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# Tests for Biologic Markers of Genotoxic Exposure and Effect

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*Abstract.* Markers of genetic damage are being used increasingly to understand and prevent environmentally related human adverse health effects. A major example has been the application of such markers to the prediction of chemical carcinogenicity. Over the past 15 years, hundreds of test systems in microorganisms, cell cultures and animals were devised and applied to this end. In spite of early successes, recent results show a discouragingly low, 60% agreement between the genetic tests and conventional, whole animal, long-term carcinogenicity assays. Corresponding efforts to predict the heritable mutagenicity of chemicals using genetic tests that do not involve heritability have given similar results.

New technologic developments for the first time are letting us make such genotoxicity measurements directly in human subjects. Examples include detection of DNA adducts, measurement of somatic mutations and improved cytogenetic methods. There is also the possibility of soon finding sufficiently sensitive methods to estimate heritable mutagenicity as a predictor of damage to future generations. These biologic markers of genotoxicity are useful for estimating human exposure and effect, for identifying toxic environments, for monitoring cancer chemotherapy and for identifying susceptible populations. They offer a major new challenge to epidemiology and public health.

*Key words and phrases:* Biologic markers, genotoxic exposure, environmental health effects, carcinogenicity, mutagenicity, genotoxicity.

Societal interest in genotoxic exposure stems from two concerns: the fear of cancer secondary to somatic mutation; and the fear of birth defects and decreasing genetic fitness secondary to heritable mutation. We need to identify agents that can cause these effects, to understand the underlying dose-response relationships, to identify exposed populations and to estimate both the magnitude of exposure and the risk of adverse health effects in such populations. Biologic markers refer either to evidence in surrogate organisms or to the expressions of exposure and effect in human populations.

## GENOTOXICITY TESTS AS IDENTIFIERS OF CARCINOGENS

A prevailing theory of carcinogenesis is that genetic damage to somatic cells initiates the cancer process. Genetic damage also has an important secondary role in promotion and progression of the early cancer cells. Based on the biologic generality of genetic mechanism and damage, one can argue that an agent's potential to cause somatic genetic damage is measurable in any of many biologic systems. These concepts received dramatic emphasis 15 years ago when Bruce Ames and colleagues showed that a simple bacterial system supported by mammalian metabolizing material was able to identify many chemical carcinogens (Ames, Durston, Yamasaki and Lee, 1973). The Ames test used the back-mutation of several different mutationally-derived histidine-dependent strains of *Salmonella typhimurium*. The strains were made defective in DNA repair and other defenses in order to maximize their sensitivity.

There are hundreds of similar genotoxicity tests using other bacteria, isolated DNA, mammalian cells (including human) and intact organisms such as Drosophila and rodents. Many genetic endpoints are tested for, including:

*Mutation*—a genetically based change, generally expressed as a change in drug resistance, a

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new metabolic requirement, or an altered gene product.

*Base substitution*—a change in the genetic 4-base code involving the replacement of one DNA base for another (e.g., ATGCTC becoming ATGATC).

*Frame shift*—insertion or deletion of bases such that there is an offset of the triplet reading frame of DNA, resulting in a string of incorrect amino acids as the gene is being translated into protein.

*Rearrangement*—deletion, insertion, inversion or translocation of DNA sequence.

*Chromosome aberration*—microscopically visible change in chromosome structure.

*Aneuploidy*—change in chromosome number.

*Sister chromatid exchange*—an easily scored exchange of chromosome strands within replicating chromosomes.

*Micronuclei*—small secondary nuclei within cells, indicating breakage of chromosomes.

*DNA repair*—evidence that one or more types of repair of DNA damage has taken place.

*DNA adduct*—a binding of exogenous chemical to DNA.

*DNA structure damage*—molecular weight change, strand breakage or other gross alteration in the structure of the DNA.

These tests collectively are known as short-term tests for genotoxicity, as contrasted to the conventional, long-term or 2-year, rodent bioassay for cancer. There are also short-term tests for transformation and promotion, two not necessarily genotoxic subcomponents of carcinogenesis, and there are short-term direct cancer bioassays, systems in which cancers are induced in animals over periods of months rather than years.

The early data from the short-term tests for genotoxicity confirmed the results from the Ames test by showing agreements greater than 90% with the results of cancer bioassays (see, for example, Purchase, Longstaff, Ashby, Styles, Anderson, LeFevre and Westwood, 1978). In addition, the various short-term tests correlated closely with each other, were stable and sensitive, and were reasonably practical on a large scale. Technically oriented countries rapidly adopted the short-term tests, using them to screen new and suspect old chemicals, and even regulating on the basis of the results. By the early 1980s, the short-term test data base contained over 10,000 mostly positive chemicals, only a small fraction of which had been tested directly for carcinogenicity.

However, subsequent analyses of the agreement between short-term tests and carcinogenicity have fallen to 60%, slightly better than chance (50%), but far from the near perfect results described initially (Zeiger and Tennant, 1986; Tennant, Margolin, Shelby, Zeiger, Haseman, Spalding, Caspary, Resnick, Stasiewicz, Anderson and Minor, 1987; Mendelsohn, 1988). There are a dozen or so explanations for why this has happened, including:

- limitations of the somatic mutation theory of cancer initiation,
- inability to predict carcinogens that act through promotion and progression by nonmutational mechanisms,
- uncertainty over which type of genotoxicity is relevant,
- difficulties in extrapolating from multiple organisms to the human,
- difficulties in extrapolating from in vitro to in vivo conditions,
- difficulties in extrapolating from one chemical class to another,
- oversensitivity of the short-term tests,
- undersensitivity of direct assays for carcinogenicity,
- inadequate or inappropriate metabolism of the chemicals under test conditions,
- inability to mimic the characteristic organ-specificity of chemical carcinogenesis.

Today there is serious questioning about both conventional cancer screening and the use of short-term tests for genotoxicity. In a world where tens of thousands of chemicals are in use, and thousands of new chemicals appear annually, conventional cancer bioassays are too slow and too expensive to be used for routine screening. They are also controversial because of their low sensitivity, their necessary reliance on high doses, disagreements in interpretation of results, the lack of good agreement between results in rat and mouse, and other issues of extrapolation to human risk. The short-term tests for genotoxicity, although much more convenient, obviously are difficult to justify when agreement with the current standard for carcinogenicity is so close to random.

Considerable attention must still be given to the careful analysis of short-term tests vis-a-vis carcinogenesis. Gaps in the data bases need filling in, chemical classes need to be expanded, the tests need further emphasis on sensitive methods that can be applied to intact animals and every effort must be made to better understand the causes of disagreement. Also, substantial effort is needed in the statistical interpretation of results, particularly when there is so much uncertainty about how either type of end point relates to human risk. Although it is clear that we must discard the original hope for good prediction of carcinogenicity by a simple test of genotoxicity, there is still reason to believe that carefully tailored, carefully interpreted,

short-term tests for genotoxicity will have a significant role in estimating carcinogenic hazard.

## SHORT-TERM TESTS FOR GENOTOXICITY AS PREDICTORS OF HERITABLE MUTAGENICITY

Fruit flies, rodents and other sexually reproductive organisms can be used to observe directly for abnormal outcomes in offspring following chemical and other exposures of the parents. End points include interrupted pregnancies, mortality, sterility, gross birth defects, heritable chromosome aberrations and specific locus mutations (i.e., changes occurring in specifically predefined genes). The closer the organisms are to the human and the better the tests simulate genetic damage of human relevance, the more difficult and expensive the tests are to carry out. They have not been used for routine screening because of this.

Again returning to principle, the generality of genetic damage suggests that the short-term tests for genotoxicity might well serve as surrogates for the heritable assays. Data on this question are in the literature, but are characterized by a heavy bias toward mutagenic rather than nonmutagenic chemicals, and a very incomplete sampling of both the short-term and the conventional heritable tests. The results, such as they are, suggest that the reproductive mechanisms have special attributes involving chemical access to the germ cells, metabolism of chemicals by the germ cells, repair of DNA damage and selection by the uniquely reproductive meiotic mechanism of cell division, all of which are poorly simulated by short-term tests. Thus the tests are marginally predictive at best (ICPEMC Committee 1, 1983; Bridges and Mendelsohn, 1986), or not predictive at all (Russell, Aaron, de Serres, Generoso, Kannan, Shelby, Springer and Voytek, 1984). The assessment is further complicated by the total absence of usable data on induction of heritable mutation in the human.

## THE PROSPECTS FOR IMPROVEMENT THROUGH APPLICATION DIRECTLY IN HUMANS

It would be easy to be discouraged about this state of affairs were it not for the incredible progress that is being made today in DNA methodologies and other approaches to studying genotoxicity. Of particular relevance to risk analysis are the impending methods that will allow us to measure markers of exposure and effect in human subjects. Genotoxicity measurements in the human are very challenging because of the inherent rarity of mutational events. Typical background rates for new mutation at a specific locus are one in a hundred thousand to one in a million depending on whether the whole gene or a specific base within the gene is the target. In addition, methods for human use have to deal with the large amount of human genetic diversity and have to work with readily sampled material. Some examples follow of what has been accomplished and of what may soon be in the offing.

### DNA and Protein Adducts

A characteristic of many genotoxic chemicals is their ability when metabolized to an active form to react covalently with DNA and other macromolecules. The methods to measure such DNA and protein adducts are rapidly increasing in sensitivity and scope, and several are now suitable to assess environmental exposure in human subjects. Adducts of ethylene oxide can be detected in the hemoglobin of exposed workers, and are biologically integrated over the 4-month lifetime of the red cell (Calleman, Ehrenberg, Jansson, Osterman-Golkar, Segerback, Svensson and Wachtmeister, 1978). Similarly, several products of cigarette smoke can be assayed in tissues and body fluids (Tannenbaum and Skipper, 1984), and numerous DNA adducts associated with smoking are detectable as well (Everson, Randerath, Santella, Cefalo, Avitts and Randerath, 1986). Some DNA adducts are measurable in homogenates at levels equivalent to one adduct per cell (Randerath, Randerath, Agrawal, Gupta, Schurdak and Reddy, 1985), and at lesser sensitivity, others can be localized by autoradiography or fluorescence to single cells in smears or tissue sections (Baan, Zaalberg, Fichtinger-Schepman, Muysken-Schoen, Lansbergen and Lohman, 1985).

Most DNA adducts are removed randomly or by repair mechanisms in a matter of hours to days, although some persist for long periods. Major difference exist between and within species in rates of repair of DNA adducts, and in the genetic consequences of adduction. In addition, the repair and the probability of mutation are greatly influenced by the location and nature of the adduct. For acute exposures, timing of samples and individual capacities to repair can be crucial to the interpretation of results. For chronic exposure, DNA adduct levels plateau at the balance point between rates of exposure and repair (Swenberg, Richardson, Boucheron and Dryoff, 1985), so that the method measures something closer to dose-rate than to cumulative dose.

### Somatic Gene Mutations

A few methods are now available to count mutant somatic cells in vivo in experimental animals and in human subjects. The first of these for the human was the detection of HPRT-deficient peripheral blood lymphocytes (Albertini, Nicklas and O'Neill, 1986). This method identifies loss of function of a gene on the X chromosome, using drug resistance as a marker. Variations of the method are available for animal

studies and for precise characterization of the DNA changes underlying the loss of function. Increases in mutant cells are observed after clinical cancer chemotherapy, but the signal fades in half a year or so because of selection against the mutant cells.

A second, more recent method, detects the loss or gain of functional gene products of human glycophorin A (Langlois, Bigbee and Jensen, 1986). Glycophorin A is an abundant protein in the red cell membrane, and is coded independently by the pair of codominant, maternally and paternally derived genes (alleles) on chromosome 4. In heterozygotes, i.e., the half of the human population in which the two alleles differ, the quantity per red cell of each allelic product can be measured independently using highly specific monoclonal antibodies and flow cytometry. A red cell demonstrating a gene loss mutation contains a normal amount of one allelic product in the absence of any of the other. A red cell containing twice the normal amount of one allelic product derives from a cell that has probably undergone mitotic recombination, a process that can convert cells to homozygosity and may be another important genetic step in cancer initiation. These are necessarily indirect inferences since the absence of DNA in red cells prevents a direct determination of the underlying genetic alteration. Recently, the glycophorin method was successfully applied to the survivors of the Hiroshima a-bomb. The response in these individuals increased linearly with dose at a slope typical of dose responses in the animal and cell culture literature, and is consistent with a somatic mutation measurement which time-integrates over four decades (Langlois, Bigbee, Kyoizumi, Nakamura, Bean, Akiyama and Jensen, 1987).

It is reasonable to expect the appearance of many more such methods involving other genes and other cell types. An ensemble of methods, with varying capacity for time-integration and representing a variety of DNA lesions and target tissues, should eventually become a major tool for assessing somatic genetic damage in human populations. When coupled to the highly refined information base available for human cancer occurrence, these methods may provide crucial evidence on the role of somatic mutation in human cancer. They can also function as biologic dosimeters in both a toxicologic and therapeutic context, and as general tools for identifying hypersusceptible people and potentially toxic environments.

### Aneuploidy and Chromosome Translocation

The application of DNA probes and hybridization to cytogenetics offers another exciting new prospect for human application of markers of genotoxicity. The probes carry fluorescence markers or enzymes that produce localized staining at the place on the chromosome that has DNA sequence complementary to the sequence of the probe. Markers based on chromosome-specific repetitive DNA are increasingly available to stain the centromeric, and, in one case, telomeric regions of specific human chromosomes (Manuelidis, 1985). In a method being developed in this laboratory, marking the centromere and telomere of chromosome 1 provides an elegant, rapid way to score for translocation, an aberration that is important but difficult to score. In the normal situation the two markers always appear on the same chromosome. When the markers are on different chromosomes, this means that a rearrangement has occurred, with one of the translocating breaks being between the two markers.

Another interesting application of such markers is based on their being visible in interphase nuclei. With single markers one can count the number of copies of a particular chromosome in nondividing cells, including human sperm (Pinkel, Straume and Gray, 1986). Because aneuploidy may be important for carcinogenesis and is the most important cause of human heritable disease, this method has several important practical applications.

Lastly, it may soon be possible to paint entire specific human chromosomes with ensembles of unique sequence probes (Pinkel, Straume and Gray, 1986). Painting of human chromosomes in hybrid cells (e.g., hamster cells with one or a few human chromosomes) is now a routine procedure and is a dramatic demonstration of the power of this method. It has already been used for rapid detection of aberrations. Extending the method to one or more human chromosomes in a human cell is much more demanding, but already two human chromosomes can be partially painted. Painting, once successful, will provide a practical way to measure all human chromosome translocations at a sensitivity that for the first time will permit the assessment of individual burdens of this long-surviving and important type of aberration.

### Heritable Mutation

There is hope that some day we may have methods suitable for measuring the degree of heritable genetic difference between parents and child. The attempt to do this with one-dimensional gel electrophoresis of red cell proteins in a-bomb survivors and their children failed to detect induced mutations because of lack of sensitivity to the type of lesion that was likely produced by radiation (Schull, Otake and Neel, 1981; Delehanty, White and Mendelsohn, 1986). Similar approaches using two-dimensional gel electrophoresis may increase the sensitivity. Meanwhile, methods are in the early stage of development to use DNA differences as the ultimate criterion of mutation

(Mendelsohn, 1986). Ideas being explored include restriction fragment length polymorphism and RNase A sensitivity to detect differences, and a novel method to probe DNA with all possible 18-mers (Delehanty, White and Mendelsohn, 1986; Myers, Larin and Maniatis, 1985). Although these methods are not yet available, and in some cases have not even been shown feasible, the rapid advances being made in DNA methodologies is enough to make one optimistic for the future.

## OVERVIEW

I hope this rapid survey of the current status of markers of genotoxicity will convince you of the importance of this field and of its current status. For hazard detection, tests for genotoxicity have fallen upon hard times, but may yet provide the validated primary screen for the carcinogenicity of particular chemicals. For epidemiologic and risk-oriented studies of human carcinogenesis and heritable mutagenesis, the rapid development of methods to make measurements of genotoxic exposure or effect directly in individual people should have a major impact. Problems of species extrapolation, estimation of exposure, heterogeneity of the human population and fundamental mechanism should be addressable with these methods. Some markers of genotoxicity are already being incorporated into epidemiologic studies, and it is easy to anticipate that these and future methods will be much sought after in the years to come. It should come as no surprise that the processes of validation and then application to epidemiology will present many interesting and important statistical challenges.

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