

Fertility, Reproduction, and Genetic Disease: Studies on the Mutagenic Effects of Environmental Agents on Mammalian Germ Cells

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Because genetically based diseases have a major impact on human health, the National Institute of Environmental Health Sciences (NIEHS) has conducted a research and testing program for more than a decade to address chemical induction of heritable genetic damage in the germ cells of mammals. Although most genetic disease results from preexisting mutations, a portion is due to the occurrence of new mutations. The supposition that exposure to mutagenic chemicals contributes to the occurrence of new mutations in the human population is strongly supported by the results from animal models. Such studies clearly demonstrate the potential of environmental chemicals to induce mutations in both somatic and reproductive cells of mammals. This NIEHS program has become a leader in the identification of genetic hazards in the environment and in the acquisition of animal model data used by regulatory agencies in assessing genetic risks to human health.

The Human Health Issue

Genetically based diseases have a major impact on human health. Of the nearly 5000 human gene loci exhibiting characteristics of Mendelian inheritance (*I*) (Table 1), many are associated with some degree of health disorder. Although the frequency of any single genetic disease is low, the total incidence of genetic disorders makes a significant impact on human health (2-5) (Table 2). Further, decreased infant mortality due to reductions in the toll of nutritional and infectious diseases has led to an increase in the contribution of genetic disorders to the overall human disease burden (9).

It has been estimated that 1% of liveborn infants express a single gene disorder and that as many as 0.6% are born with a major chromosome abnormality. An additional 1% may manifest a serious genetic disorder sometime after birth. Emphasizing the impact of genetic disease on childhood health is the estimate (10) that nearly 10% of pediatric hospital admissions in North America are for care and treatment of genetic disorders. A higher proportion of humans may exhibit late-onset disorders associated with polygenic traits as a result of preexisting or new mutations in any one of several genes. These mutations may lead to

Table 1. Number of human gene loci identified in McKusick's Ninth Edition (*I*).

Mode of inheritance	No. of loci
Autosomal dominant	1864 (1183)*
Autosomal recessive	631 (923)
X-Linked	161 (175)
Total	2656 (2281)

*Additional loci not fully identified or validated in parentheses.

Table 2. Incidence of genetic diseases in human newborns (6-8).

Mode of inheritance	Incidence per 1000 newborns
Autosomal dominant	1.4-10
X-Linked	0.4-0.8
Recessive	1.7-2.5
Multifactorial	47-90
Chromosomal	1.9-6.2

increased susceptibility to some forms of heart disease, diabetes, or cancer (*II*).

In a study by Baird et al. (4) with a sample of more than 1 million consecutive live births, almost 8% developed some type of genetically based disease before age 25. In a study on the human health impact of 351 Mendelian disorders compatible with survival beyond early childhood, Costa et al. (9) found that 25% of the disorders were apparent at birth and more than 90% by the end of puberty. In addition, life span was reduced in 57% of the disorders, and reproductive capacity was impaired in 69%. Treatment prognosis was found to be poor for most of these disorders, with only 10-15% of the phenotypes exhibiting significant improvement in response to treatment (*J2*).

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Likewise, the impact of chromosomal disorders is severe, and prognosis following medical treatment is poor. Of all recognized pregnancies (> 5 weeks gestation), 15% are lost before term; as many as 50% of these conceptuses have detectable chromosome anomalies (13) (Table 3). Loss of conceptuses before recognized pregnancy, which is estimated to be 30–50% (14), is likely to involve a high frequency of chromosomal anomalies.

The total contribution of *de novo* mutations to the incidence of genetic disease is not well established but, in the case of dominant or X-linked disorders, it is possible to determine if the mutation was preexisting or newly arisen. In addition, most chromosome anomalies that are lethal or cause infertility are obviously not preexisting. Based on a review of the literature, it has been estimated that 81% of all chromosome abnormalities and 20% of single gene disorders are due to *de novo* mutations in reproductive cells (11,15). In a more recent examination of 1549 malformed infants of 20 weeks gestation or greater, Nelson and Holmes (6) found that of the 48 malformations resulting from identifiable single mutant genes (i.e., autosomal dominant, autosomal recessive and X-linked traits), 44% were due to a presumed new mutation (Table 4).

The scientific evidence that exposure to environmental mutagens can induce mutations in both somatic and germinal

tissues is clear. This fact has been thoroughly demonstrated in a variety of organisms, including mammals. Determining whether the offspring of individuals exposed to germ cell mutagens will subsequently develop an observable disease is, however, much more problematic. Unequivocal epidemiological evidence that exposure to mutagenic chemicals can produce heritable genetic effects in humans is lacking for several reasons. First, it is difficult to identify exposed populations. Second, exposure levels are often low. Third, the population size of potentially affected offspring is likely to be small. Further, the fact that the exposed and affected individuals are not of the same generation requires more than a routine surveillance system (16–19).

For these reasons, animal models have been used to estimate the heritable effects of environmental mutagens. A variety of *in vivo* genetic assays have been conducted in rodents, including tests for germ cell mutagenicity on more than 20 chemicals (20). These tests are supplemented with mechanistic studies to increase our understanding of the mutation process. The National Toxicology Program (NTP) has taken a prominent role in the identification of genetic hazards in the environment and in the acquisition of data that are used by regulatory agencies in assessing genetic risks to human health. For example, NTP-sponsored germ-cell mutagenesis studies have been responsible for the identification of acrylamide as a reproductive hazard (21–23), and dose–response and dose-rate data on ethylene oxide have been used by the EPA in their hazard assessment efforts. The evaluation of various aspects of germ-cell mutagenesis such as dose, dose rate, sex, and strain effects; investigations of mutation induction in cells such as oocytes and zygotes; and identification of models of human genetic diseases such as those for β -thalassemia (26,27) and carbonic anhydrase-2 deficiency (28) are among the contributions made to the understanding of mammalian germ-cell mutagenesis. Germ cell genotoxicity assays used by the NTP are described here along with highlights of results using those assays.

Table 3. Conception loss and cytogenetic abnormalities in recognized human pregnancies (%II).

Gestational age, weeks	% Conceptions lost	% of lost conceptions with cytogenetic abnormality
5–7	6.3	17.5
8–11	3.3	50.6
12–15	2.5	47.0
16–19	1.0	32.8
20–27	1.4	10.7
Stillborn	1.0	5.7
Liveborn		0.6
Neonatal death		5.4
Death < 12 months		6.9
Childhood death		7.4

Table 4. Incidence of malformations among 69,277 infants of at least 20 weeks gestational age (6).

Origin	Cases/1000 (% of malformations)
Genetic causes	11.34 (50.7)
Chromosomal	2.27
Nonchromosomal	9.08
Single gene	0.69
Autosomal dominant	0.30
Presumed new mutation	0.14
Affected parent	0.16
X-linked	0.07
Presumed new mutation	0.01
Affected parent	0.06
Autosomal recessive	0.14
Presumed new mutation	0.07
Affected parent	0.07
Uncertain pattern	0.17
Familial	3.25
Multifactorial inheritance	5.14
Other causes	
Teratogens	0.71 (3.2)
Uterine factors	0.56 (2.5)
Twinning	0.09 (0.4)
Unknown cause	9.66 (43.2)

Mouse Assays to Study Germ Cell Mutations

Dominant Lethal Test

The mouse dominant lethal test (DLT) is the primary test used to detect the genetic effects of chemicals in germ cells. Damage induced in the genetic material of the developing male germ cell may not affect the ability of resulting sperm to fertilize the ovum, yet can result in the death of the conceptus early in development. Experimental evidence has shown that the primary class of genetic damage responsible for dominant lethality is chromosomal aberrations and that the death of the conceptus results from an imbalance in the normal chromosomal complement following cell division (e.g., loss of chromosome fragments) (29). In addition to chromosomal aberrations, aneuploid gametes (having too few or too many chromosomes) may lead to dominant lethality and hence constitute a second class of genetic damage that is detected by this assay.

The basic design of a DLT involves treatment of one parent, usually the male, with the agent under study. Treated and control males are mated with untreated females, and the uterine contents of females are analyzed 13–17 days after mating to quan-

titate the incidence of living and dead implants. Definitive dominant lethal effects are evidenced by a significant increase in dead implants and a concomitant decrease in live implants. Females may also be used as the treated parent in dominant lethal studies to assess the genetic effects of chemicals on oocytes. Special measures must be taken in female dominant lethal tests because early embryonic survival may be affected by chemical-induced changes in female reproductive physiology and because of the limitations on the numbers of litters and offspring obtained from treated females. Nonetheless, it is important that genetic effects be assessed in female germ cells, especially following the recent demonstrations of female-specific germ cell mutagens (30,31).

Because the event detected in the DLT is death of the conceptus, it is clear that the test does not assess a biological end point that reflects a potential health risk to future generations. Nonetheless, the DLT plays a key role in initial attempts to determine whether an agent reaches the germ cells and induces genetic damage. The induction of dominant lethality provides clear evidence that the integrity of the germ-cell genetic material has been affected. Thus, positive results in a dominant lethal study are adequate basis for concern that other more serious, heritable effects may also be induced by the agent under study (20,32).

Heritable Translocation Test

The heritable translocation test (HTT) is designed to detect induced chromosomal damage in germ cells resulting in reciprocal exchanges of chromosome segments between nonhomologous chromosomes. Gametes bearing reciprocal translocations normally are capable of fertilization; the resulting offspring have a complete complement of chromosomes and are viable. The primary biological effect in the translocation-carrying offspring is reduced fertility or, in some cases, complete sterility. The effect on fertility results from abnormalities in the pairing of homologous chromosomes at the first meiotic division, an event unique to meiosis. Because the two translocated chromosomes have no complete homolog, pairing involves more than two chromosomes and can result in abnormal meiotic segregation. When chromosomes are distributed into the germ cells, theoretically, half of the resulting gametes will bear a duplication of one chromosomal segment (the distal material on one translocated chromosome) and a deletion of another (the distal material on the second translocated chromosome).

As in most of the tests of germ-cell mutagenicity, either males or females can be used as the treated parent, but for practical reasons, the tests are routinely conducted using treated males. These males are mated to untreated females and the resulting F_1 males are mated to identify those that exhibit reduced fertility. Such males are then assessed cytogenetically to determine if the effect is indeed the result of a translocation. In semisterile males, preparation of testicular tissue to permit visualization of chromosomes in first-division spermatocytes will reveal translocations as multivalent chromosome configurations. In fully sterile males where total disruption of meiosis may preclude cytogenetic analysis of spermatocytes, it is necessary to evaluate metaphase preparations from somatic cells, such as kidney epithelium or lymphocytes. Due to the resources (at least 500 F_1 males should be screened per dose group) and expertise required to conduct the HTT, a limited number of laboratories routinely conduct the test. Therefore, a limited historical database exists on the spon-

taneous incidence of heritable translocations in mice. Nonetheless, this rare event is estimated to occur spontaneously in about one in 5000 male gametes.

Morphological Specific Locus Test

The major concern in genetic risk considerations has been gene mutations in spermatogonial stem cells. The stem cells are the origin of germ cells throughout the male's reproductive life, and their permanence provides the only germ-cell stage wherein genetic damage can accumulate through time and thereby pose an increasing risk of genetic damage to the progeny. Further, gene mutations are less likely than chromosomal damage to be eliminated by selection during the cell divisions of gametogenesis and are, therefore, more apt to persist to the mature germ cells. As shown in Figure 1, matings that occur in week 7 or later following treatment of the male mouse sample sperm that were in the spermatogonial stem-cell stage at the time of treatment. By doing serial matings after treatment, one can thus determine the germ-cell stage(s) sensitive to the effect of a test agent.

The most extensively used method for studies of mutations induced at specific loci in mammalian germ cells is the mouse morphological specific locus test for visible markers (MSLT) developed by W.L. Russell in the early 1950s. This test was developed to assess the germ-cell mutagenicity of ionizing radiation and provided the bulk of data used in radiation genetic risk analysis. More recently, the test has been used to study the germ-cell mutagenicity of chemicals.

The basic concept of the MSLT is relatively simple. Seven genes (or loci) that affect morphological characteristics of the mouse, namely, hair color, eye color, and ear shape, are assessed for mutations. In the standard test, males homozygous for the wild-type alleles of these seven genes are treated with the test agent and then mated to untreated females homozygous for recessive alleles at the same seven loci. Normal male gametes produce progeny that are heterozygous at these seven loci and will have the same wild-type appearance as their sires. If treatment has induced mutations that affect the expression of any of the seven loci, sperm containing such mutations will give rise to offspring that express the phenotype of the recessive allele. At 2–4 weeks of age, progeny are examined for variant appearance; variants are bred to confirm that the variant phenotype is genetically based, i.e., is transmitted to the offspring.

The loci studied in these mouse assays are not necessarily related to loci that affect murine or human health but are assumed to be representative of other loci in the genome. They were chosen to provide a convenient measure of the frequency with which mutations are induced by a test agent. The types of genetic damage recovered in the MSLT include intragenic changes such as basepair substitutions, small additions or deletions of genetic material, and deletions of larger segments of the DNA that span one of the loci studied along with adjacent DNA that may contain other genes.

Because of the long history of the MSLT, an immense historical database has accumulated on the frequency of spontaneous mutations at the seven loci studied. This database offers the assay two great advantages. By virtue of the evidence for low variability across time, it mitigates the need for large concurrent controls. It also provides data needed for the development of sensitive statistical methods for analysis of test results.

Day of Mating Following Treatment	Treatment 0
Male	
Female	

FIGURE 1. Approximate relationships between the days of mating following acute treatment with a test chemical and the stage of male and female germ cells at the time of treatment.

Biochemical Specific-Locus Test

The biochemical specific-locus test (BSLT) is similar in many respects to the MSLT; only the unique characteristics of the BSLT will be discussed here. Two features make the BSLT particularly desirable for germ-cell mutagenesis studies. First, the BSLT screens for mutations in a large number of known genes, thereby providing an efficiency that is not available in other gene mutation tests. At present, more than 30 genes can be screened in each F_1 animal as compared with seven genes in the MSLT. In addition, these genes are distributed throughout the genome, having been mapped to 15 different chromosomes and, theoretically, a much larger number of genes could be screened as technological developments make such screening feasible. Second, many of the genes screened in the BSLT are the same as those that are associated with genetic disease in humans, again providing a closer link between rodent test results and possible human health effects. Mutations recovered in genes that are related to genetic disease in humans may also provide invaluable mouse models for human diseases (e.g., Cooley's anemia and carbonic anhydrase II deficiency) and may contribute to the further understanding and treatment of genetic diseases. Thus, more confidence may be placed in the extrapolation of mouse test results to potential genetic risk in humans.

Two inbred strains of mice are used in the BSLT and, as in the MSLT, the standard test involves treatment of males followed by mating with untreated females. The F_1 progeny are then analyzed for evidence of mutations. Both blood and kidney tissue are taken from each F_1 animal and are used to prepare samples that are subjected to either starch gel electrophoresis or isoelectric focusing. Mutations are detected that affect the electrophoretic characteristics of defined protein products.

Contributions to Assessing Genetic Hazard

Chemical Testing

In meeting the responsibilities of the NTP to increase the number of chemicals tested for potentially hazardous effects, the heritable effects program has added significantly to the number of chemicals for which mammalian germ-cell mutagenicity data are available. In 10 years, the program has doubled the number of chemicals tested for the induction of

specific locus mutations and, as presented in Table 5; a substantial number of chemicals has also been tested for other mutational end points such as dominant lethal mutations and heritable translocations.

Chemicals have been selected for testing for a variety of reasons, but the two primary considerations are known human exposure and research-based interest. In general, chemicals are not chosen for germ-cell mutagenicity studies unless they are known to be mutagenic *in vitro* and have been shown to induce genetic effects in somatic cells of rodents using mouse or rat bone marrow cytogenetic assays.

Table 5 summarizes the results of NTP's mammalian germ-cell mutagenesis studies. Chemicals such as dibromochloropropane, ethylene dibromide, and phenol were tested because of concern for occupational exposures, but showed no evidence of germ cell mutagenicity, whereas others such as ethylene oxide, acrylamide, and methylene *bis*-acrylamide have been shown to induce high levels of genetic damage in germ cells. With acrylamide, it was demonstrated for the first time that dermal exposure could result in induced genetic damage in germ cells (34).

Several cancer chemotherapeutic agents have also been tested to provide animal data to support human epidemiology studies that assess the reproductive outcomes of cancer survivors who have received radiation and/or chemical therapy. Adriamycin, bleomycin, chlorambucil, melphalan, and platinol are among the chemotherapeutic chemicals tested to date. The results of these studies have been particularly interesting. Chlorambucil and melphalan were found to be the most potent mutagens ever discovered in postmeiotic male germ cells. Molecular analysis of the mutations induced by these and other chemicals has revealed that mutations recovered from germ cells exposed as stem cells or during later premeiotic stages are almost all small, intragenic changes whereas those induced in later stages are predominantly deletions of the target gene and varying amounts of flanking DNA (32).

Three other chemotherapeutic agents, adriamycin, bleomycin, and platinol, have been shown to induce dominant lethal mutations in female germ cells, but show no such effect in males. Both the relation between germ-cell stage and molecular lesions and the demonstration of female-specific effects are important considerations in the design and interpretation of epidemiological studies relating chemotherapy and reproductive outcomes.

Table 5. Summary of results from National Toxicology Program mouse germ cell mutagenicity studies.

Chemicals	Dominant lethal			Heritable		Specific locus		
	Male	Female	Reference ^a	translocations	Reference	Postmeiotic	Premeiotic	Reference
2-Acetylaminofluorene		-	(33)					
4-Acetylaminofluorene		-	(33)					
Acrylamide	+	-	Unpublished; (21,34)	+	(22)	+	-	(23)
Adriamycin	-	+	(35)	- ^b	Unpublished			
3-Azido-3-deoxythymidine	-	-	Unpublished					
Benzo(a)pyrene		+	(33)					
Bleomycin	-	+	(36)	- ^b	Unpublished			
Chlorambucil	+		Unpublished	+		+	-	(37)
Corn oil	-	-	(38)					
Dibromochloropropane	-	-	(39)			-	-	(40)
Diethyl sulfate		-	(41)					
Dimethyl sulfate	+	-	Unpublished; (41)	+	Unpublished			
Divinyl sulfone	-		(42)					
Ethylenebisacrylamide	+		Unpublished	+	Unpublished			
Ethylene dibromide	-		(43)				-	Unpublished; (43)
Ethylene oxide	+	+	(41,44)	+	(45)	+	-	(46,47)
Ethyl nitrosourea	+	+	(48)	+	(48)		+	(32)
Hexamethylphosphoramide	-	+	Unpublished				-	Unpublished
Hycanthone	-	+	(31)					
Melphalan	+		Unpublished	+	Unpublished	+	+	(49)
6-Mercaptopurine	+		(50)	-	(50)	-	-	(50)
Methylmethane sulfonate		-	(41)					
Methylnitrosourea	+		(48)	+	(48)	+	+	(51)
Methylvinyl sulfone	-		(42)					
Methylenebisacrylamide	+		(52)	+	(52)			
Nocodazole		-	(53)					
Phenol	-		Unpublished					
Platinol	-	+	(30)			-	-	Unpublished
Pyrene		-	(33)					
Tetrahydrocannabinol	-		(54)	-	(54)			
Urethane	-		(55)			-	-	(55)

^aUnpublished test results.

^bResults from treated females.

Effects on Male Postmeiotic Germ Cells

Spermatogonial stem cells are the germ cells of greatest concern for genetic risk because they constitute a permanent, dividing-cell population that can accumulate induced genetic damage throughout the reproductive life of the male. Subsequent germ-cell stages, such as differentiating spermatogonia, spermatocytes, spermatids, and spermatozoa, are all transient and thus can only accumulate genetic damage over a relatively brief period. However, because it was known that some germ-cell mutagens exhibit their peak mutagenic activity in postmeiotic germ cell stages, it was decided early in the development of this program to evaluate both pre- and postmeiotic germ-cell stages for induced mutations. The testing of chemicals for mutagenicity in postmeiotic germ cell stages permits the identification of chemicals that were only mutagenic in these stages, an important consideration when evaluating the genetic risk associated with continuous or recurrent exposures of reproductively active individuals.

As can be seen in Table 5, several chemicals have been identified that are solely or primarily mutagenic in postmeiotic stages of spermatogenesis. In fact, with the exception of melphalan, chemicals of environmental significance that have been shown to be male germ-cell mutagens are specific for postmeiotic germ-cell stages. These include chemicals such as acrylamide and ethylene oxide that are present in occupational environments as well as chemotherapeutic agents such as chlorambucil.

Expansion of Test Systems

Although the MSLT, BSLT, DL, and HT have provided a wealth of information toward the identification and characterization of mammalian germ-cell mutagens, each is limited in the types of mutations detected and in the relevance of these mutations to health effects in the first generation following mutagen exposure. In an effort to overcome some of these limitations, two projects were recently initiated to expand the classes of mutational events recovered by an assay.

The first is a combined end point assay based on the BSLT. It retains the desirable characteristics of this system while incorporating other mutational end points that can be detected in the same progeny (56). In this system, progeny are screened for dominant morphological variation, recessive morphological variation, and dominant cataracts. The system is thus designed to detect a broader range of induced genetic variants including both dominant and recessive mutations in both selected and unselected genes. By obtaining more information from each F₁ mouse, more effective use is made of the experimental animals, and the resulting data should provide a clearer indication of the overall genetic risk associated with exposure to the test chemical. In addition, efforts are underway to detect induced DNA damage such as insertions, deletions, and rearrangements through the use of DNA probes for repetitive DNA sequences.

The second system is based on the MSLT. The assessment of dominant damage system (57) employs several indicators of

induced genetic damage expressed in the first generation following exposure. These indicators are all associated with survival or impaired health or fitness in the offspring. They include cataracts, skeletal abnormalities, litter sizes, and survival to 11 weeks. Using the germ-cell mutagens X-rays, ethylnitrosourea, and chlorambucil, preliminary experiments are being conducted to determine the induced frequencies of dominant effects relative to recessive mutations detected in the MSLT.

Effects of Zygote Exposures

In a series of experiments conducted to determine the effects of germ-cell mutagens on the zygote, it was discovered that chemical exposure of the pronuclear-stage zygote could result in a range of developmental abnormalities distinct from what is observed following either treatment of germ cells or of the developing embryo or fetus (44,58). Preliminary evidence indicates that these effects may not be the result of gene or chromosomal mutation, and it has been speculated that they may result from phenomena such as the induction of transposable elements or disruption of normal gene regulation (59). Results presented in Table 6 show that these zygote-exposure effects are mutagen specific.

Although the seven chemicals included in Table 6 induce high frequencies of early deaths (deciduomata or "resorption moles"), there is a dichotomy with regard to frequency of induced mid- and late gestation deaths. Ethylene oxide, ethylmethane sulfonate, dimethyl sulfate, and diethyl sulfate induce high frequencies of mid- and late gestation deaths, and among the surviving fetuses there is a notably high incidence of developmental abnormalities. With the other three chemicals (methylmethane sulfonate, ethylnitrosourea, and trimethylene melamine) early deaths are induced but the incidence of mid- and late gestation deaths and the proportion of live fetuses with abnormalities are low. In addition to defining the unique mutagen sensitivity of an early developmental stage of the conceptus, these studies offer a promising opportunity to elucidate, at the molecular level, genetic and epigenetic processes required for normal mammalian development.

Female-Specific Effects

In the course of our dominant lethal studies, four chemicals were identified that appear to induce genetic damage in oocytes but not in male germ cells (Table 5). The unique vulnerability of female germ cells to genetic damage by hycanthone, platinol, adriamycin, and bleomycin emphasizes the need to direct more

effort toward assessing induced genetic damage in female germ cells as well as the importance of incorporating data from such studies into assessments of genetic risk.

The Future

In recent years, technical advances have provided efficient methods for molecular analysis of mutations in defined target genes (61). The polymerase chain reaction (PCR), DNA sequence analysis, and the isolation of molecular probes to various loci have been used to efficiently recover and identify mutations. Most studies have focused on the identification of new mutations in somatic cells; however, such techniques may also be useful in screening for mutations in disease-related genes in the progeny of mutagen-exposed individuals. The limitations of screening large numbers of individuals for a specific mutational change, however, have led to the development of methods for assessing genomic variations that are not associated with specific loci.

There are several promising molecular techniques that have the potential to detect newly arising genetic changes and thereby to provide insight into the etiology of spontaneous or induced human genetic disease. These techniques include *a*) application of chromosome-specific fluorescent probes, *b*) identification and screening of hypervariable regions of the mammalian genome using probes for repetitive DNA sequence, and *c*) the use of PCR and standard nucleic-acid blotting techniques. These procedures are not limited to elucidation of changes in specific genes but can be used to identify changes that might occur over a large portion of the genome as a result of a germline mutation. Experimental approaches already in use in defining germline variation include the use of chromosome specific probes to identify aneuploidies, Southern blot analysis of genomic insertions, deletions, and rearrangements (1/D/R; 62), and assessment of meiotic recombination as detected by PCR in individual human sperm cells (63). Some of these techniques as well as methods for determining locus specific variation, have been reviewed recently (64).

Probes specific for each of the 24 human chromosomes are rapidly becoming available. Such probes can be tagged with fluorescent reporter molecules, and the site of hybridization (and therefore the chromosome of interest) can be observed directly by fluorescence microscopy. This approach to identifying specific human chromosomes has been used widely in human gene mapping studies and is being developed for use in detection of chromosomal gain or loss in individual sperm cells. Such procedures may be useful in determining the incidence of aneuploidy in occupationally or environmentally exposed individuals. A

Table 6. Summary of results from treated zygote experiments.*

Test chemical, dose	% Early deaths	% Mid- and late gestation deaths	% Live fetuses with abnormalities	Reference
Ethylene oxide, 1200 ppm	53	29	37	(44,60)
Ethylmethane sulfonate, 250 mg/kg	31	31	29	(28,44)
Diethyl sulfate, 150 mg/kg	24	37	24	(41,60)
Dimethyl sulfate, 25 mg/kg	57	11	25	(41,60)
Methylmethane sulfonate, 75 mg/kg	25	2.5	3.8	(41,60)
Ethylnitrosourea, 75 mg/kg	49	2.0	15	(44)
Triethylene melamine, 1.2 mg/kg	57	2.2	10	(44)
Control ^b	3.6	1.2	0.6	(41)

*Exposure of females 6 hr after mating.

^bA typical control.

similar set of probes specific for each of the mouse chromosomes is currently being isolated and characterized. These will permit laboratory studies to be conducted using animals treated with selected chemicals and dose levels so that observations made in humans can be extended or confirmed.

DNA hybridization techniques have been proposed for the study of genomic I/D/R (62). The I/D/R technique uses a variety of probes that may hybridize to repetitive sequences, hyper-variable regions, or specific genes and pseudogenes. Experimentally, all of the probes are used to detect alterations in hybridization patterns following Southern blotting of genomic DNA. These techniques allow the detection of large-scale genomic alterations. Once again, such studies can be applied to human populations and similar I/D/R studies are underway to assess induced rearrangements in mice.

Likewise, DNA probes to linked genes can be used as PCR primers to detect meiotic recombination events (63). Specific genetic sequences in individual sperm can be screened and variations can be quantitated, thus providing a measure of meiotic recombination frequencies in control and exposed populations. Similar probes can be used to quantitate meiotic recombination frequencies in mice or other experimental organisms.

Methods for producing and studying transgenic animals comprise another emerging technology that can be applied to studies of induced heritable effects. It is now possible to construct transgenic animal models that carry specific genes or an altered (or modified) genetic background to allow the study of induced mutations *in vivo*. At least three transgenic mouse models for detecting point mutations *in vivo* are at various stages of development. All use bacterial genes that have been inserted into the mouse genomes as mutation targets. These provide a convenient means to study the induction of mutations in different tissues, including the germ cells, of an experimental animal. Two bacterial genes, *lac Z* (65) and *lac I* (66) have been used as targets for forward mutations. Both provide colorimetric assays for mutations. Thus, transgenic mice used in these assays can be treated with a test chemical, and after a period to allow for DNA repair and fixation of any new mutations, different organs can be removed and the DNA prepared for the recovery of λ phage carrying mutated *lac Z* or *lac I* genes. Mutations are identified by the color of individual plaques following infection of *E. coli* and the precise nature of the mutation can then be determined by sequencing the target gene. The third transgenic mouse system has been developed to detect reversion mutations of an amber codon of bacteriophage Φ X174. In this system, Φ X174 is recovered from the mouse genome, and mutations are detected by their ability to form plaques on an *E. coli* host (67).

All the techniques discussed above have the ability to detect genetic variation in progeny as compared to the parental generation, and many of these techniques may be noninvasively applied to cohorts of mutagen-exposed populations. Thus, such techniques provide a basis for the development of sensitive assays for newly induced germline mutations. Routine use of these methods to screen for induced genetic effects will, however, require additional development and experimental validation. Any clear association between chemically induced changes in the human genome and specific environmental exposures will likely require the development of parallel animal assays. Data derived from molecular genetic studies in humans, along with verifiable methods to extrapolate from corresponding animal data, will

permit a more informed assessment of the biological and medical consequences of mutagen exposure. Without such comparative data, extrapolation from animal data to estimates of the genetic risk for humans will be difficult. Continued animal studies, with greater emphasis on the use of molecular techniques to detect and characterize mutants and elucidate the structure and function of the genome are needed to increase our understanding of the mutational process in mammals and to improve our ability to assess genetic risk.

Collaborations are being developed within and outside of NIEHS to pursue the detection of induced mutations in human germ cells while refining methods that permit detection of the similar effects in mice. Further, studies to determine the effects of induced germ-cell mutations on tumor frequencies in subsequent generations and to investigate novel processes such as the induction of mobile genetic elements and alterations of genetic imprinting that influence mammalian heritable effects will be pursued.

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REFERENCES

1. McKusick, V. A. Mendelian Inheritance in Man: Catalogs of Autosomal Dominant, Autosomal Recessive and X-linked Phenotypes, 9th ed. Johns Hopkins University Press, Baltimore, MD, 1990.
2. Czeizel, A., and Sankaranarayanan, K. The load of genetic and partially genetic disorders in man: I. Congenital anomalies: estimates of detriment in terms of years of life lost and years of impaired life. *Mutat. Res.* 128: 73-103 (1984).
3. Mohrenweiser, H. W. Functional hemizyosity in the human genome: direct estimate from twelve erythrocyte enzyme loci. *Hum. Genet.* 77: 241-245 (1987).
4. Baird, P. A., Anderson, T. W., Newcombe, H. B., and Lowry, R. B. Genetic disorders in children and young adults: a population study. *Am. J. Hum. Genet.* 42: 677-693 (1988).
5. Czeizel, A., Sankaranarayanan, K., Losonci, A., Rudas, T., and Keresztes, M. The load of genetic and partially genetic diseases in man: II. Some selected common multifactorial diseases: estimates of population prevalence and of detriment in terms of years of lost and impaired life. *Mutat. Res.* 196: 259-292 (1988).
6. Nelson, K., and Holmes, L. B. Malformations due to presumed spontaneous mutations in newborn infants. *N. Engl. J. Med.* 320: 19-23 (1989).
7. Mohrenweiser, H. W. Germinal mutations and human genetic disease. In: *Genetic Toxicology* (A. P. Li and R. H. Heflich, Eds.), CRC Press, Boca Raton, FL, 1991, pp. 67-92.
8. Lyon, M. F. Estimation of genetic risks and increased incidence of genetic disease due to environmental mutagens. *Mutat. Res.* 115: 255-291 (1983).
9. Costa, T., Scriver, C. R., and Childs, B. The effect of Mendelian disease on human health: a measurement. *Am. J. Med. Genet.* 21: 231-242 (1985).
10. Hall, J. G., Powers, E. K., McIlvaine, R. T., and Ean, V. H. The frequency and financial burden of genetic disease in a pediatric hospital. *Am. J. Med. Genet.* 1: 417-436 (1978).
11. U.S. Congress, Office of Technology Assessment. Technologies for Detecting Heritable Mutations in Human Beings. Report no. OTA-H-298. U.S. Government Printing Office, Washington, DC, 1986.
12. Hayes, A., Costa, T., Scriver, C. R., and Childs, B. The effect of Mendelian disease on human health: response to treatment. *Am. J. Med. Genet.* 21: 243-255 (1985).
13. Hook, E. B. Perspectives in mutation epidemiology 3. Contribution of chromosome abnormalities to human morbidity and mortality and some comments upon surveillance of chromosome mutation rates. *Mutat. Res.* 114: 389-423 (1983).

14. Wilcox, A. J., Weinberg, C. R., O'Connor, J. F., Baird, D. D., Schlatterer J. P., Canfield, R. W., Armstrong, E. G., and Nisula, B. C. Incidence of early loss of pregnancy. *N. Engl. J. Med.* 319(4): 189-194 (1989).
15. Crow, J. F., and Denniston, C. The mutation component of genetic damage. *Science* 212: 888-893 (1981).
16. Carter, C. O. Genetics of common single malformations. *Br. Med. Bull.* 32: 216 (1976).
17. Falconer, D. S. The inheritance of liability to diseases with variable age of onset with particular reference to diabetes mellitus. *Ann. Hum. Genet.* 31: 1-20 (1967).
18. Fraser, F. C. The genetics of common familial disorders-major genes or multifactorial. *Can. J. Genet. Cytol.* 23: 108 (1981).
19. Czeizel, A., Belyei, A., Kranicz, J., Mocsai, L., and Tusnady, G. Conformation of the multifactorial threshold model for congenital structural talipes equinovarus. *J. Med. Genet.* 18: 99-100 (1981).
20. Bishop, J. B., and Shelby, M. D. Mammalian heritable effects research in the National Toxicology Program. In: *Banbury Report 34: Biology of Mammalian Germ Cell Mutagenesis* (J. Allen, B. Bridges, M. Lyon, M. Moses, and L. B. Russell, Eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990, pp. 425-435.
21. Shelby, M. D., Cain, K. T., Hughes, L. A., Braden, P. W., and Generoso, W. M. Dominant lethal effects of acrylamide in male mice. *Mutat. Res.* 176: 35-40 (1985).
22. Shelby, M. D., Cain, K. T., Cornett, C. V., and Generoso, W. M. Acrylamide: induction of heritable translocations in male mice. *Environ. Mutagen.* 176: 363-368 (1987).
23. Russell, L. B., Hunsicker, P., Cacheiro, N. L. A., and Generoso, W. M. Induction of specific-locus mutations in the germ cells of the mouse by acrylamide monomer. *Mutat. Res.* 262: 101-107 (1991).
24. Generoso, W. M., Cain, K. T., Hughes, L. A., Sega, G. A., Braden, P. W., Gosslee, D. G., and Shelby, M. D. Ethylene oxide dose and dose-rate effects in the mouse dominant-lethal test. *Environ. Mutagen.* 8: 1-7 (1986).
25. Rhomberg, L., Dellarco, V. L., Siegel-Scott, C., Dearfield, K. L., Valcovic, L., and Jacobson-Kram, D. Quantitative estimation of the genetic risk associated with the induction of heritable translocations at low-dose exposure: ethylene oxide as an example. *Environ. Mol. Mutagen.* 16: 132-134 (1990).
26. Skow, L. C., Burkhardt, B. A., Johnson, F. M., Popp, R. A., Popp, D. M., Goldberg, S. Z., Anderson, W. F., Barnett, L. B., and Lewis, S. E. A mouse model for beta-thalassemia. *Cell* 34: 1043-1052 (1983).
27. Goldberg, S. Z., Kuebbing, D., Trauber, D., Schafer, M. P., Lewis, S. E., Popp, R. A., and Anderson, W. F. A 66 base pair insert bridges the deletion responsible for a mouse model of beta thalassemia. *J. Biol. Chem.* 261: 12368-12374 (1986).
28. Lewis, S. E., Erickson, R. P., Barnett, L. B., Venta, P., and Tashian, R. Ethylnitrosourea-induced null mutation at the mouse *Car-2* locus: an animal model for human carbonic anhydrase II deficiency syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 85: 1962-1966 (1988).
29. Brewen, J. G., Payne, M. S., Jones, K. P., and Preston, R. J. Studies on chemically induced dominant lethality. I. The cytogenetic basis for MMS-induced dominant lethality in postmeiotic male germ cells. *Mutat. Res.* 33: 239-250 (1975).
30. Katoh, M., Cain, K. T., Hughes, L. A., Foxworth, L. B., Bishop, J. B., and Generoso, W. M. Female-specific dominant lethal effects in mice. *Mutat. Res.* 230: 205-217 (1990).
31. Sudman, P. D., and Generoso, W. M. Female-specific mutagenic response of mice to lycanthone. *Mutat. Res.* 246: 31-43 (1991).
32. Russell, L. B., Russell, W. L., Rinchik, E. M., and Hunsicker, P. R. Factors affecting the nature of induced mutations. In: *Banbury Report 34: Biology of Mammalian Germ Cell Mutagenesis* (J. Allen, B. Bridges, M. Lyon, M. Moses, and L. B. Russell, Eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990, pp. 271-289.
33. Generoso, W. M., Cain, K. T., Hughes, L. A., and Braden, P. W. Tests for induction of presumed dominant lethal effects in female mice. In: *Evaluation of Short-Term Tests for Carcinogens: Report of the IPCS Collaborative Study on In Vivo Assays* (J. Ashby, F. J. deSerres, M. D. Shelby, B. H. Margolin, M. Ishidate, and G. C. Becking, Eds.), Cambridge University Press, Cambridge, UK, 1988, pp. 189-193.
34. Gutierrez-Espeleta, G. A., Hughes, L. A., Piegorsch, W., Shelby, M. D., and Generoso, W. M. Dermal exposure to acrylamide induces germ cell mutations in mice. *Fundam. Appl. Toxicol.* 18: 189-192 (1992).
35. Generoso, W. M., Cain, K. T., Hughes, L. A., and Foxworth, L. B. A restudy of the efficacy of adriamycin in inducing dominant lethals in mouse spermatogonia stem cells. *Mutat. Res.* 226: 61-64 (1989).
36. Sudman, P. D., Rutledge, J. C., Bishop, J. B., and Generoso, W. M. Bleomycin: female-specific dominant lethal response in mice. *Mutat. Res.*, in press.
37. Russell, L. B., Hunsicker, P. R., Chacheiro, N. L. A., Bangham, J. W., Russell, W. L., and Shelby, M. D. Chlorambucil effectively induces deletion mutations in mouse germ cells. *Proc. Natl. Acad. Sci. U.S.A.* 86: 3704-3708 (1989).
38. Generoso, W. M., Cain, K. T., Hughes, L. A., and Foxworth, L. B. A restudy of the efficacy of adriamycin in inducing dominant lethals in mouse spermatogonia stem cells. *Mutat. Res.* 226: 61-64 (1989).
39. Generoso, W. M., Cain, K. T., Hoskins, J. A., Washington, W. J., and Rutledge, J. C. Pseudo dominant lethal response in female mice treated with plant oils. *Mutat. Res.* 129: 235-241 (1984).
40. Russell, L. B., Hunsicker, P. R., and Cacheiro, N. L. A. Mouse specific locus test for the induction of heritable gene mutations by dibromochloropropane (DBCP). *Mutat. Res.* 170: 161-166 (1986).
41. Generoso, W. M., Shourbaji, A. G., Piegorsch, W. W., and Bishop, J. B. Developmental response of zygotes exposed to similar mutagens. *Mutat. Res.* 250: 439-446 (1991).
42. Shelby, M. D., Gutierrez-Espeleta, G. A., Generoso, W. M., and McFee, A. F. Mouse dominant lethal and bone marrow micronucleus studies on methyl vinyl sulfone and divinyl sulfone. *Mutat. Res.* 250: 431-437 (1991).
43. Barnett, L. B., Felton, C. F., Givson, B. J., Sharpe, D. S., Shelby, M. D., and Lewis, S. E. No heritable mutations are induced by ethylene dibromide at electrophoretically expressed loci. *Mutat. Res.* 282: 127-133 (1992).
44. Generoso, W. M., Rutledge, J. C., Cain, K. T., Hughes, L. A., and Braden, P. W. Exposure of female mice to ethylene oxide within hours after mating leads to fetal malformation and death. *Mutat. Res.* 176: 269-274 (1987).
45. Generoso, W. M., Cain, K. T., Hughes, L. A., and Foxworth, L. Concentration-response curves for ethylene oxide-induced heritable translocations and dominant lethal mutations. *Environ. Mol. Mutagen.* 16: 126-131 (1990).
46. Lewis, S. E., Barnett, L. B., Felton, C., Johnson, F. M., Skow, L. C., Cacheiro, N., and Shelby, M. D. Dominant visible and electrophoretically expressed mutations induced in male mice exposed to ethylene oxide by inhalation. *Environ. Mutagen.* 8: 867-872 (1986).
47. Russell, L. B., Cummings, R. B., and Hunsicker, P. R. Specific-locus mutation rates in the mouse following inhalation of ethylene oxide and application of the results estimation of human genetic risk. *Mutat. Res.* 129: 381-388 (1984).
48. Generoso, W. M., Cain, K. T., Cornett, C. C., and Cacheiro, N. L. A. DNA target sites associated with chemical induction of dominant-lethal mutations and heritable translocations in mice. In: *Genetics: New Frontiers. Proceedings of the Fifteenth International Congress of Genetics.* Oxford and IBH Publishing Co., New Delhi, 1983, pp. 347-355.
49. Russell, L. B., Hunsicker, P. R., and Shelby, M. D. Melphalan, a second chemical for which specific-locus mutation induction in the mouse is maximum in early spermatids. *Mutat. Res.* 282: 151-158 (1992).
50. Russell, L. B., and Hunsicker, P. R. Study of the base analog 6-mercaptopurine in the mouse specific-locus test. *Mutat. Res.* 176: 47-52 (1987).
51. Russell, W. L., and Hunsicker, P. R. Extreme sensitivity of one particular germ cell stage in male mice to induction of specific-locus mutations by methylnitrosourea. *Environ. Mutagen.* 5: 498 (1983).
52. Rutledge, J. C., Cain, K. T., Kyle, J., Cornett, C. V., Cacheiro, N. L. A., Witt, K., Shelby, M. D., and Generoso, W. M. Increased incidence of developmental anomalies among descendants of carriers of methylenebisacrylamide-induced balanced reciprocal translocations. *Mutat. Res.* 229: 161-172 (1990).
53. Generoso, W. M., Katoh, M., Cain, K. T., Hughes, L. A., Foxworth, L. B., Mitchell, T. J., and Bishop, J. B. Chromosome malsegregation and embryonic lethality induced by treatment of normally ovulated mouse oocytes with nocodazole. *Mutat. Res.* 210: 313-322 (1989).
54. Generoso, W. M., Cain, K. T., Cornett, C. V., and Shelby, M. D. Test for induction of dominant-lethal mutations and heritable translocations with tetrahydrocannabinol in male mice. *Mutat. Res.* 143: 51-53 (1985).
55. Russell, L. B., Hunsicker, P. R., Oakberg, E. F., Cummings, C. C., and Schmoyer, R. L. Test for urethane induction of germ-cell mutations and germ cell killing in the mouse. *Mutat. Res.* 188: 335-342 (1987).
56. Lewis, S. E. Biochemical specific-locus test and a new multiple-endpoint mutation detection system: considerations for genetic risk assessment. *Environ. Mol. Mutagen.* 18: 303-306 (1991).
57. Selby, P. B. The importance of the direct method of genetic risk estimation and ways to improve it. In: *Banbury Report 34: Mutation Induction and Heritability in Mammalian Germ Cells* (J. Allen, B. Bridges, M. Lyon, M. Moses, and L. B. Russell, Eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990, pp. 437-449.

58. Generoso, W. M., Rutledge, J. C., Cain, K. T., Hughes, L. A., and Downing, D. J. Mutagen-induced fetal anomalies and death following treatment of females within hours after mating. *Mutat. Res.* 199: 175-181 (1988).
59. Katoh, M., Cacheiro, N. L. A., Cornett, C. V., Cain, K. T., Rutledge, J. C., and Generoso W. M. Fetal anomalies produced subsequent to treatment of zygotes with ethylene oxide or ethyl methanesulfonate are not likely due to the usual genetic causes. *Mutat. Res.* 210: 337-344 (1989).
60. Generoso, W. M., Rutledge, J. C., and Aronson, J. Developmental anomalies: mutational consequence of mouse zygote exposure. In: *Banbury Report 34: Mutation Induction and Heritability in Mammalian Germ Cells* (J. Allen, B. Bridges, M. Lyon, M. Moses, and L. B. Russell, Eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990, pp. 311-319.
61. Meuth, M. The structure of mutation in mammalian cells. *Biochem. Biophys. Acta* 1032: 1-17 (1990).
62. Mohrenwiser, H. W., Larsen, R. D., and Neel, J. V. Development of molecular approaches to estimating germinal mutation rates: I. Detection of insertion/deletion/rearrangement variants in the human genome. *Mutat. Res.* 212: 241-252 (1989).
63. Li, H., Gyllensten, U. B., Cui, X., Saiki, R. K., Erlich, H. A., and Arnheim, N. Amplification and analysis of DNA sequences in single human sperm and diploid cells. *Nature* 335(6189): 414-417 (1988).
64. Mohrenwiser, H. W., Perry, B. A., and Judd, S. A. Molecular approaches to the estimation of germinal gene mutation rates. In: *Banbury Report 34: Biology of Mammalian Germ Cell Mutagenesis* (J. Allen, B. Bridges, M. Lyon, M. Moses, and L. B. Russell, Eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1990, pp. 335-349.
65. Gossen, J. A., de Leeuw, W. J. F., Tan, C. H. T., Zwarthoff, E. C., Berends, F., Lohman, P. H. M., Knook, d. L., and Vijg, J. Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 86: 7971-7975 (1989).
66. Kohler, S. W., Provost, G. S., Fieck, A., Kretz, P. L., Bullock, W. O., Sorge, J. A., Putman, D. L., and Short, J. M. Spectra of spontaneous and mutagen-induced mutation in the *lacI* gene in transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 88: 7958-7962 (1991).
67. Burkhart, J. G., Winn, R. N., VenBeneden, R., and Mailing, H. V. The Φ X174 bacteriophage shuttle vector for the study of mutagenesis (abstract.) *Environ. Mol. Mutagen.* 19(S20): 8 (1992).