Alterations of Germ Cells Leading To Mutagenesis and Their Detection

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Germ cell risk is an essential component of estimating the deleterious results of environmental mutagens. The present set of in vitro model systems appears to have limited ability to measure germ cell risk from all types of genetic lesions. The assays most practical for use in genetic testing measure the induction of chromosome alterations but not specific locus gene mutations. Quantitative estimation of germ cell risk based on such available assays is difficult to make and may lead to incorrect assumptions of risk. Better assessment of germ cell genetic damage will require expansion of present procedures to increase sensitivity and the development of improved assay systems.

This paper is primarily an analysis of risk assessment using the types of assays which are currently available to us, and will attempt to describe the genetic alterations that can be detected using these systems and the significance of these alterations on the human gene pool. A description of the model systems, whether they are considered useful or not, and some of the problems involved in risk assessment will be noted, again trying to relate this as much as possible to human risk assessment.

In the broadest sense, when one considers germ cell mutation, it is possible to identify a number of effects. Consequences such as dominant lethality, genetically based diseases, and loss or gain of sex chromosomes are only a few. The individual in which germ cell mutations are induced is not actually at risk. Only the progeny of the organism would be at risk. This is in contrast to the effects of somatic mutations.

Somatic mutations are those which would be induced in non-germ cell tissue and result in direct risk to the affected individual. One potential effect from somatic mutations would be a teratogenic (developmental alteration) alteration. If the mutation were induced prenatally during early development, normal cell division and differentiation might be altered, resulting in a biological effect described as terata (/). It has also been proposed that somatic mutation may lead to the induction of neoplasia as well (2).

Figure 1 illustrates the potential effects of germ and somatic mutations. Although substantial indirect data show a high correlation between mutations and cancer, there is not as much direct evidence for the somatic mutation theory of carcinogenesis. It appears that about 50% of teratogenic effects have a hereditary basis (Table 1). The remaining discussion, however, will be confined to germ cell alterations and the effect that these mutations have on subsequent generations.

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Table 1. Comparison of the carcinogenic, mutagenic and teratogenic effects of several chemical classes.\(^a,b\)

| Agent                  | Mutagen | Carcinogen | Teratogen |
|------------------------|---------|------------|-----------|
| Nitrogen mustard       | +       | +          | +         |
| Cyclophosphamide       | +       | +          | +         |
| Triethylenemelamine (TEM) | +    | +          | +         |
| 6-Mercaptopurine        | +       | +          | +         |
| Hycanthone             | +       | +          | +         |
| Captan                 | +       | +          | +         |
| Folpet                 | +       | +          | +         |
| X-irradiation          | +       | +          | +         |
| Cigarette smoke (condensate products) | + | + | (+) |
| Triazines              | +       | +          | +         |
| Androgenic hormones    | -       | ?          | +         |
| Rubella virus          | -       | -          | +         |
| Quinacrine             | (+)     | -          | +         |
| Halothane              | (+)     | ?          | (+)       |
| 5-Fluorouracil         | -       | -          | +         |
| Cytosine arabinoside   | (+)     | -          | +         |
| Hydroxyurea            | -       | -          | +         |
| Methotrexate           | (+)     | (+)        | +         |
| Actinomycin D          | (+)     | -          | +         |
| Benzene                | (+)     | (+)        | +         |
| Urethane               | (+)     | +          | +         |
| Colchicine             | -       | -          | +         |
| Chlorpromazine         | (+)     | -          | +         |

\(^a\) From Brusick (3).

\(^b\) Among a diverse group of recognized teratogens it can be seen that the relationship between carcinogenic, mutagenic, and teratogenic effects holds for only about 50% of the compounds.

The remaining agents that are teratogenic appear to act through nongenetic mechanisms or possibly multiple mechanisms some of which may have an indirect genetic basis. \((\_\_)\) = Data questionable, conflicting, or results obtained only under very specialized treatment procedures.

What are the possible types of effects that can occur in germ cells? If a definition of mutation in a broad sense of the term is used, two different classes can be examined. One would be visible changes (macrolesions) in chromosomes and the other would be nonvisible (microlesions) (Fig. 2). Macrolesions would be the type of alterations that would be microscopically visible in the chromatin material of the germ cells. These are detected as either changes in chromosome number or as structural changes in chromosomes. Microlesions are point mutations and these would be changes at the molecular level, that is, alterations at the nucleotide level of the DNA double helix.

These two classes of mutations have a very different impact on the gene pool of an exposed population. Macrolesions include chromosome breaks, gaps, fusions of chromosomes, translocations from one part of the chromosome to another (either reciprocal or nonreciprocal), deletions of chromosomes and also various nondisjunction effects. Except for a few of these types such as balanced translocations, certain types of nondisjunction effects, for example, trisomy 21, which could lead to mongolism and some small deletions, macrolesions are not transmissible. That is, following the induction of a macrolesion in a cell, the next cell division, whether it be meiotic or mitotic, would normally result in cell death due to the inability of the cell to undergo a complete meiotic or mitotic division, to the loss of some biochemical property because of the deletion of a chromosome, or to a nonuniform distribution of the chromosomes to daughter cells. This lethality would then be expressed directly upon the subsequent cell division or at the time of fertilization. Following fertilization, a resultant zygote may be unable to undergo any further divisions because of the lack of chromosomes or because too much chromosomal material is present. Because macrolesions are not transmissible, they would be expected to have little or no impact on the gene pool, that is, our concern for detecting these types of mutations would be considerably less than for the transmissible types.

The effects of macrolesions such as balanced translocations, which could be transmitted to the F\(_1\) progeny, certain types of small deletions, and certain nondisjunction events, appear to be expressed immediately in the F\(_1\) progeny. Nondisjunction results in some easily identifiable phenotypes, such as in trisomy 21 (mongolism). Balanced translocations would generally result in reduced fertility in F\(_1\) progeny so that they would also be identified. In some cases dominant alterations may have more phenotypic effects than just reduced fertility and will produce other functional problems in F\(_1\) progeny (\(d\)). Reduced life expectancy as well as reduced fertility may result. Thus, macrolesions which are transmissible can be identified very early, probably in the next generation, and their presence would provide a tool for assessing overall impact of chemical mutagens on the human gene pool. There would probably be high negative selection for these mutations if they affected reproductive performance. It can be concluded therefore that this class
of mutations has little impact on genetic vitality of the human population on a long-term basis. The alterations represent short-term detrimental impacts because of increased health costs associated with fetal wastage, human miscarriage, and nondisjunctional effects.

Under the class of genetic effects designated microlesions we have nucleotide events resulting in base pair substitution mutations or in frameshift mutations (5). Base-pair substitutions result in the substitution of one nucleotide pair for another in the DNA helix, resulting not in any net quantitative change in DNA, but in a qualitative change in the nucleotide sequence which may then lead to a mutation (Fig. 2). Frameshift mutations, however, result in net quantitative differences in the amount of DNA by the addition or deletion of one or a small number of nucleotides. The loss or the addition of one or more base pairs to the DNA molecule would result in a frameshift mutation (Fig. 2). Both types of microlesions result in small changes in the DNA which permit them to be transmitted without cell death. Microlesions of the base-pair substitution or the frameshift type can be subdivided again into two classes designated dominant mutations or recessive mutations. With dominant mutations the effect is generally identified morphologically and would be expressed in the F1 generation. A dominant mutation in the F2 generation can be transmitted to the F3 generation in up to 50% of the offspring, but its expression may be less, depending upon the penetrance of the gene. An affected individual can often be recognized in the population, thus offering the opportunity of reducing the transmission of this effect to the offspring by good genetic counseling. Dominant mutations also serve another function; they can be used in population monitoring (4). The effects are expressed in the heterozygous individual, and thus any new mutations that arise in a given generation would be easily detected. The data can be used to monitor that population for any fluctuations in mutation frequencies or mutation rates over a given chronological period. This requires large-scale epidemiological investigations, but at least it can provide information regarding the effects of environmental mutagens.

Recessive mutations represent the true health problem in terms of induced mutations. They do not result in the immediate expression of visible mutations; they will not necessarily be expressed in the F1 or even in the F2 generation. Two mutant alleles at the same locus are necessary to express the mutant phenotype. Therefore, the induction of recessive point mutations by environmental mutagens could result in the accumulation of these mutations in the population as heterozygotes, and it may take many generations before they are expressed in a homozygous state leading to increases in genetic-based diseases. By the time such a change is evident, it would be too late to make corrective adjustments, and mutants would be expressed in future generations even if the mutagens that initiated the mutations were eliminated. Herein lies the real problem from a long-term standpoint with respect to the human gene pool and the vitality of human hereditary potential.

With this as a rationale for the development of mammalian test models, the types of mammalian models that are available to detect mutations will be discussed, again on emphasizing that these tests are for detecting germ cell mutations. An attempt will be made to show how these tests can be applied to the problems that must be faced in terms of monitoring the human gene pool for mutation induction. There are several types of animal models that could be used. These range from cytogenetic investigation of spermatogonia cells (spermatocytes or oocytes) in treated individuals (6, 7) to large-scale studies involving the specific locus gene mutation test in mice (8). The most commonly employed are somewhere between these two extremes. Examples of routine tests would be the dominant lethal test (9) and the heritable translocation test (10).

The Mouse Specific Locus Test

The specific locus test can measure, theoretically, the type of mutation that is of the most concern, that is, point mutation. This technique is used where one of the parents has a series of homozygous recessive mutant alleles. The normal parent would be exposed to the mutagen and then mated to the homozygous recessive parent. F1 progeny would then be looked at for the presence of one or more of the expressing mutant alleles that could be carried by the tester strain (Fig. 3). This can be done either morphologically through the use of coat color alleles and a number of other types of morphological alleles in the specific locus test that was developed by Russell (8). Malling and Valcovic (11) have developed a technique to detect electrophoretic variants among several biochemical mutants in mice as a specific locus test. In general, if the specific locus test is employed, the size of the test itself will be determined by the number of genes that can be monitored. When measuring rare events occurring at only a small number of targets (monitored genes) a large population must be examined to see a significant change in mutation frequency. The number of alleles generally available in specific locus tests with mice ranges between 5 and 10 so
that, relatively speaking, out of the total genome of the mouse, 5 to 10 genes represent a relatively small group of targets, necessitating a relatively large number of animals be examined to measure effects that would be induced in one or more of these target loci. Thus, these tests can be used to monitor for the point locus type of mutation, but they cannot be practically applied to the evaluation of large numbers of chemicals. However, specific locus tests are important, in that they can identify mutagens that can produce either point mutations or small deletions which are transmissible and can be verified as such by additional matings to show that the phenotype is a true mutation. These tests are suitable for risk assessment of special chemicals. It is difficult to establish the type of mutation definitively, even though one can do subsequent mating experiments to show that the effect is transmissible. The verification system cannot differentiate between a true point mutation and a small deletion. There is also a problem involved in distinguishing mutation from nonmutagenic events such as mitotic recombination or gene conversion events which would mimic mutational alterations, but which would be induced by mechanisms other than those for gene mutation.

**Direct Cytological Examination of Germ Cells**

It is possible to conduct cytogenetic assays which directly examine the germinal cell lines of exposed male or female animals for clastogenic effects. There is a problem in this approach if one looks at exposed germ cells, for example spermatogonial cells, treated with a chemical or with radiation. Experiments have been conducted in which somatic cells were compared to spermatogonial cells for visible chromosomal aberrations \((12, 13)\). In somatic cells a certain frequency of chromosomal aberrations was observed. When the spermatogonial cells of the same treated animal were examined, there was a reduction by as much as 75% in the same type of alteration that would be induced in the somatic cells, indicating that within the germ cell line there is a screening mechanism which will eliminate aberrant cells during cell division. These cells may be cleared out because they have reduced viability compared to the normal cells. A similar loss of transmissible balanced translocations in mouse spermatogonia following x-irradiation also suggested a meiotic screening mechanism. Thus there appears to be a mechanism for reducing the transmission of aberrations to the F1 progeny. Therefore, using spermatogonia or spermatocytes

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**Figure 3.** Specific locus test.
to make a risk-assessment evaluation of the mutagenic capability of a chemical would be very misleading in terms of the actual quantitative expression of these types of events in F1 progeny because most of the events that would be detected cytologically in early stage germ cells will be eliminated prior to the formation of viable sperm. Cytological examination may be a quick and inexpensive procedure, but it is an uncertain procedure when one begins to extrapolate observed effects in the treated animal with predicted effects in the F1 progeny.

**Sex Chromosome Loss**

Sex chromosome loss is another endpoint that is applicable to germ cell analysis (14). This consists of screening for XO individuals following treatment. These effects would be due in most cases to nondisjunction mechanisms. Such assays can be performed quite readily by using strains with X chromosome-linked markers, so that loss of a sex chromosome would result in a morphological change in the F1 progeny.

There is another assay performed with the use of a type of field mouse, in which the sex chromatin material is stained more densely on cytological examination than other types of chromatin, so that one can quickly screen with this particular animal whether sex chromosomes have been gained or lost from the germ cells (15).

**Dominant Lethal and Heritable Translocation Tests**

Direct cytogenetic analysis and the specific locus test are not used as frequently as the dominant lethal and heritable translocations assays to provide a risk assessment for germinal cells.

The following paragraphs present a closer look at the dominant lethal test and the heritable translocation test; these are the most widely used tests for measuring germ cell mutations. A relatively large body of data exists from these assays, and they provide in many cases the basis for current risk assessment of industrial chemicals and drugs.

The dominant lethal test can be conducted in both male and female animals, although typically it is conducted in males. A group of males, anywhere from 10 to 50 depending upon the design of the experiment, would be treated with the chemical. Generally the animals would be treated either acutely in a single exposure or subchronically for 5 days and then mated sequentially to groups of virgin females over the period of spermatogenesis. The females would be caged with the treated males, for, in the case of rats 5 or 6 days and in the case of mice 4 days. After mating, the females are removed, and then at midpregnancy fertilized animals are examined for the number of living or dead embryos and the number of implants (Fig. 4). Fetal mortality is used as an indication of germ cell mutation. Pre- and postimplantation effects can be measured.

![Diagram of the male dominant lethal assay](image)

**Figure 4.** The male dominant lethal assay.

In the heritable translocation test, males are also treated (Fig. 5). This test is generally conducted in the mouse. The males are mated to virgin females (two per male), but, instead of sacrificing the females, they are allowed to litter and the F1 male progeny are retained. These F1 males are then mated sequentially to groups of virgin females and an estimation of sterility or semisterility is made to presumptively identify F1 male progeny carrying balanced translocations. The translocation carriers, once identified presumptively, can be verified cytogenetically by looking at germ cell arrested in meiotic metaphase for translocations.

In general, these are the types of protocols available to study germ cell risk. Evaluating the utility of these tests with respect to meeting the following criteria is important in adopting them as risk-assessment tests: (a) what is the level of the background effect; (b) what endpoints are used to identify mutation (what types of events are detected); (c) can the mutants be verified; (d) what is the general level of sensitivity of the test; (e) what is the ability of the test to detect alterations during different stages in the spermatogenic cycle; and (f)
what is the transmissibility of the event and what are the sex or strain differences. The limitations and advantages of the dominant lethal and heritable translocation assays will become obvious based on these criteria.

First of all, with respect to spontaneous background, dominant lethal tests, at least in mice, have a background of about 6 or 7%, which is relatively high. The reason that the background is relatively high is that alterations in a number of different genes can lead to dominant lethality as an endpoint. Therefore the opportunity for fetal wastage is high even in untreated animals. In the case of heritable translocation, the background is relatively low; at most about one carrier per thousand normal animals (14). This is a comparatively low background and results because the test is measuring a very specific type of chromosome event.

The types of events that can be detected in the dominant lethal test will be gross chromosomal alterations: changes in chromosome structure, changes in chromosome number or nondisjunction events. In the case of the heritable translocation test, only one type of chromosomal structural event, a balanced translocation, is detected. The endpoint criteria in the dominant lethal test can be preimplantation or postimplantation loss. Preimplantation loss is generally derived by comparing the total number of corpora lutea to the total number of implants per pregnant female. The number of implants and the number of living and dead embryos are used in postimplantation evaluation of dominant lethality. For the heritable translocation assay, the endpoint criterion would be either sterility or semisterility in the F1 males followed by the verification of translocations by cytogenetic techniques. Because the heritable translocation technique is a two-generation test, verification of the genetic origin and transmissibility of the effect is possible.

Verification of the genetic origin of dominant lethal effects cannot be accomplished, and is one of the limitations of this test.

The level of sensitivity of an assay is an important consideration for its utilization. What magnitude of animal numbers would one need to detect a significant effect? Statistically, there is a sizable difference between the dominant lethal and the heritable translocation assay. For example, using the mouse dominant lethal test one can provide an acceptable level of sensitivity with something like 40 male animals per treatment group, dosed and mated one male to one female, over twelve 4-day sequences. Approximately 12 of these sequences cover spermatogenesis (Fig. 4). This protocol can give a reasonable level of sensitivity. A two- to threefold increase over the background can be detected with a 95% level of confidence, and roughly 1100 animals would be required. The problem is that because the background is so high to begin with, only a potent germ cell mutagen will lead to a two- to threefold increase. For the heritable translocation, however, to detect roughly an equivalent increase over the background, one would need roughly 4600 animals to achieve a 95% confidence level. Thus, there is a tremendous difference, about four- to fivefold, in terms of the number of animals to achieve comparable levels of sensitivity. Theoretically, however, the heritable translocation assay might detect weaker mutagens. The dominant lethal test is less reliable as a genetic assay for germ cell risk assessment, because it lacks verification as to the genetic nature of the effects. The ability to verify the genetic lesion in the translocation test gives it a weighted advantage.

Detection of cell stage specificity can be obtained with the dominant lethal assay by sequentially mating as outlined. For example, in a mouse, twelve 4-day cycles would cover the route of a cell from speratogonia to mature sperm in the mouse. In the heritable translocation test, the protocol specifies
a continued dosing period over spermatogenesis followed by a single mating period, which will not permit any investigation into the specific cell sensitive stage in spermatogenesis.

The dominant lethal test can be performed on both males and females (16). Female dominant lethal tests are not done routinely simply because: (a) the oocytes are generally less sensitive to chemicals than sperm; and (b) one cannot easily discriminate between cytotoxic effects and true genetic effects particularly in preimplantation loss. Artifactual preimplantation loss also occurs in male-treated dominant lethal tests but would be expected to be less than that found in the female-treated procedure. Good female dominant lethal studies can be performed by taking the time to identify the estrus cycle in the females to be treated and then setting up a regimen in which the treatment can be commenced following ovulation, so that one will be treating more sensitive cells than the oocytes (17).

The heritable translocation test is exclusively conducted in male animals. Strain differences in sensitivity have been reported for both the dominant lethal and heritable translocation assays (Generoso, personal communication).

Both the dominant lethal and the heritable translocation tests have decided advantages and disadvantages with respect to sensitivity and general utilization for evaluating the mutagenic effects of chemicals. Neither of them, in the author's opinion, are optimum tools to do risk assessment because neither of them detect specific locus gene mutations which are the type of alterations of major concern. Studies in Drosophila melanogaster, where both point mutation induction and dominant lethal effects can be scored in the same exposed population, have shown two distinct induction curves for point mutations and dominant lethal mutations (18). At low concentrations of the test mutagens the induced mutations were almost exclusively point mutations (microlesions), and not until significantly high concentrations were dominant lethal (macrolesions) induced. This may be the case in mammals as well. If so, the mammalian assays that are used to screen for mutagenic effects of chemicals may well produce negative results, because it may not be possible to expose the animals to sufficient concentrations of the test chemical to produce dominant lethality (chromosomal effects). At levels of the test material that are relatively nontoxic, the results would appear to be negative by the dominant lethal and translocation assays, although significant numbers of specific locus point mutations might have been produced. Mutagens which induce significant levels of point mutations would be negative in the types of assays that we use as model systems, and only those chemicals which are potent chromosome mutagens will show up as positive. Among a large group of chemicals tested in dominant lethal assays, specifically a group of chemicals on the GRAS list screened by the Food and Drug Administration, 50% fell into the questionable range. That is, one could not make either a clearly positive or a clearly negative assessment from the particular test. This points out another weakness of the dominant lethal procedure as a sensitive measuring device. The inherent resolution of the technique is not good unless large numbers of animals are used or the chemical is a potent mutagen.

If there is some way to quantify the number of point mutations that might lead to chromosomal aberrations, then the use of the dominant lethal assay or heritable translocation assay which measures the chromosomal aberrations would have more reliability in assessing germ cell risk for all types of genetic damage. Most investigators state that the dominant lethal test is used as a signal. If dominant lethality is observed, it can be assumed that other types of mutations are being induced. This may be somewhat naive, since there is no way to relate gene mutations or other types of genetic lesions quantitatively to dominant lethality.

Another point regarding germ cell toxicity concerns negative results and the ability to measure whether any of the administered chemical actually reaches the germ cells. As an example, the amount of administered dimethylnitrosamine (DMN) was measured in the testicular fluid of a treated animal. No alkylation of sperm DNA was measured, but there were measurable amounts of DMN in the testes. Most likely this observation was related to the metabolic capacity of the testes. Certainly one might find DMN in the testes, but DMN is not the molecule that will alkylate DNA. The active metabolite of DMN is an alkylating agent. Activation systems that are found in the testes are not capable of forming the molecule that will alkylate the DNA and produce germ cell mutations. Using systems to radiolabel materials and then looking for the presence of label in germ cells does not give a true measure of whether or not a negative result is true or false. The chemical might be present, but there may be no biological effect of the chemical because the testes cannot produce the necessary bioactivation.

In conclusion, one has the choice of developing better mammalian test systems, using submammalian test systems such as Drosophila to measure germ cell effects, or trying to gain an understanding of the relationship between point mutation (microlesion) induction and chromosome (macrolesion) induction in mammals. If such a relationship can be
quantified in some way, the accuracy and reliability of the dominant lethal and heritable translocation assays will be increased considerably, making these tests, which this author now considers to be inappropriate models for determining risk, useful tools in genetic toxicology.

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