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THE POTENTIAL FOR NEW METHODS TO ASSESS HUMAN REPRODUCTIVE GENOTOXICITY

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Running Title: **NEW METHODS FOR REPRODUCTIVE TOXICITY**

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There is a very great need to develop a better understanding of the nature and risk of heritable damage to the human genome. As this symposium amply demonstrates, medical treatment, environmental exposure and human heritable predisposition are important, poorly understood sources of risk. We sorely lack the methods to make timely and sensitive observations of heritable mutagenicity in the human. Measurements in experimental mammals can be done to a limited extent and at great expense. However, the extrapolation of such results to the human is very uncertain, particularly when there is no background of human information to use as corroboration or calibration.

To me, the definitive, heroic attempt to measure human heritable mutations has been in a-bomb survivors in Japan as described in this volume by J. Neel. This large, heavily mutagenized population is perhaps the most likely place one could find induced human mutation, and yet the extensive search for chromosomal and electromorphic variants has so far been dramatically negative (18,19). Conceivably this is because humans are blessed with low heritable mutagenicity, but more likely this result is a realistic reflection of the inadequacy and insensitivity of our current methods.

What then are the prospects that suitable methods can be developed and that we can someday readily measure the background of human heritable mutagenicity and identify and quantify induced effects in large and small populations?

GENERAL PRINCIPLES

Heritable mutations are induced in the germ cells of exposed individuals and are expressed in the immediate and future progeny through such impacts as birth defects, the loss or alteration of specific gene function, and the evolution, heterogeneity or degradation of the human genome. The initial events in the DNA of the germ cell are analogous to corresponding DNA-damaging events in somatic cells. However, male and female germinal cells differ substantially from each other and from somatic tissue, and one would not be surprised to find that the cells of these tissues have differing pharmacologic accessibility, metabolic activation, DNA damage, DNA repair, and probability of survival with various types of DNA change. Animal studies indicate that few somatic mutagens are spermatogonial mutagens, and that some in vivo mutagens are not heritable mutagens at all. Substances that are pan-mutagenic may well produce very different amounts and spectra of genotoxicity in somatic and heritable contexts.

Measurement strategies differ dramatically between somatic and heritable mutation detection. The specific-locus somatic mutant is a rare event among cells. Background rates range from one cell in a hundred-thousand to one cell in ten million, depending on the target size and susceptibility to the effect. Detection is necessarily at the level of the single mutant cell, but is in a setting where there are millions of cells in which to search for the rare event. In contrast, the specific-locus heritable mutant is a rare individual among many. Each cell of that individual is marked with the same mutational change of DNA, and the phenotype, if it is expressed, can range from a subtle local change in metabolism to a gross or systemic alteration. Detection need not be cellular, and material to sample is generally plentiful. Unless the individual presents clinically, the challenge in measurement is to study enough individuals or genes to find the rare, sporadic mutational events. The analysis generally requires confirmation of paternity and detailed testing of both parents to distinguish between a transmitted and an induced event.

Intermediate between somatic and heritable effects, are effects in germ cells. The target material is obviously germinal, as is the metabolic setting, repair, etc. However, germ cells, particularly sperm, express rarity and cellularity in much the same way that somatic cells do. Thus one can conceive of measuring the rate of mutation in the millions of sperm in a single ejaculate, but to do this one must be able to identify the mutants at the single-cell level.

DNA METHODS

The prospects for identifying novelty in the DNA of a child with respect to the DNA of both parents was reviewed several years ago by a working group of experts (2). The technical limit of such an approach would be to sequence the three sets of DNA and to examine for any coding patterns that appear in the child's genome but not in any of the four haploid genomes of the parents. The task of error-free sequencing of 2×10^{10} bases is so overwhelming, that the scientific world is currently heavily debating whether it is worth it or even feasible to sequence a single reference human genome. Mutations in the human genome will run at background rates of approximately one per 10^8 bp. Given the present expense, capacity, and error-rate of DNA sequencing, it is incomprehensible to recommend mutation detection by sequencing all or part of the DNA of multiple families. However, the likely commitment in the near future to order and sequence the human genome should promote rapid development of DNA processing capability. Perhaps someday soon it will be possible to consider sequencing as the method of choice.

Restriction fragment length polymorphism (RFLP) is technically a more tractable approach than direct sequencing, but it too is probably also beyond practical application (2). With this method, base changes that either create new restriction sites or eliminate old restriction sites can be signaled by the appearance of novel restriction fragments when comparing the child to the parents. The combination of a single probe and single 6-cutter restriction enzyme can test for change in 18 bp per haploid genome. Thus it would require something like 10 restriction enzymes, 278 probes, 1000 children and 14,000 gels to find one background event at the expected normal mutation rate.

Efficiency is somewhat better for two other DNA methods. Myers *et al* (12) have shown that single base substitutions and other DNA changes can be detected by using RNASE A to cleave mismatches within RNA:DNA heteroduplexes. Standard, labeled, human genomic RNA fragments are hybridized to parental and child's DNA, and digested with RNASE A. Fragmentation of the labeled RNA is tested by sizing with gel electrophoresis, and the patterns are compared between parents and child. A variant of this method has recently used RNA:RNA duplexes to identify base changes in mutant human messenger RNA (3), an approach that might allow focusing a screening effort exclusively on functional exons. The second DNA method uses DNA:DNA duplexes and identifies the mismatches by melting lability as judged by mobility on gradient denaturation gels (2). While these two methods are the most efficient that have been tested to date, they probably fall one or a few orders of magnitude below the level that is practical enough to implement.

Finally an approach referred to as subtractive hybridization has been suggested by Church (2). While not yet bench-tested, this approach promises large improvements in efficiency, but raises many technical uncertainties. The method is based on genomic screening using a library of all possible DNA 18-mers. Tetramethylammonium chloride (TMCL) is used during hybridization to minimize heterogeneity of complementation due to at:gc differences. To begin, one reduces the combined DNA of both parents to fragments of 100 bp or so, hybridizes the fragments in large excess to the library of 18-mers near the Tm in the presence of TMCL. Unbound 18-mers are then salvaged and represent sequences presumably not present in either parent. The child's genome is similarly fragmented and then hybridized to the salvaged 18-mers. Any 18-mer duplexed to the child's DNA is separated and treated as a presumptive novel sequence. The potential efficiency of this method stems from parallel processing of the entire genome. However, judging from yet to be published analyses done at this laboratory by E. W. Branscomb, one crucial limitation is that perfectly matching 18-mers will bind only about 100 fold greater than will single-base mismatches. This ratio is insufficient to achieve rare event

detection in a single pass through the genome. It remains to be seen whether successful multiple pass methods using this principle can be devised.

It seems unlikely that we have heard the last word about DNA methods to identify mutation, and the working panel was optimistic that variants of the above methods or perhaps entirely new methods will be discovered as the DNA technologies continue on their rapid development. In anticipation of this, DNA from a-bomb families is being collected and stored by the Radiation Effects Research Foundation in Hiroshima.

One other point about DNA methods is the possibility of applying them at the single-cell level. Present DNA technology is unable to clone or otherwise amplify a single diploid or haploid amount of DNA. However, the polymerase chain reaction can now amplify specified sequences up to levels suitable for conventional DNA analysis (17). Its present lower limit of input DNA is equivalent to the genomes of 50 cells. Thus with only modest improvement, it may well be possible in the near future to apply this DNA method to the analysis of mutation rates in sperm.

PROTEIN METHODS

Specific locus electrophoretic methods, such as the one-dimensional gel techniques described in detail elsewhere in this volume (Neel), give a background rate of 0.5×10^{-5} mutations per locus per generation in the Japanese offspring studied at Hiroshima and Nagasaki. Applied to 30 loci per offspring, these methods have essentially exhausted the available population of children and have resolved no significant change between offspring of control and exposed parents. Why this is so is a matter of debate, but my current opinion is that the lack of an observed radiation effect is because this method requires a mutationally derived change of charge in the face of residual enzyme function. Ionizing radiation is most likely to produce its effects as deletions which will often result in absence of gene product or strikingly altered function.

Two-dimensional electrophoresis adds a significant amount of new information about each protein, and introduces parallel processing of perhaps hundreds of proteins at once (13,14). It is subject to the same inefficiency as the one-dimensional gels if, for normally homozygous gene products, one cannot recognize loss of half of the signal due to null mutation or dramatically altered gene product. A recent report by Giometti *et al* (4) applied this method to the offspring of irradiated and chemically mutagenized mice and gave very promising results, including the identification of null mutants. Confir-

mation of this result and establishing the availability of sufficient numbers of suitable proteins in readily sampled human material could well make this a practical method.

Another approach to detection of mutant proteins is to use monoclonal antibodies either to identify specific protein abnormalities or to demonstrate absence of a normal protein (8). The first of these, using known abnormalities, has the advantage of giving a positive signal when the mutant is present. However, typical abnormal mutant proteins, such as sickle hemoglobin, generally have single amino-acid or other very small changes in the protein. Antibodies can be found to recognize such changes (7), but the genetic target size for a single amino-acid change (corresponding to a single base change in the DNA) is 100 to 1000 times smaller than that for the whole gene. Thus these mutants are extremely rare, and for some types of damage, such as ionizing radiation, unlikely to be produced. The alternate strategy is to use the antibodies to search for the absence of gene product. Here the problem will be using a negative signal and being subject to all of the technical and biological reasons why a particular gene product may be phenotypically turned off or modified beyond recognition. Perhaps the only way to make this strategy work is to have an allelic or otherwise closely related gene product to act as a control. The best example of this narrowed approach is the somatic mutation test for glycophorin A (9).

Half the human population is heterozygous for glycophorin A, having both the M and the N allele of the gene. The two alleles differ in the first and fifth terminal codons and are located on chromosome 4. Each allele is codominantly expressed as 250,000 copies of protein found in the membrane of every red blood cell regardless of what is happening to the other allele. Monoclonal antibodies easily discriminate the two allelic proteins, and one can literally fluorescently stain each red cell both red and green with the respective allelic antibodies and thus test whether or not each cell has a normal complement of the two proteins. Studies on normal subjects indicate that two in every hundred-thousand red cells are missing one of the proteins while expressing the other normally. Chemotherapy patients show increased levels of such hemizygously expressed cells (21), and a-bomb survivors, 40 years after radiation exposure, show a linear, nonthresholded response of glycophorin variants to radiation dose (9).

Glycophorin A could be measured in the blood of children of a-bomb survivors and presumably at rates approximating two per hundred-thousand children (increasing with dose) one would find children with hemizygous expression defects in all of their red cells. But such a single-locus application

would make little sense. Rather this test is an example of a cellularly-based specific-locus test, originally designed for somatic mutation, and possibly a model for methods that could be applied to sperm.

The two protamines are a corresponding paired set of proteins that might serve as mutant markers in human sperm. These proteins are expressed in the haploid state during the late stages of spermiogenesis. They replace histones as neutralizing and stabilizing proteins of DNA. Monoclonal antibodies have been developed to mark the two protamines, and experiments to determine whether either one can be missing from individual sperm heads are just getting under way (20).

OTHER METHODS

Chromosome analysis of fetal and newborn cells is a well established genetic method for studying heritable damage. Several variants of these approaches may add new dimensions to future studies. The first of these is the search for circulating fetal cells in maternal circulation during early stages of pregnancy. Herzenberg and colleagues (6) showed that this can be done with HLA markers based on differences in maternal and paternal genotypes. However, a more general approach perhaps can be based on developing good cytochemical markers directly for embryonically expressed proteins. Once identified, the cells can be studied by cytogenetics if they can be brought into cell division, or by DNA hybridization (15,16), polymerase chain reaction (17), or surface markers if they cannot be made to divide. That such methods should be able to identify trisomy 21 has been shown in several studies (5,22).

The second cytogenetic method returns once more to sperm, and is a new, increasingly used technique to karyotype the haploid chromosome set of single human sperm after they undergo decondensation in a hamster oocyte (1,11). This is a labor intensive method, ill suited to population monitoring, but the only technique we have to measure paternal genotoxicity directly in easily sample material. Available results have already shown the appearance of abnormal chromosome number for each of the human chromosomes, response to ionizing radiation and other genotoxicants, and the expected types of segregation in men with balanced translocations. The method has also shown an unexpectedly large number of structural and numerical chromosome abnormalities in otherwise normal men.

DISCUSSION

The immediate prospects are not good for practical methods for measuring the human heritable mutation rate. The methods I have discussed range from speculative to impractical, and at best are

sensitive enough only for large numbers of subjects. They are clearly not suitable for contemporary environmental problems, such as evaluating the potential mutagenic effects of a drug, an accident or a dump site. Given the rapid development of DNA methods and the current status of two-dimensional gel electrophoresis, there is some hope that the intermediate prospects may be better. Perhaps with an imaginative leap and the diligent application of resources we will have reasonable methods in another decade.

In contrast, the prospects for useful cellular-based male germinal methods seem more promising and immediate. Effective specific locus methods for sperm are already conceivable and may be practical in a few years. Obviously such methods will not predict heritable effects definitively, but they will provide direct information on reproductive genotoxicity and should contribute significantly to many current medical and environmental situations where genetic damage is suspected.

In the meanwhile, one hopes that funding and research interest into reproductive genotoxicity will continue at a reasonable pace. At issue is of course the development of new methods, but also the better understanding of mechanism, species extrapolation, and general reproductive biology. We owe it to the genetic well being of future generations to remain dedicated to the solution of these problems and to the protection of the human genome.

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