Future Research Directions to Study Genetic Damage in Germ Cells and Estimate Genetic Risk

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The late Frits Sobels developed a parallellogram model to estimate genetic risk to humans based on experimental data in somatic cells (peripheral blood) of exposed animals and humans and on data from progeny studies of exposed animals (mice). Recently, an extension to the original parallellogram model was proposed to bridge the gap of extrapolation between rodent and human germ cells by studying sperm samples. The comparison in the parallellogram of rodent/human sperm data with data from rodent progeny tests to derive at an estimate of human progeny at risk is more promising. Therefore, data on all possible end points, DNA adducts, mutations, chromosomal aberrations, and aneuploidy, should be obtained in sperm of exposed rodents and humans. The technology from somatic cell studies is available or adaptable to sperm studies. Sperm samples lend themselves to automated analyses because they are a homogeneous cell population. By flow cytometry or image analysis, large cell samples can be studied per individual. Animal experiments could be conducted in the actual range of chronic human exposure to low doses. The acceptability of extrapolation from the high acute doses so far used in animal experiments to low chronic doses of human exposure could be assessed. Proof could be obtained in human germ cells for the assumption that data from animal experiments can be extrapolated to humans. Data from transgenic rodent systems may play an important role in the extension of the parallellogram approach to genetic risk estimation by providing a link between cancer and genetic risk estimates. — Environ Health Perspect 104(Suppl 3):619-624 (1996)

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Introduction

Why genetic risk estimates?

During a recent IPCS (International Program on Chemical Safety) Workshop on the Harmonization of Risk Assessment for Carcinogenicity and Mutagenicity, the subgroup dealing with germ cell mutagenic risk assessment developed a scheme for the identification of germ cell mutations (1). In this scheme, the male rodent dominant lethal test was identified as the method of choice to recognize germ cell mutations. Based on information from the male rodent dominant lethal test, preventive measures against exposure to mutagens are possible by classifying the chemical as a germ cell mutagen. Many chemicals have been or are being used unavoidably such as ionizing radiation and cancer therapeutic agents, to name the most obvious; for these agents, germ cell mutagenicity in rodents is well established by demonstrating induced transmissible mutations. It is an often-cited fact that the induction of genetic disease in humans has not been proven by epidemiological studies (2); there is a gap between demonstrating induced mutational events (3) and assessing an increased incidence of genetic disease in progeny of exposed humans, which we are currently not able to bridge (4). It will require a huge effort to improve genetic epidemiology in order to provide reliable information on environmentally induced genetic disease. Major improvements are needed in estimating spontaneous mutation frequencies underlying the prevalence of genetic disease and in designing sensitive parameters to determine increases in genetic disease such as sentinel phenotypes (5). Although available epidemiological studies of radiation-exposed populations or cancer patients do not demonstrate induced genetic disease, we should not dismiss the serious danger that environmental exposure to mutagens increases the genetic burden of humans.

In the absence of direct proof of a human genetic risk and its magnitude, the extrapolation from animal models to humans is the only method of choice. Several studies have shown that radiation or chemicals can increase chromosomal aberrations in human germ cells (6-10). These studies provide proof that the human germ cell genome is mutated similar to the rodent germ cell genome; however, the methodologies (human testicular biopsies or human sperm/hamster egg in vivo fertilization) are too impractical to be employed routinely. Modern molecular techniques provide tools to detect genetic alterations including DNA damage, mutations, and aneuploidy in rodents and humans. The sperm studies have the advantage that they cover the extrapolation gaps between rodents and humans, but they have the disadvantage of assessing the genetic alterations in germ cells as opposed to genetically affected progeny. Incorporating the sperm studies into the parallellogram approach of genetic risk assessment (11) will be an important step to improve the validity of quantifying human genetic risk based on animal experiments.

Present Methods of Germ Cell Studies

The dominant lethal test (12) has been mentioned above as the tool to detect germ cell mutagenicity. It is commonly performed with acutely treated male animals that are mated at different intervals after treatment to cover the entire period of male germ cell development (13). This protocol provides valuable information on sensitive stages, which can be different from chemical to chemical even if they are structurally related (14). Because the dominant lethal assay determines the frequency of genetic alterations (unstable chromosomal aberrations) that lead to embryonic death, it does not lend itself to genetic risk quantification. Quantification of germ cell mutagenicity is performed by testing progeny for various marker genes, whereby the information from the dominant lethal
assay can serve as a guide in respect to acute dosing and possible sensitive germ cell stages.

The present methods of progeny studies employ almost exclusively the mouse as the model animal (Table 1). The classical progeny test is the specific locus test that employs six or seven recessive marker genes developed in Oak Ridge and Harwell and uses mouse tester stocks which are homozygous for these genes (16, 17). All progeny of crosses between the tester strain parent (usually the female) and the wildtype parent (usually the treated male) will be heterozygous, displaying the dominant wild type phenotype. If a mutation, spontaneous or induced, leads to a recessive allele at one of the respective wildtype loci in a paternal germ cell, the resulting offspring will express the mutant phenotype. Originally, this test has been used to determine the mutation frequencies in spermatogonial stem cells by permanently mating the treated males to tester-stock females. From the point of genetic risk assessment, spermatogonial stem cells are the most important germ cells because mutations induced in the stem cells will generate mutated sperm throughout the reproductive life of an individual (mouse or man); however, only few chemical mutagens and ionizing radiation induce mutations in stem cells. Most chemical mutagens are effective in later developmental stages such as spermatocytes, spermatids, or spermatozoa (28). It is comparatively difficult to test the differentiated post-stem cell stages with their limited life span. Repeated experiments are necessary to obtain the recommended sample size of 12,000 to 18,000 progeny (29). This is one of the characteristics and, at the same time, the main disadvantage of all progeny tests. Due to the low spontaneous mutation frequency (in the order of 1/10^6) of any of the employed marker genes, large numbers of progeny have to be observed. Clear and easy-to-detect phenotypes are necessary to screen the required numbers of progeny, and large animal facilities are a prerequisite. They only exist in less than half a dozen laboratories around the world. It becomes understandable that only a small database exists for the specific locus assay even though it has been used for decades.

Another disadvantage of the specific locus assay is the fact that only six to seven loci are being tested. Their mutability varies widely, and the question is often raised of how representative these loci are for the entire genome. To increase the number of tested loci by a factor of 10, the multiple end point approach was developed by Ehling et al. (30). In this approach, the progeny are screened for the 7 specific recessive loci and for 23 loci controlling protein-charge changes (22–24). 12 loci for enzyme activity alterations (25), and about 30 loci coding for dominant cataract mutations (21). The results with ionizing radiation and two selected chemicals, ethyl-nitrosourea (ENU) and procarbazine, indicate that the mutability of the different marker genes varies by an order of magnitude, with the dominant cataract genes being 10 times less mutable than the morphological specific locus genes.

Other genetic end points used for progeny testing have certain disadvantages because they are extremely laborious. The test for dominant skeletal abnormalities (19, 31) has been modified to ease the amount of animal breeding, but the modified test procedure does not prove the heritability of the observed variant phenotypes (20). Histocompatibility loci have also been used, but the identification of variants by skin graft rejection is extremely difficult (26). Dominant visible mutations should be and are assessed along with other phenotypes; however, they require highly experienced observers and are easily overlooked (18).

Transmissible chromosomal damage is tested in the heritable translocation assays. The most arduous procedure by far is the determination of the translocation carriers among the progeny by reduced fertility, followed by cyrogenetic confirmation. Because the spontaneous translocation frequency is two orders of magnitude higher than for the phenotypic markers, it requires fewer progeny to be screened (27).

### Present State of the Art of Risk Estimates

Genetic risk estimates can be seen in two ways: counseling exposed individuals and determining the risk for a certain population. Two major methods of genetic risk quantification for mutagenic agents can be discriminated (32). The indirect method expresses the genetic risk ensuing a certain exposure as a relative increase above the spontaneous mutation frequency and predominantly uses data of the specific locus test or the heritable translocation test. The doubling dose is determined in animal experiments as the theoretical dose of a chemical mutagen that induces as many mutational events per generation as occur spontaneously. The relative genetic risk for humans is then calculated as the ratio between the population or individual exposure dose and the doubling dose derived from animal experiments. The indirect method is applicable to determinations of individual risks, as well as to population risks. It can only be expected that the indirect risk estimate states orders of magnitude rather than precise values. This information on increment over spontaneous mutation frequencies has two major drawbacks. The uncertainties about the spontaneous mutation frequencies leading to genetic disease in humans have already been mentioned. The second problem is the assessment of the population dose.

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Table 1. Tests developed for systematic screening of progeny for transmitted mutations in the mouse (Mus musculus). *

| Tests                          | References | Generation, methods, number of loci screened | Spontaneous mutation rate \^1 |
|-------------------------------|------------|--------------------------------------------|-------------------------------|
| Morphological specific loci   | (16, 17)   | F\(_1\), externally visible traits, 6 or 7 recessive loci | 0.9                           |
| Dominant visible mutations    | (18)       | F\(_1\), externally visible traits, 0.008  |                               |
| Dominant skeletal mutations   | (19, 20)   | F\(_1\), skeletal defects, 12 indicator anomalies | 27*                          |
| Dominant cataract mutations   | (21)       | F\(_1\), lens opacities, −30 loci           | 0.07                          |
| Mutations to altered protein  | (22–24)    | F\(_1\), variant electrophoretic patterns of proteins, 20–30 loci | 0.07                          |
| Specific enzyme activity      | (25)       | F\(_1\), blood enzyme activity changes, 12 loci | 0                            |
| Mutations at histocompatibility loci | (26) | F\(_1\), skin graft rejection by 80 days, 30–50 loci | 1.0                           |
| Reciprocal translocations     | (27)       | F\(_1\), fertility reduction and cyrogenetic confirmation | 40*                          |

*Data modified from Favor (15). \^1 Mutations per locus \times\ gamma (\times 10^{-5}). \^2 Reciprocal translocations per gamete (\times 10^{-5}). \^3 Variants, heritability not proven.
The direct method gives the expected number of newly occurring cases of genetic disease in a certain exposed human population independent of the spontaneous mutation frequencies. It is based on data from the cataract test or other tests employing dominant marker genes. The mutation frequency to dominant traits is determined experimentally for a particular organ system and multiplied by a factor for all possible dominant mutations in humans and the human exposure dose.

The application of both methods to calculations of human genetic risks requires certain assumptions: the various biological and exposure factors affect the magnitude of the induced mutation frequency in a similar way and to a similar extent in mice and humans, and there is a linear or linear quadratic dose response.

The main uncertainty lies in the second assumption. The extrapolation occurs from high acute exposure to low chronic exposures of the human environmental exposure situation. There are no data with any of the above approaches on chronic exposure to chemical mutagens. Fractionation experiments with ENU have shown that the data gave the best fit to a threshold model (33). Saturation of spermatogonial repair capacities is the most likely reason for a threshold to occur. At a low chronic exposure situation, repair capacities might not be affected and all DNA lesions may be dealt with appropriately; however, repair can make mistakes leading to mutations. With chronic exposure, these mutations, albeit rarely induced, can accumulate in the stem cell population. Therefore, it is urgent that exposures other than acute high dose exposures are tested in germ cells.

Mutation data from spermatogonial stem cell studies for a quantification of genetic risk are only available for radiation and a few chemical mutagens such as the alkylating agents ENU, mitomycin C, procarbazine, and triethylenemelamine. First attempts have been made to quantify the genetic risk by calculating the doubling dose based on heritable translocation induction for the occupational mutagens acrylamide and ethylene oxide (34,35). These scanty data demonstrate the need for improved methodologies that allow a quicker gathering of quantitative germ cell information and require fewer animals.

Future Directions of Germ Cell Testing

Future work on germ cell mutagenesis will probably extend in two areas. The relevance of the mouse data will have to be improved by using genetic markers that are relevant to human hereditary diseases, and the development and use of mouse models for human genetic diseases and developmental defects will be a major goal of future work with the traditional progeny test. Another trend for future developments includes molecular techniques to assess mutational processes. One promising development is the introduction of transgenic mice in mutation research. Another emerging application of molecular techniques is directed towards the analysis of sperm from experimental rodents and humans for mutations, DNA damage, and aneuploidy.

Transgenic Mouse Germ Cell Studies

With the transgenic mouse systems using the λ shuttle vector lacI, lacZα (Big Blue mice), it was shown that mutations induced by ENU in male germ cells could be successfully assessed (36). Transversions, transitions, and deletions of base pairs were identified (37). Interestingly, the spontaneous mutation frequencies are an order of magnitude higher in somatic tissues than in male germinal tissue (38); however, the mutation frequencies induced by ENU and chlorambucil are mostly in the same order of magnitude in testicular tissue as in somatic tissues (39). The database contains only these two model germ cell mutagens so far (40).

The advantage of the transgenic mouse system is that somatic and germinal cell samples can be studied within an animal to quantify and compare the mutational events that may lead to cancer or genetic disease. The mutations can be sequenced and the nature of the mutational process can thus be determined. A collaborative study on transgenic germ cell mutation assays, which has been initiated recently (41), will generate data for ENU (as the standard), methyl methanesulfonate, and isopropyl methanesulfonate. Details of the sampling protocol (site and timing) for male germ cells and procedures of lambda packaging of DNA will be tested and standardized. It remains to be determined how well the transgenic mouse systems recognize germ cell mutagens qualitatively, in regard to germ cell stage sensitivity, and quantitatively.

Sperm Assays

Molecular techniques can now be applied to studies of sperm for all classical mutagenicity end points, mutation induction, DNA breakage, and aneuploidy. These methods for the first time provide tools to compare germ cell effects of external exposure between rodents and human subjects. The comparison, on a quantitative basis, will substantiate or disprove the validity of the classical extrapolation of animal data to human genetic risk.

A method developed by Parry et al. (42) can detect base changes in restriction enzyme recognition sites (RSM). This method is based upon the detection of DNA sequences resistant to the cutting action of specific restriction enzymes and the amplification of these resistant sequences using polymerase chain reaction. The RMS method can be used to study induced base changes in any species, tissue, and genes of known DNA sequence for which unique DNA primers are available, and which contain a number of unique restriction enzyme recognition sites. The RMS method has been adapted to testicular samples, and experiments using the technique to compare mutation induction between somatic and sperm cells and between rodent and human tissue samples are presently under way (JM Parry, personal communication).

To detect DNA breakage, the single cell gel electrophoresis assay, the comet assay, has gained worldwide recognition (43,44). DNA breakage is required for the formation of structural chromosome aberrations (clastogenicity), and thus the comet assay can serve as an indicator test to assess the potential clastogenic effect of a chemical. The application of the comet assay to sperm encounters certain technical problems that are being addressed and that will be solved soon. It is very likely that this technique will be much quicker than the laborious and time-consuming analysis of structural chromosomal damage in first cleavage divisions of the mouse sperm genome after in vivo fertilization (45) or of the human sperm genome after in vitro fertilization with hamster eggs (7-10). Researchers hope that the comet assay in sperm will give a qualitatively similar answer to cytogenetic sperm assays as it already does in somatic cells.

Aneuploidy is a major cause of human genetic disease; however, this mutational end point has been neglected in genotoxicity testing guidelines and in genetic risk assessment exercises because validated test procedures were not available. In recent years, molecular cytogenetic techniques have brought a major breakthrough. Labeled repetitive chromosome-specific DNA probes were employed to detect the
absence or additional presence of individual chromosomes in nondividing cells. The fluorescence in situ hybridization (FISH) technique using these probes has been successfully employed to mouse and human sperm for the detection of nullisomy or disomy of individual chromosomes and diploidy of the sperm genome (46–49). At present, several laboratories in Europe have started to use the FISH method to analyze human sperm from individuals after chemotherapy or unsuccessful self-poisoning attempts and to compare the data with those of rodents (rats and mice), which were treated according to the human exposure scheme. This technique bears great potential to finally provide a validated internationally accepted procedure for aneuploidy testing in genotoxicity test guidelines and to generate data for the hitherto missing end point in genetic risk estimates.

The Parallelogram Approach
A parallelogram model to estimate genetic risk to humans was developed by F.H. Sobels (11); this model was based on experimental data in somatic cells (peripheral blood) of exposed animals and humans and on data from progeny studies of exposed animals (mice). In this parallelogram, DNA adduct measurement is regarded as the internal dose assessment (50). Recently, an extension to the original parallelogram model was proposed (Figure 1) to bridge the gap of extrapolation between rodent and human germ cells by studying sperm samples (51).

It is very difficult to extrapolate from mutation rates in somatic cells to rates of genetically altered progeny. It is more promising to compare rodent and human sperm data with data from rodent progeny tests to derive an estimate of human progeny at risk. Therefore, data on all possible end points, DNA adducts, mutations, chromosomal aberrations, and aneuploidy, should be obtained in sperm of exposed rodents and humans. Sperm samples lend themselves to automated analyses because they are a homogeneous cell population. By flow cytometry or image analysis, large cell samples can be studied per individual. Animal experiments could be conducted in the actual range of chronic human exposure to low doses. The acceptability of extrapolation from the high acute doses so far used in animal experiments to low chronic doses of human exposure could be assessed. Proof that data from animal experiments can be extrapolated to humans could be obtained in human germ cells.

Conclusion
The area of mutation research developed from radiation genetics, and the concern was that heritable damage could be induced not only by ionizing radiation but also by mutagenic chemicals. Thus, in the beginning, the genetic burden to future generations was the driving force for the development in the research area. With the recognition of correlations between somatic mutations and cancer development and with the observation in the mid-1970s that rodent carcinogens were also bacterial mutagens, the concern and the efforts were directed towards carcinogen identification. Mutation research benefited from this concern by the introduction of international guidelines for mutagenicity testing; however, the original concern of chemically increased genetic ill health in the human population was only maintained by a few researchers. Presently, the technologies of molecular genetics provide tools to open new horizons to germ cell mutagenicity testing, and the field of mutation research is receiving new impulses for improvement of genetic risk estimation.

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