Chapter 5

New Methods for Measuring Somatic Mutations

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INTRODUCTION

All the techniques for learning about heritable mutations described in chapter 4 rely on comparing DNA or proteins of parents with DNA or proteins of their children to infer the kinds and rates of mutations that occurred in parental germ cells. While that information is of great value in learning about heritable mutations, it comes well after the mutations have actually occurred. Heritable mutation tests are not useful for determining rapidly that an individual or a population has been exposed to a mutagen, so that people who have been exposed can be appropriately monitored and others, who have not been exposed, can be warned and spared exposure to that same mutagen. For this reason, it is desirable to develop tests for somatic cell mutations that might allow inferences to be made about possible heritable effects of some mutagenic exposure. Instead of providing an exhaustive catalog of somatic cell mutation assays, this chapter provides examples of developing technologies that may soon be available to measure somatic mutations in human populations.

It *is not known whether the rates and kinds of somatic mutations in blood or any other nongerminal tissue have any predictable relationship with rates and kinds of germinal mutations; it is only with further development and testing of those methods, together with methods for detecting germinal mutations, that such relationships might be elucidated.* However, even without knowing the precise relationships between somatic and germinal mutations, somatic tests may provide an early warning that people are being exposed to environmental mutagens. Furthermore, identifying and characterizing somatic mutants will provide important guidance for reducin$_g$ exposures to mutagens and carcinogens, and is relevant to the study of aging and of carcinogenesis.

Exposure to mutagens may increase the risk of developing some kinds of cancer. In addition, increases in the frequencies of second cancers occurring years after treatment of a first cancer with radiation therapy have been noted (see e.g., ref. 11). In a study of survivors of cervical cancer, the risk of developing a second cancer was found to be highest for organs close to where radiation therapy was delivered, strongly suggesting that the development of second cancers maybe related to the mutagenic effects of radiation (11).

Measurement of somatic mutants offers the possibility of identifying differences in somatic mutation rates over time within individuals, among different individuals, and among different populations. Such studies can be used to examine possible associations between exposures to particular environmental or occupational agents and the frequency of mutations. Ultimately, they maybe used to identify individuals or populations who are exposed to mutagens. This information could be useful to the regulatory agencies in identifying genotoxic compounds and their potencies.

To be useful for studying human populations, somatic tests must be based on reasonably accessible cells (e.g., blood cells) and on recognizable markers for mutation (e. g., variant proteins or enzymes). The most easily sampled human tissue is blood, and thus far, all the somatic tests that have been developed for studying mutations have used red and white blood cells. Somatic cell mutation assays focus on two types of cellular changes that result from mutations: 1) alterations in phenotypic properties of cells, such as changes in cells' resistance to drugs added to their growth media; l and 2) alteration in a gene product, such

'Studying drug resistance in humans is inherently limited to studying single copy genes, such as genes on the X chromosome (including the hprt gene) or dominant selectable markers.

as a protein. The first type of change has been most extensively studied using the mutation assay based on 6-thioguanine (6-TG) resistance, and the second type has been studied using two types of proteins found in circulating red blood cells.

Instead of examining DNA directly, current and developing somatic tests detect rare mutant cells based on marker phenotypes among large numbers of mostly identical normal cells of a specific type. Although somatic cell mutation assays detect phenotypic events that represent, as closely as possible, actual mutations in DNA, the data are expressed as numbers of *mutants,* rather than in numbers of *mutations*. Various analytic techniques have been used to estimate the mutation frequency from the observed mutant frequency (97).

DETECTION OF SOMATIC MUTATIONS IN WHITE BLOOD CELLS

Albertini and his colleagues (3) and Morley and his colleagues (79) have developed assays using human peripheral blood lymphocytes to detect mutations that result in a phenotypic change in these cells. The assays are based on the ability of mutant T-lymphocytes, which are present in the peripheral blood at the time the sample is drawn, to replicate DNA and to grow under conditions that do not allow growth of normal cells. The mutant cells are identified by their resistance to 6-TG, a drug that is normally toxic to human T-lymphocytes.

Normal cells produce an enzyme, hypoxanthine-guanine phosphoribosyl transferase (HPRT), that metabolizes 6-TG to a toxic chemical, killing the cells. Mutations in the *hprt* gene (the gene that directs the production of HPRT), can result in cells that do not produce HPRT. The mutation from the normal gene *(hprt^{+})* to the mutant gene *(hprt-)* makes the cell resistant to 6-TG (6-TG') because they cannot metabolize 6-TG into its toxic form.

This particular selective system has been shown to meet all the necessary criteria for a valid genetic assay: 1) the 6-TG^{r} mutants are genetic variants that "breed true": whether grown with or without 6-TG, they remain resistant to 6-TG when challenged with the drug (3,79); 2) exposure of laboratory cultures of T-lymphocytes to known mutagenic agents such as X-rays or ultraviolet radiation increases the frequency of 6-TG^{r} mutants, indicating an increase in mutation in these cells (17,160); 3) there is a demonstrable change in the gene product—a deficiency in the enzyme HPRT; and 4> there is a demonstrable change in the DNA sequence of the *hprt* gene—a direct demonstration that the *hprt* gene has mutated (4,143).

Selection for HPRT Mutants in T= Lymphocytes

Nicklas (97) described two methods for identifying mutant T-lymphocytes in human blood samples. The first is the autoradiographic assay. After blood is drawn, the T-lymphocytes are purified and frozen. On thawing, the cells are split into two cultures, both of which are treated with phytohemagglutinin, a chemical that stimulates DNA synthesis. One of the cultures is also treated with 6-TG. The other culture is an untreated control.

A label, radioactive thymidine, is added to both cultures, and all cells capable of DNA replication and growth incorporate the labeled thymidine into their DNA. In the the control culture not exposed to 6-TG, essentially all the cells divide and incorporate thymidine, In the culture exposed to 6-TG, normal cells are killed and therefore do not incorporate the radioactive thymidine into their DNA. The cells from both cultures are then fixed on microscope slides, covered with an X-ray film or emulsion, and then developed using standard radiographic techniques. Cells that have incorporated radioactive thymidine emit radiation, causing a black, exposed spot on the developed film. The developed films are then viewed under a microscope. This technique is quite sensitive; radioactive cells can be counted at a rate of 2 per 10,000 to 1 per 10 million cells. The procedure can also be automated to make it relatively effi-

cient for identifying rare mutants. Dividing the fraction of radioactive cells in the 6-TG-exposed culture by the fraction in the culture not exposed to the drug produces an estimate of the frequency of 6-TG-resistant mutants.

The second method, called the clonal assay, selects for cells that are resistant to 6-TG. White blood cells are dispensed in small test tubes or wells in a small plastic device resembling a tiny muffin tin, each test tube or well containing 6-TG in the growth medium. Only the mutant 6-TG-resistant cells grow and divide, forming visible colonies within 10 to 14 days. These colonies can then be analyzed further by isolating their DNA and analyzing it for mutations. The clonal assay allows characterization of mutations found in these cells, whereas the autoradiographic method permits only determination of their frequency.

Mutational Spectra From Human T= Lymphocyte HPRT Mutants

Cariello (16) described a technique for analysis of the *hprt* gene that may correlate mutational patterns with particular mutagenic exposures. In this method, all of the mutant *hprt*- T-lymphocytes in a small blood sample are isolated and grown in culture for 2 weeks to produce enough DNA for analysis. DNA of these clones of different *hprt*- mutants is then examined by first cutting it into fragments with restriction enzymes, adding a group of different DNA probes and separating DNA bound to the probes by gradient gel electrophoresis. DNA with different mutations will bind to different probes. The pattern of mutations in the original blood sample appears as the pattern of DNA probe positions on a gel. Different mutagens are known to produce clearly different patterns of mutation in human as well as bacterial cells, so the aim of this technique is to identify the probable causes of mutations in human blood cell samples by using these patterns, or "mutational spectra" (139). This approach includes studies of the pattern of spontaneous mutation in human T-lymphocyte samples as well as studies of the mutational spectra produced by potentially mutagenic chemicals to which human beings may be exposed. The goal of this approach is to "fingerprint" for the presence of a particular mutagen in an individual's life history.

The *hprt* Gene

One of the advantages of studying the *hprt* gene is that much has been learned about it from studies of a particular genetic disease. Heritable mutations in the *hprt* gene located on the X chromosome lead to Lesch-Nyhan syndrome, a severely debilitating disorder characterized by mental retardation and self-mutilation. The *hprt* gene has been isolated from blood samples taken from patients with Lesch-Nyhan syndrome, and the mutations in the *hprt* gene have been studied. Comparisons have been made between the types of mutations present in cells of Lesch-Nyhan patients and those present in rare, mutant *hprt*- T-lymphocytes selected from blood samples of people without the disease. DNA from 5 of 28 Lesch-Nyhan patients were found to have major *hprt* gene alterations—deletions of DNA, amplification of DNA, and detectable changes in DNA sequence (172). Studies of mutant T-lymphocytes selected from normal blood samples have shown similar gene alterations in several cases (4,143). These results show that the *hprt*- T-lymphocyte assay detects spontaneous mutations in the *hprt* gene in somatic cells that are qualitatively similar to clinically apparent heritable mutations in humans, and also demonstrates that the clonal assay in T-lymphocytes detects true mutations, not phenotypic changes that mimic mutations.

Results of Studies of *hprt* ⁻ T-lymphocytes

A number of studies have measured frequencies of T-lymphocytes resistant to 6-TG in normal individuals. Average mutant frequencies for the groups of people tested fall within a fifteenfold range, between about 1 and 15 mutations per million cells (table 7). However, mutant frequencies for different individuals vary widely. For instance, Albertini reports a range of frequencies from 0.4 to 42 mutants per million T-lymphocytes from 23 individuals, representing a hundredfold range in somatic mutant cell frequencies among a small number of people (2). This wide range in

Table 7.—Frequencies of hprt⁻ T-Lymphocytes in Human Beings

Average mutant frequency	Range	Number	Reference
1.1×10^{-6}	NR[a]	12	(70)
2.2×10^{-6}	NR	NR[a]	(1)
2.9×10^{-6}	NR	11	(117)
3.0×10^{-6}	NR	14	(79)
3.8×10^{-6}	—	1	(2)
4.6×10^{-6}	$3.9-6.0\times10^{-6}$	2	(137)
6.1×10^{-6}	NR	45	(26)
1.2×10^{-5}	—	1	(3)
1.4×10^{-5}	$0.4-42\times10^{-6}$	23	(2)
1.5×10^{-5}	$0.8-2.5\times10^{-5}$	24	(160)

[a]Number of individuals not reported.

SOURCE: Office of Technology Assessment

mutant frequencies may be due to different ages and other characteristics of the T-lymphocyte donors. Two investigators have shown that there is a linear increase of *hprt-* mutants in T-lymphocytes with increasing age (141,160). Donor characteristics, such as smoking behavior, have been shown to increase somatic mutant frequency; genetic variation in sensitivity to mutagens may also exist among individuals.

The degree to which the observed frequency of mutants reflects the actual frequency of somatic mutations in this assay is not fully understood. Descendants of the original mutant cells increase the observed frequency, so growth of mutants in vivo could falsely lead to a conclusion of higher mutation rates than is actually the case, unless the data are corrected for this problem. In addition, *hprt-* cells maybe shorter-lived than *hprt*⁺ cells. Before the technique can be used to estimate possible increases in somatic mutation rates from environmental exposures, the wide range of background rates of these mutants has to be investigated, including discrimination between true genetic events and any possible phenocopies. While imprecision of the assay may contribute to this wide range of mutant frequencies, such a range is not unexpected for genetically and geographically diverse human populations.

DETECTION OF SOMATIC MUTATIONS IN RED BLOOD CELLS

Unlike white blood cells, mature red blood cells have no nuclei and contain no DNA. They cannot be grown in the laboratory since they do not have the capacity to replicate. Red blood cells can, however, be used to detect mutations expressed in altered proteins on their surface. Red blood cells are also easily obtained from a blood sample. There are about 1 billion red blood cells per milliliter of whole blood. Branscomb (13) described two methods to detect variant red cells, using high-speed automated microscopy and flow cytometry to analyze and sort hundreds of red blood cells per second to detect rare red cell mutants. These two methods are described below.

Glycophorin Somatic Cell Mutation Assay

The glycophorin assay detects gene-loss mutations expressed by the absence of cellular proteins corresponding to those genes. Such mutations can be detected where two or more variants of a particular protein normally exist, where the genes for the alternative forms are said to be codominant (both variants are expressed when a gene for each is present), and where different variants are expressed in the same cell. A mutation causing the loss of function of the gene for one of the variant forms results in a cell that expresses only the other form of the protein (9,10,5o).

One such protein is glycophorin A, a glycoprotein present on the surface of red blood cells. Two variants of glycophorin A normally exist, called "M" and "N," which differ from each other by 2 out of a total of 131 amino acids. The M and N serotypes have no known biological function, and each allele functions independently of the other. The M and N variants of glycophorin A can be labeled independently using monoclinal antibodies carrying different fluorescing molecules. As a result of the labeling, the M variant appears green and the N appears red when viewed under a fluorescent microscope. A flow cytometer, a machine through which cells are passed at

very high speed, can sort and count the number of cells that are green, containing only the M variant, and the number that are red, containing only the N variant.

Individuals whose red cells display both variants of glycophorin are heterozygous for the M and N alleles (i. e., they inherited an M gene from one parent and an N gene from the other). As used currently, this assay detects gene loss mutations at glycophorin genes in individuals who are heterozygous for the M and N variants. Red blood cells from heterozygotes show both red and green fluorescence; if a mutation inactivates one of the glycophorin A alleles, only the other variant would be present on the cell surface, hence only one color would appear. Single-color cells are recognized and distinguished from the majority of double-colored cells, and are counted as somatic mutant cells.

The glycophorin A assay is currently being used at the Lawrence Livermore Laboratory to study mutations in two populations: cancer patients' blood cells examined before and after treatment with known doses of chemotherapeutic drugs, and Japanese atomic bomb survivors whose dose of radiation can be estimated (69).

Hemoglobin Somatic Cell Assay

The frequency of particular hemoglobin variants that exist at low frequencies in red blood cells of normal individuals can be measured. Cells containing these variants presumably arise in bone marrow stem cells, the precursors of red blood cells. Methods have been developed using monoclonal antibodies, prepared for each type of variant, to label particular mutant hemoglobin molecules. This method detects small mutations, such as single nucleotide changes resulting in amino acid substitutions, frame shift mutations, etc., in the gene for beta-globin, a constituent of hemoglobin.

Investigators at the University of Washington (132,133) and at Lawrence Livermore National Laboratory (50) have developed an approach to detect hemoglobin mutations in red blood cells. In this technique, an antibody to the mutant hemoglobin is bound to a chemical called a fluorochrome, which is then mixed with red cells in suspension. The fluorochrome emits fluorescent light of a specific color when light of a particular wavelength is shone on it. The cells are screened under a microscope that allows the investigator to direct light of a particular wavelength on the sample. If any of the cells contain the mutant hemoglobin bound to fluorescing antibody, they can be viewed and counted.

Stamatoyannopoulos and his colleagues have used this technique on blood samples from 15 individuals using antibodies specific for Hemoglobin S (the mutant form of hemoglobin present in sickle cell anemia) and Hemoglobin C. An average of 50 million cells per individual was screened, and an average mutant frequency of 1.1 in 10 million cells per subject was obtained, with a range of 4 in 100 million to 3 in 10 million. These investigators also screened the red blood cells of 10 individuals who had been exposed to known mutagens—X-rays or mutagenic drugs used to treat cancer. The frequencies of mutant hemoglobins obtained from these samples were within the range found for normal individuals.

This technique is labor-intensive because it relies on visual screening of the cells: it takes one technician nearly 1 month to screen 100 million red cells. The investigators have since collaborated with Dr. Mendelssohn and his associates at the Lawrence Livermore Laboratory to use their automated fluorescence-activated cell sorter to screen the cells. Although some technical difficulties were encountered with this instrument, results obtained on red cells from six normal individuals (on screening 500 million cells per individual) yielded an average mutant frequency of 1 in 10 million, about the same as the average frequency determined from visually counted cells.

With an array of different mutant hemoglobins to use for production of antibodies, a variety of mutations can be detected in hemoglobins found in rare cells present in normal individuals: single nucleotide substitutions, deletions, frame-shift mutations, or virtually any mutation that causes the production of mutant hemoglobins.

There are currently two technical problems with this technique. First, a significant number of false positives are generated. Better methods of fixa-

tion and antibody staining of the hemoglobins are necessary, along with verification of each variant found. Second, the method is not yet fully automated, but research in several laboratories is progressing toward full automation. Significant progress has already been made; Mendelson (68) reported that with present capabilities using flow sorting, 88 antibody-labeled red cells can be identified in a mixture that contains a known quantit y of 100 such labeled red cells and 1 billion unlabeled red cells, a relatively good retrieval rate.

CONCLUSIONS

The methods to detect and quantify somatic mutations suggest some additional approaches for studying the nature and mechanisms of mutation in human beings. Somatic tests offer the possibility of drawing associations between exposures to specific mutagens and particular genetic events in the cell, and they may help to identify individuals and populations at high risk for mutations. They may lead to understanding the relationships between the frequency and nature of somatic and germinal mutations, and if those relationships can be identified, somatic tests may contribute significantly to understanding why human heritable mutations occur and may help predict their future occurrence. At present, however, it is not possible to directly extrapolate from mutations in somatic cells to risks of heritable mutations in offspring.

Animal studies can be used to measure quantitatively the correlations between exposure to mutagens and rates of somatic mutation, as well as to investigate possible associations between somatic and heritable mutations. Coordinated tests of somatic and germinal mutations in experimental animals may help guide such research in humans. Such data, combined with data on human somatic mutation rates, may suggest specific risks of heritable mutations in human populations.

In this regard, the tests for somatic mutation described in this report appear promising. None of these methods is currently ready for application to human population studies, however, even though many of the technical details have been worked out. Since the assays do not detect DNA mutations directly, it is particularly important to verify the results from these methods by isolating variant cells and confirming that the altered proteins do indeed reflect primary changes in the genes coding for these proteins. It is also necessary, although more difficult, to determine how often these methods miss the mutations they are designed to detect,

In general, the most useful somatic cell mutation assay may be one that detects a spectrum of mutations (single amino acid substitutions to large deletions and translocations) and that can detect mutations in different cell types. Different tissues may demonstrate different frequencies of somatic mutants, and equally as important, some types of mutant cells may have longer lifespans in the body than others. Consideration of such characteristics could be useful in choosing the most appropriate assay for particular circumstances. Short-lived mutants could be useful in studying the effects of short-term exposures to potential mutagens, while longer lived mutants could be better indicators of mutagenic effects of long-term, chronic exposure to environmental mutagens.

The genetic analysis of white blood cell DNA offers the possibility of identifying genetic “fingerprints, ” or particular patterns of mutations induced by specific mutagenic agents. Such patterns are an essential first step toward understanding the molecular mechanisms of genetic change in human DNA,