specific locus test, the wild-type mouse is exposed to radiation or to a known or suspected chemical mutagen and then mated to the tester strain. In most cases, only male wild-type mice are exposed, and the tester strain mice are female. The reason for this is largely a practical one: one exposed male can sire a large number of progeny

when mated to many females, but an exposed female can have only one litter at a time. In addition, the information so far available suggests that male germ cells are more sensitive to mutagens than are female germ cells. After mating exposed wild-type and tester mice, the offspring are examined for the easily seen morphological features related to the seven loci. Since the morphologic variants are readily identified, large numbers of offspring can be examined quickly by an investigator. The frequency of mutant offspring of exposed parents is compared with offspring of unexposed parents. The importance of the ease of recognizing mutants *is* underscored by the low frequency at which mutations occur spontaneously —as few as **50** mutations per 1 million offspring of unexposed mice. (Because mutations can be detected at any of the seven loci, examination of one mouse provides information about one observation at each of the seven loci.)

Estimates for the rate of spontaneous mutations in mice based on specific locus test results are summarized in table 8. These data are based on examining almost 1 million progeny of exposed mice, *or* about 7 million loci. Though the reliability of the estimate is limited by the small number of mutations seen, the calculated rate of between two and eight mutations per 1 million genes is not inconsistent with the human rate estimated by one-dimensional electrophoresis of blood proteins (see ch. **3).**

Although the "average" rate of mutations is a useful touchstone, it is used in the full knowledge that the average includes very different rates at the seven loci. For instance, there is a 35-fold difference in radiation-induced mutation rates between the most and least sensitive of these particular seven loci. The choice of a different set of loci for examination might yield quite different results.

Other Studies in Animals

There is a family of animal experiments called dominant mutation tests, which use the appearance of dominant features, such as skeletal anomalies or cataracts, to detect mutations (108). Many of the endpoints used resemble known human genetic disorders, and the results of these tests have been used to estimate human genetic risk.

In addition to the types of studies described above, some of the techniques used to study human beings have been applied to experimental animals. Electrophoresis of blood proteins and quantitative enzyme assays in human beings, described in chapter 3, have been used to study corresponding proteins in mice. The electrophoretic approach has been used in studies of mutagenized mice at the National Insitute of Environmental Health Sciences (51,52,53,63) and also at the Institute for Genetics, Neuherberg, Germany (19,19a). A small effort in electrophoretic analysis has also been conducted at the Oak Ridge National Laboratory (107). Similar studies have been reported in Drosophila (80,103,140,161).

Mutagenized mice have been tested for enzyme deficiency variants using quantitative enzyme assay techniques similar to those used to study human populations (19a,73). Enzyme assays have also been used in mouse and Drosophila experiments that involve selective mating, similar to the specific locus test strategy. By selecting parental strains that have electrophoretically distinct alleles at a locus, enzyme deficiency variants can be detected in offspring by the absence of one activity staining band that, in the absence of a

Table 8.—Spontaneous Mutation Rates in Mice[a]

Sex	Number of mutants	Number of progeny[a]	Mutations/locus[b]	Reference
Female	3 or 8[c]	204,639	2.1 or 5.6 x 10^{-8}	(1 10)
Male	39	727,319	7.6 x 10^{-6}	(1 13)

[a]Data about spontaneous mutation rates were collected from animals used as controls in experiments to determine the effects of radiation or chemical agents on males and females separately, There are more progeny from males because more experiments have been done in males
[b]A rate of 1×10^{-6} means that, on average, a mutation occurs once in a million loci
[c]The choice of three or eight mutants exemplifies a problem in counting mutations Three litters from female mice had mutant Off Spring Two litters had one mutant each, the third had six mutants, all of which may have resulted from a single mutation The result is three probable mutations and eight mutant offspring

SOURCE Office of Technology Assessment

mutation, would be inherited from one of the parents (51,52,103,161).

Data from animal experiments using electrophoretic and quantitative enzyme assays are limited, but at this stage a general, cautious conclusion can be drawn. The background mutation rate in the mouse derived from these experimental data is in the same range as the estimate of the spontaneous rate derived from studies in human beings (77), recognizing, however, that these estimates are based on small numbers and may be heavily influenced by chance fluctuations.

FACTORS THAT INFLUENCE INDUCED MUTATION RATES IN MICE

When radiation or a chemical is tested for mutagenicity, the experiment does not necessarily produce definitive answers to the questions: "Is this agent mutagenic'?" And "if so, how mutagenic is it?" The answers differ depending on several factors, two important ones being whether the animal is male or female and the stage of development of the exposed germ cell. There is ample evidence from radiation studies that male and female germ cells differ in their sensitivity to mutagens. In males, germ cells appear to be more likely to sustain a mutation the further along the developmental path they are. Overall, female germ cells appear to be more resistant to mutagens than are male germ cells.

Effects in Males

The male testis, from puberty on, contains a mixture of germ cells at different developmental stages, including spermatogonial stem cells, differentiating spermatogonia, and intermediate stage cells, the spermatocytes and spermatids, which are maturing into spermatozoa. The mechanisms of sperm production are qualitatively very similar in mice and men, differing largely in timing: in mice, sperm production from spermatogonia to spermatozoa takes 35 days; in men, it takes 74 days (60). When a male is irradiated, germ cells at all stages of development are exposed. By varying the times of mating, the effects of radiation on different stages can be examined. For instance, if male mice are irradiated and mated immediately, any mutants that appear in the progeny must result from mutations that were caused in fully mature sperm. In a mating two weeks after exposure, eggs are fertilized by spermatozoa that were spermatids at the time of irradiation. If seven or more weeks are allowed to pass, sperm in the ejaculate would have been irradiated at the spermatogonial stem-cell stage. Experimental evidences shows that cells at each stage have varying sensitivities to induction of mutations by radiation, the later, postspermatogonial stages being most sensitive.

While spermatogonial stem cells are more resistant to the effects of mutagens than are germ cells in later stages of development, mutations in stem cells are of greater concern. Stem cells persist throughout the lives of males, constantly giving rise to new generations of sperm cells. A mutation in stem cell DNA results in every cell that buds off from the stem cell bearing a mutation. The lifespan of later germ-cell stages constitutes only a small fraction of the total reproductive lifespan (especially in humans). Thus, in the case of acute exposure to these later germ-cell stages, only conceptions occurring during a short postexposure interval are at risk. For these reasons, the effects of radiation and some chemicals have been especially closely studied in spermatogonial stem cells in animals.

The most common way for people to be exposed to ionizing radiation is through acute exposure to medical X-rays, or long-term, low-level exposure to natural background radiation. Certain groups are exposed occupationally (e.g., uranium miners, nuclear powerplant workers, some medical personnel); and some may have special low-level environmental exposures from living near nuclear installations, nuclear waste sites, uranium mine tailings, or areas of high natural radon concentrations.

in mice, exposure of males to X-rays or other forms of ionizing radiation at low dose rates causes a measurable increase in the number of mutations, while such exposures do not measura-

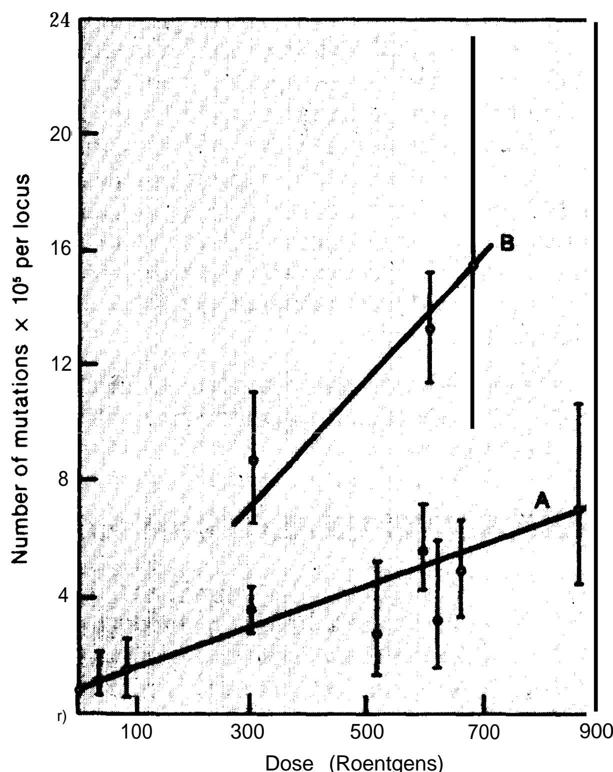
bly increase mutation rates in females. Russell and Kelly (113) counted the numbers of mutants caused by varying doses of radiation in male mice. They find no evidence for a dose so low that it is not mutagenic, but the number of mutations decreases with lower and lower doses (see fig. 13). The data in the figure also show the differing results of exposure at high and low dose *rates.* For example, the number of mutations caused by a total dose of 300 roentgens (R), I administered at a dose rate of between 72 and 90 R per minute is about 80 X 10^{-6} per locus; the same *total* dose administered at dose rates of 0.08 R per minute or lower causes fewer mutations, about 30 X 10-' per locus. These data may be interpreted by saying that low dose rates allow time for repair of some of the damage caused by the radiation. (For further discussion of dose and dose-rate relationships, see ch. 7.)

Effects in Females

Female mammals differ from males in the biology of germinal stem cells. Whereas male spermatogonial stem cells divide constantly during adulthood, females are born with their total complement of egg precursor cells. Shortly after birth, all cells destined to become eggs (ova) are present in the ovaries as "arrested oocytes. " Exposure of arrested oocytes to radiation has caused no increase in mutation rates in mice (110). During the six weeks before ovulation an arrested oocyte matures, undergoing meiosis and, in a mature state, travels from the ovary to the uterus. Irradiation during this maturation period does increase mutation rates in female germ cells.

Two different explanations can be advanced for the failure to recover mutations from irradiated arrested oocytes. First, it might be that arrested oocyte DNA is somehow protected from the mutagenic effects of radiation, perhaps by having very efficient repair mechanisms that correct radiation-induced mutations. On the other hand, in mice, arrested oocytes are very sensitive to the killing effects of radiation (27). These lethal effects could also account for the absence of mutagenic effects in female mice. It is argued that since

Figure 13.—Spermatogonial Mutation Rates in Mice After Low-Dose Rate and High-Dose Rate Radiation



Straight lines fitted to experimental results from a number of specific-locus tests involving single doses of radiation. Line A: **Radiation delivered at low dose rates (0.8 Roentgens/minute and lower). Line B: Radiation delivered at high dose rates (72 to 90 Roentgens/minute). The figure shows that a dose of radiation delivered at a faster** rate causes more mutations per locus than the same total dose of radiation delivered at a slower rate. (90°10 confidence limits are shown.)

SOURCE: W. L. Russell and E. M. Kelly, "Mutation Frequencies Male Mice and the Estimation of Genetic Hazards of Radiation in Men, " *Proceedings of the National Academy of Sciences* (U. S.) 79:542-544, 19s2.

radiation kills oocytes very efficiently, oocytes would either die before they were mutated or they would die with mutations that would never be expressed.

The extreme sensitivity to the killing effects of radiation in mouse arrested oocytes is not common to all animals. In the squirrel monkey, high sensitivity is limited to oocytes irradiated during fetal life. Arrested oocytes in other monkey species are no more sensitive to lethal effects of radiation than are other cells. Similarly, human oocytes, at least those that are irradiated after

[1] A measure of radiation. In human terms, a chest X-ray examination produces a gonadal radiation dose of less than 0.0005 R (148).

birth, appear resistant to the lethal effects of radiation.

The differing sensitivities of oocytes of different species to lethal effects of ionizing radiation complicate efforts to extrapolate from mutation rates in female mice to the expected rates in human females (28). In particular, radiation may result in more mutations in human females than would be predicted from female mice because human arrested oocytes are less likely to be killed by radiation. That might allow a human oocyte to survive a mutagenic dose of radiation and to be fertilized.

Using experimental data from female mice, Russell (1977) made several calculations relating effects of radiation in mice to potential effects in human females. In each calculation, he made the "worst case" assumption that all stages of female germ cells were as sensitive to the mutagenic effects of radiation as are the most sensitive stages—maturing and mature oocytes-in mice. Because there are different methods of handling the data, he made four different calculations. Three produced estimates indicating that radiation would not increase the mutation rate in human arrested oocytes. The fourth estimated that human arrested oocytes would be somewhat less than half as sensitive (from 17 to 44 percent as sensitive) to radiation as are human spermatogonia.

ESTIMATING EFFECTS IN HUMAN BEINGS FROM ANIMAL DATA

Most extrapolations from animal data to human mutagenic risks are based on studies in male mice because: 1) there are more data for male mice than for female mice, 2) there appear to be fewer biological differences in germ cell development between males of the two species than there are between females (see above), and 3) male germ cells appear to be more sensitive to radiation than female germ cells and may provide a more sensitive indicator of genetic risk.

The dose-response curves in figure 13 are derived from experiments on male mice and they provide information for estimating human risks. The absence of a detectable threshold for genetic effects in male mice suggests that humans are at some increased risk for mutations at any level of radiation exposure. This finding increases the importance of making quantitative estimates of the effects of radiation; since risk does not go to zero except at zero dose, what is it at levels of human exposure? The "doubling dose, " which is the dose of radiation that induces an additional number of mutations equal to the number that occur spontaneously (resulting from all mutagenic influences, known and unknown), can be derived from the information in figure 13. The doubling dose differs depending on the radiation dose *rate.* The doubling dose in male mice for high dose rates is about 40 R; for low dose rates, about 110 R. This is of practical importance for human beings, since some exposures are at high dose rates—e.g. the populations of Nagasaki and Hiroshima and people receiving radiation therapy for some types of cancer—but most population exposure to radiation is delivered at a low dose rate—e.g., people who live near radioactive mines. About three times as many mutations are caused by radiation delivered at a high dose rate as opposed to an equal total radiation dose delivered at a low dose rate (114).

In chapter 3, the effects on mutation rates of acute irradiation of the populations at Nagasaki and Hiroshima are discussed. The exact doubling dose for those populations is still being debated because of uncertainties about the total dose and because of insensitivity of the methods used to detect mutations. Using the best information available about those populations, however, humans appear to be less sensitive than mice to high dose rate exposures.

Current radiation exposures of United States citizens average about 200 millirem (mrem) per year. On a population basis, this exposure is generally divided about equally between high dose rate medical exposures and low dose rate exposures to natural sources of radiation. These exposures do not approach the doubling dose for

mutations. If the average rate of human mutations is now on the order of one mutation per 1 million genes per generation, we can estimate the effect of a hypothetical increase in human radiation exposure to twice the current level. If exposures increased to 400 mrem and if that exposure were as mutagenic as the high dose rate delivered to mice, it would increase the average human rate to 1.005 mutations per 1 million genes per generation. Such an increase would be undetectable by any current method.

Animal studies have proven useful for learning about the various relationships of dose, particularly for radiation, and germ-cell and heritable mutation rates. They are limited, however, in providing information directly applicable to human beings. First and most important, human beings differ from animals in ways that we know about and in more ways that are not understood. Second, the endpoints measured in animal tests do not generally correspond directly to endpoints measurable in human beings under nonexperimental conditions. Third, the available animal tests, like the currently available methods for studying human beings, detect mutations arising in specific, selected, loci which may not be representative of other loci. Evidence from Russell's specific locus test, cited above, shows a 35-fold difference between the least sensitive and most sensitive of the loci tested. A fourth consideration, again similar to the limitations of studies in humans, is that each test does not detect more than a fraction of the mutations that can occur, and the exact nature of the mutations that are detectable cannot be characterized with these methods.

When the new technologies discussed in this report are further developed, they presumably will be applied to experimental animals as well as to human beings. Studies that examine corresponding genes with the same techniques in humans and animals may provide some of the interspecies comparisons that now are lacking.