Environmental Health Perspectives
Vol. 99, pp. 135–141, 1993

In Vivo Mutations in Human Blood Cells: Biomarkers for Molecular Epidemiology

by Richard J. Albertini,[1] Janice A. Nicklas,[2] James C. Fuscoe,[2] Thomas R. Skopek,[3] Richard F. Branda,[1] and J. Patrick O'Neill[1]

Mutations arising *in vivo* in recorder genes of human blood cells provide biomarkers for molecular epidemiology by serving as surrogates for cancer-causing genetic changes. Current markers include mutations of the glycophorin-A (GPA) or hemoglobin (Hb) genes, measured in red blood cells, or mutations of the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) or HLA genes, measured in T-lymphocytes. Mean mutant frequencies (variant frequencies) for normal young adults are approximately: Hb (4×10^{-8}) < *hprt* (5×10^{-6}) = GPA (10×10^{-6}) < HLA (30×10^{-6}). Mutagen-exposed individuals show decided elevations. Molecular mutational spectra are also being defined. For the *hprt* marker system, about 15% of background mutations are gross structural alterations of the *hprt* gene (e.g., deletions); the remainder are point mutations (e.g., base substitutions or frameshifts). Ionizing radiations result in dose-related increases in total gene deletions. Large deletions may encompass several megabases as shown by co-deletions of linked markers. Possible *hprt* spectra for defining radiation and chemical exposures are being sought. In addition to their responsiveness to environmental mutagens/carcinogens, three additional findings suggest that the *in vivo* recorder mutations are relevant *in vivo* surrogates for cancer mutations. First, a large fraction of GPA and HLA mutations show exchanges due to homologous recombination, an important mutational event in cancer. Second, *hprt* mutations arise preferentially in dividing T-cells, which can accumulate additional mutations in the same clone, reminiscent of the multiple hits required in the evolution of malignancy. Finally, fetal *hprt* mutations frequently have characteristic deletions of *hprt* exons 2 and 3, which appear to be mediated by the VDJ recombinase that rearranges the T-cell receptor genes during thymic ontogeny. Illegitimate events such as these also appear to occur in human leukemias.

Introduction

Molecular epidemiology is an attempt to combine the disease relevance of traditional epidemiology with the use of biomarkers to overcome limitations in studying diseases that are infrequent or have long incubation periods. Biomarkers measure the *in vivo* effects of deleterious environmental agents and may even serve as surrogates for disease. However, interpretation of human studies is dependent on the biomarker employed. Does it sensitively detect exposures? Is there any specificity? Does the biomarker detect only exposure or does it reveal *in vivo* toxic effects that, while not disease effects, help to predict them? Answers, of course, require an understanding of the biology of the biomarkers.

Some human cancers are undoubtedly caused, at least in part, by genotoxic carcinogens. Human exposures to these agents can be monitored by biomarkers of chromosome or gene level damage, or through direct assessments of DNA damage, chemical adduction, or repair. This review will consider a particular subset of these, i.e., gene level damage. Specifically, we will focus on the measurement and analysis of somatic cell gene mutations arising *in vivo* in human blood cells. Although all of the assays for human monitoring will be considered, the emphasis will be on *in vivo* hypoxanthine-guanine phosphoribosyltransferase (*hprt*) mutations in T-lymphocytes.

Cells Used for Studies of Current Biomarkers of *in Vivo* Somatic Cell Mutations in Humans

At present, mutations are scored as mutant cells in peripheral blood, i.e., red blood cells (RBCs) or T-lymphocytes (T-cells) (Table 1). However, the mutations giving rise to these mutants arise in progenitor cells. For the RBC assays, mutations arise in multipotent stem cells or in somewhat differentiated hematopoietic precursor cells in the bone marrow. Continual proliferation and differentiation of these mutant progenitors produce the peripheral blood RBCs scored in the assays. For the T-cell assays, mutations in adults arise in differentiated, mature T-cells (*1*), probably in lymph nodes or other body tissues. Although fully differentiated, these cells retain the capacity for limited

[1]VCC Genetics Laboratory, University of Vermont, Burlington, VT 05401.
[2]Environmental Health Research and Testing, Inc., Research Triangle Park, NC 27709.
[3]Pathology Department, University of North Carolina at Chapel Hill, NC 27599.
Address reprint requests to J. A. Nicklas, Genetics Laboratory, 32 N. Prospect Street, Burlington, VT 05401.

Table 1. Characteristics of cells used in assays for human *in vivo* somatic mutations.

Mutant cells	Target cells for mutation	Body compartment for mutation	Metabolic competence of target cell
RBC	Multipotent stem cell, differentiated precursor cell	Bone marrow	Probable, also, stem cell surrounded by competent macrophages
T-cell	Post-thymic (adults) Prethymic (fetus)	Blood Most tissues Bone marrow Thymus (?)	Competent (partial?) Proximity to other tissues

but repetitive cell division after stimulation *in vivo* (usually by antigen), providing expanded clones derived from original progenitors. Mutant T-cells deriving from mutations in expanding clones make up a greater or lesser fraction of these clones, depending on when they occur during clonal expansion. In the fetus, however, and presumably in children and adolescents, mutations may arise in pre- or intrathymic pre-T-cells. The prethymic progenitors of T-cells may even be bone marrow stem cells, in which case the kinetics of appearance and persistence of T-cell mutants in peripheral blood will be similar to what is seen for RBC mutants.

The differentiation stage of mutational target cells relative to the mutants scored in an assay determines some characteristics of that assay's results [reviewed in Albertini et al. (*2*)]. For example, mutations arising in stem cells continuously produce mutant progeny. Therefore, a characteristic of stem cell mutations for human monitoring is memory, i.e., mutations arising in the distant past can be detected. Costs of memory, however, include variability, determined in part by the number of stem cells producing the population of mature cells sampled at any given time, and loss of temporal relationships between induced mutational events and the detection of mutants in peripheral blood. In contrast, mutations arising in differentiated T-cells are more closely related in time to the presence of mutant cells in the peripheral blood, especially if there is a finite life span of mutants. The cost, of course, is memory.

Mature RBCs have lost their nucleus. There is, therefore, no possibility for molecular studies to define molecular spectra of mutations. The term "variants" acknowledges this. T-cells, however, can be propagated *in vitro* and studied at the molecular level. Also, the T-cell receptor (TcR) genes, which rearrange during thymic differentiation of individual T-cells, may be analyzed in these cells relative to mutations (*1*). This allows decisions to be made regarding the *in vivo* independence of different mutant isolates and the differentiation stage *in vivo* when the mutation occurred.

There is probably metabolic competency for the target cells for both the hematopoietic and T-cell mutations. Hematopoietic precursor cells are surrounded by metabolically active macrophages in the bone marrow. Mature T-cells are partially metabolically competent and circulate through all of the tissues, where they are probably exposed to mutagenic metabolites.

Table 2. Human *in vivo* somatic mutation assays.

Genetic assay	Target gene	Target size	Chromosome location	Assay method	End point	Range of mean values
HbS (RBC)	*B chain of* hemoglobin (Hb beta)	2 kb, 3 exons (*50*)	*11p15.5*, autosome (*51*)	*HbA (normal) → HbS (sickle cell Hb) mutations are* detected by fluorescent anti-HbS antibody treatment of fixed RBCs on slides. The rare fluorescent cells in a HbA/HbA homozygous individual are scored by an image analyzer (*52*).	Vf	4×10^{-8}[a] (*17*)
GPA (RBC)	Glycophorin A (MN blood group antigen)	44 kb, 7 exons (*53*)	4q28–31, autosome (*51*)	Anti-M and anti-N antibodies, labeled with green and red fluorophors, respectively, are used to double label wild-type (nonmutant) RBCs from M/N heterozygous individuals. A single or dual beam cell sorter detects the loss of either antigen (M*θ* or N*θ*) (hemizygous) or double expression of the other (MM or NN) (homozygous). The first is due to simple allele loss; the second is due to somatic recombination or gene conversion (*54*).	Vf	10×10^{-6} (hemizygous)[b] $10–20 \times 10^{-6}$ (homozygous)[c] (*3*)
hprt (T-cells)	Hypoxanthine-guanine phosphoribosyl transferase (*hprt*)	44 kb, 9 exons (*55*)	Xq26, X-chromosome (*51*)	The short-term assay measures first-round DNA synthesis of stimulated T-cells *in vitro* in 6-thioguanine (TG) by either autoradiography or BrdU incorporation. Labeled cells presumably have undergone *in vivo hprt* mutation (*19*).	Vf[d]	$2–9 \times 10^{-6}$ (*2*)
				For the cloning assay, direct cloning of T-cells is performed with or without 6-TG in the presence of T-cell growth factor and feeder cells (*56–58*).	Mf	$3–10 \times 10^{-6}$ (*2*)
HLA (T-cells)	HLA-A (major histocompatibility genes)	5 kb, 7 exons (*59*)	6p21.3, autosome (*51*)	HLA mutants are detected by direct cloning of T- cells after their treatment with anti-HLA monoclonal antibodies to eliminate wild-type cells (*60*).	Mf	30×10^{-6} (*6*)

Abbreviations: Vf, variant frequency; Mf, mutant frequency.
[a]Single study.
[b]Hemizygous mutant = single loss (see text).
[c]Homozygous mutant = loss with gain of homologous allele (see text).
[d]Short-term autoradiographic assay (see text).

Assays Used for Studies of Current Biomarkers of *in Vivo* Somatic Cell Mutations in Humans

Four assays are currently available for measuring *in vivo* somatic cell gene mutations in humans (Table 2). An understanding of the biology of each assay is important for its interpretation, especially for comparing results across assays. The mutational target sizes of the four genes studied in these assays differ. The Hb β gene is 2 kb in length; however, the functional target size for the mutation currently scored is a single base change. The glycophorin A (GPA) and *hprt* genes are approximately equal in size; however, for GPA, the immunological methods used in the assay define target size by the antibody used and the protein structures required for recognition, whereas for *hprt*, chemical selection for mutants requires loss of catalytic activity of the HPRT enzyme, which may not occur following all possible mutations of the gene. For HLA, although this is, in principle, another large target gene, the immunological methods used for mutant detection make it difficult to define target size.

Autosomal genes (hemoglobin [Hb], GPA, HLA) allow for the detection of interhomologous chromosome mutational events, such as somatic recombination and gene conversion, in addition to the more commonly considered mutational events, thus providing surrogates for events that may be important in the genesis of cancer. In contrast, the *hprt* gene is on the X-chromosome, which is either actually or functionally hemizygous in all mammalian cells; therefore, interhomologous chromosome mutational events cannot occur at *hprt*. Results, to be considered below, should be interpreted in light of these biological characteristics.

Quantitative Aspects

Studies from several laboratories have reported background mutant or variant frequency values (Mf; Vf) for normal adults (Table 2). Background mutations are not synonymous with spontaneous mutations because the causes are not known. However, the terms are often used interchangeably. In summary, the relative order of background Mf (Vf) values from normal adults for the several markers is: Hb > *hprt* = GPA > HLA. Age-related increases in these values have been reported for GPA, *hprt*, and HLA (*3–7*).

Qualitative (Molecular) Aspects

T-cells can be recovered from cloning assays and propagated *in vitro* into populations of sufficient size for molecular analyses. Although this has been accomplished for both *hprt* and HLA mutations, the largest database at present is for *hprt*.

hprt. There has been quite good aggreement among laboratories that approximately 15% of *hprt* mutations in normal adults result from gross structural alterations (*2,8–11*). These include deletions, insertions, and rearrangements visible on Southern blots. The breakpoints of the alterations are distributed randomly within the gene, with no "hot spot" thus far identified (*2*). Total deletions of *hprt* may show co-deletion of linked anonymous markers, which map several centiMorgans distant in both 5′ and 3′ directions, indicating that deletions can be quite large (*12*).

The remaining 85% of the background *in vivo hprt* mutations in adults consist of point mutations. Because of the importance of *hprt* as a genetic target, a repository has been established to collate information on *in vivo* and *in vitro* mutations in human cells developed in all laboratories engaged in such studies worldwide (*13*). More than 700 such mutants have been sequenced to date. The mutations characterized include more than 200 somatic mutations derived from normals, smokers, chemotherapy patients, and patients with the DNA repair disorders xeroderma pigmentosum and ataxia telangiectasia. Fifty-seven germinal *hprt* mutations (Lesch-Nyhan syndrome) also have been characterized. In addition, several hundred *hprt* mutations generated *in vitro* by agents including ethyl nitrosourea methyl-*N*-nitroguanidine, formaldehyde, ICR-191, cyanoethylene oxide, cisplatin, benzo[a]pyrene diol epoxide, and UV light have been sequenced. The data collected to date suggest that *hprt* is sensitive to all mutation types, including base pair substitutions, frameshifts, and deletions of various sizes. A total of 227 different base substitutions have been observed at 172 of the 657 bases of the coding region. Of these, approximately 85% were missense mutations, 13% nonsense mutations, and 2% did not lead to any amino acid change. (The latter class of mutations may express phenotypically due to codon usage problems or due to decreased stability of the resulting *hprt* mRNA.) Every class of base substitution has been observed at similar frequencies and was distributed throughout the coding region in all nine exons of *hprt*. Thirty-eight frameshifts (25 −1, 13 +1) involving both GC and AT base pairs have been observed. In general, frameshift mutations occurred at or adjacent to sequences containing two or more consecutive identical bases. Thirty-one examples of small deletions (2–49 bases) were recorded. Most of these deletions were flanked by 2-5 base direct repeat sequences. More than 150 mutants displaying splicing aberrations have been identified. These include examples of single, multiple, and partial exon loss and involve all exons except exon 1. (Exon 4 [28%] and 8 [30%] are most frequent.) Ten of fourteen sequenced exon loss mutants displayed base substitutions at the splice acceptor or donor region of the missing exon. The remaining mutations most likely contain point mutations or small deletions within other intron sequences involved in splicing.

Considering only the *in vivo* mutations (46 Lesch-Nyhan germinal, 51 normal adult somatic, 86 exposed adult somatic), several hotspots of mutation were observed. In particular, four basespair sites have been observed to be mutated in all groups. However, an additional 132 sites have been recorded with only a single observation per site. This suggests that there are many more sites in *hprt* that routinely mutate *in vivo* (perhaps 100–200 additional sites), although the total number of *in vivo* mutations analyzed to date is not large enough to have observed them all.

HLA. Approximately 30% of the background mutations in adults result from total deletions of the gene as detected on Southern blots (*14*). At HLA also, some deletions appear to be quite large, as demonstrated by the associated phenotypic loss of the HLA-B antigen coded by the allele in "cis" with the deleted HLA-A allele. Thus, deletions of > 1000 kb are seen. More importantly, recent molecular analyses of *in vivo*-derived HLA loss mutants show that approximately 30% also arise from mitotic recombination (*15*). Therefore, this important class of mutations can be recognized at the molecular level. At present, there are no

published sequence data on *in vivo* HLA mutations defining the nature of point mutations at this locus.

Somatic Cell Gene Mutations Induced *in Vivo* by Environmental Mutagens

The utility of the assays of *in vivo* somatic mutation for environmental monitoring is being assessed by defining their ability to detect human exposures to model mutagens, e.g., smoking, cytotoxic chemotherapy and ionizing radiations, or to genotoxic agents in well-defined accidents. Most investigations have shown clear increases in Vf or Mf values in exposed individuals.

Effects of Smoking

Definite increases in *in vivo hprt* Mf values due to smoking have been reported by several laboratories (*5,16–18*). In fact, this increase was greater than 50% in smokers compared to nonsmokers in one study (*16*). Other studies, however, have reported weaker or no effects (*19,20*). Smoking also results in somewhat higher GPA Vf values than for nonsmokers, but, in most studies, this increase does not achieve statistical significance (*3*). Preliminary results in ethylene oxide workers and ataxia telangiectasia and xeroderma pigmentosum heterozygotes suggest that in smokers the frequency of HbS variant cells is doubled as compared to nonsmokers (*21*).

Exposure to Cytotoxic Chemotherapeutic Agents

Most studies of cancer patients undergoing chemotherapy have shown clear increases in *hprt* T-cell Mf values (*22–24*). A similar increase has been seen for GPA mutations (*25*). However, a recent study of 31 breast cancer patients receiving either chemotherapy, radiotherapy, or both reported no significant increase in *hprt* Mf attributable to chemotherapy (*26*). The significant increases seen in treated patients could be accounted for solely by radiotherapy. Two of the agents received by these patients, cyclophosphamide and adriamycin have been implicated as mutagens in other studies of *in vivo* somatic mutation (*25,27*).

We have recently completed a similar study of women with breast cancer receiving potentially mutagenic therapies, and we obtained quite different results (*20,28*). All patients in our study had surgery with curative intent; subsequent therapy was in the adjuvant mode. Group 1 patients had no therapy beyond surgery, or had hormonal therapy, group 2 patients had local radiotherapy with or without hormonal therapy, and group 3 patients had chemotherapy with or without radiotherapy and/or hormones. The first blood sample was obtained after surgery but before the onset of adjuvant therapy; subsequent samples were obtained at 6-month intervals. Women presenting with noncancerous breast masses served as controls.

In contrast to the study noted above, there was a significant increase in mean Mf at 6 months in women receiving chemotherapy ($18.2 \pm 1.7 \times 10^{-6}$) compared to prechemotherapy ($11.1 \pm 1.4 \times 10^{-6}$). A more modest increase in mean post-treatment Mf was seen after local radiotherapy, but neither this, nor the slight increase in the group patients, reached statistical significance. Following chemotherapy, *hprt* Mf values returned toward baseline, but remained somewhat elevated.

Comparison of the two *hprt* studies of breast cancer patients reveals factors that may be of importance in interpreting *in vivo* mutagenicity studies. In the study showing no significant chemotherapy effects, the time of assay relative to chemotherapy was variable. Timing is probably extremely important for optimally detecting *hprt* mutant T-cells in peripheral blood. Sampling long after an exposure may well underestimate *hprt* mutation induction. Also, some of this difference was due to the kind of chemotherapy received, with fluorouracil and methotrexate producing little mutagenicity. In addition, individual factors may also be of importance, such as serum folate levels in the deficiency range, which were particularly common in the women who had the most marked Mf elevations after chemotherapy. At present, there are no published reports of molecular analyses of *hprt* mutations following a specific cancer chemotherapy or other form of *in vivo* chemical mutagenesis. However, several studies are in progress (see above for summary of current sequence information). The expectation is that different chemical mutagens will be shown to induce different molecular spectra of mutations, thereby producing signatures for specific recognition.

Exposures to Ionizing Radiation

hprt. Mfs have been studied in several groups of humans exposed to ionizing radiation (*24,27,29–32*). Induced mean frequencies in exposed individuals have varied from a low of 7.5×10^{-9} to a high of 5.5×10^{-6}/cGy. This range of almost three orders of magnitude illustrates, in the extreme, the importance of timing in measuring *in vivo*-induced *hprt* mutations in peripheral blood T-cells. The rate of induction determined in servivors of the atomic bomb explosion is 7.5×10^{-9} and was determined almost 45 years after the mutants were induced (*32*). Eliminating this study reveals the range of *hprt* mutant induction by ionizing radiation to be approximately $1–5 \times 10^{-6}$/cGy for high-dose, low linear energy transfer irradiations.

The GPA assay was also used in studies of atomic bomb survivors 45 years after the event (*33,34*). These studies revealed an increment of approximately 0.4×10^{-6} induced GPA mutant RBCs/cGy, even at this late date, with a good deal of interindividual variability. Such results in RBCs are in accord with stem cell mutations, discussed above.

In an effort to better define the *in vivo* mutagenic effects of ionizing radiation, our laboratory has determined *hprt* Mfs in cancer patients exposed to an internal γ-emitting radionuclide (^{131}I) (radioimmunotherapy [RIT]) producing total body irradiation in an exponentially decaying dose (*35,36*). We determined Mf values in 13 patients before and 36 patients after treatment. The post-treatment group included 11 samples obtained 2 months after a first treatment of 62–104 cGy, 18 samples 2 months after the last of a series of multiple treatments (cumulative total of 128–669 cGy), and 7 samples obtained 10–48 months after the last of multiple treatments (cumulative total of 128–324 cGy). Based on either the dose of the first single treatment or the last of the multiple treatments, a dose–response relationship of $0.9–1.1 \times 10^{-6}$ mutants/cCy was seen. Based on the total cumulative exposure, this induction fell to $0.2–0.3 \times 10^{-6}$ in the 2-month samples, and to 0.09×10^{-6} in the 10–48-month samples. (This dose response compares with a value of 0.2×10^{-6} mutants/cGy in T-cells acutely irradiated *in vitro*.) These time-dependent declines in the *in vivo* Mf point again to the

Table 3. Gross structural alteration in *in vivo hprt* mutations.

	Change on Southern blot						
Subject	Total deletion	Partial deletion	Single breakpoint	New fragment only	No change	Total	% Gross alteration
Normals	9 (22%)	20 (49%)	9 (22%)	3 (7%)	246[a]	287[b]	14.2
pre-RIT	3 (15%)	10 (50%)	6 (30%)	1 (5%)	98	118	16.9
post-RIT	37 (41%)	27 (30%)	22 (24%)	4 (4%)	145	235	38.3

RIT, radioimmunotherapy.
[a]Independent mutations without visible alteration on Southern blot.
[b]Total independent mutations studied for the group.

importance of sampling time in human *in vivo* mutagenicity studies. Nonetheless, *in vivo* exposures to whole-body ionizing radiation clearly result in dose-dependent increases in *hprt* Mf.

Ionizing radiation is known to induce gross structural alterations in *hprt* and other recorder genes in cultured human and other mammalian cells. Bradley et al. (*8*) found an increased frequency of mutants with gross structual alterations on Southern blots in patients who had received radionuclides for diagnostic purposes (33%) as compared to patients before receiving radionuclides (13%). In atomic bomb survivors, the frequencies of mutations showing gross structural alterations at *hprt* were essentially the same in individuals exposed to radiation from the bomb compared to nonexposed controls (*9*); however, as noted, the study was conducted almost 45 years after the exposure.

Our laboratory recently completed an analysis of 241 independent *in vivo hprt* mutational events, isolated from 19 RIT patients (*35,36*). The *hprt* molecular lesions in these mutations were compared with the *hprt* molecular lesions in 118 *in vivo* mutations from 4 cancer patients before their therapy and in 287 background *in vivo* mutations in normal young males (Table 3). The mutations from post-RIT patients showed clearly greater frequencies of gross structural alterations than those from pre-RIT or normal individuals. The latter two frequencies are quite similar, suggesting that cancer per se does not produce this sort of damage at *hprt*.

This difference in the kinds of *in vivo hprt* mutations after ionizing radiation, compared to *in vivo* mutations before exposure or in normals, extends to the sizes of the molecular lesions. Post-RIT patients have much higher frequencies of total gene deletions. Therefore, not only is the frequency of *in vivo* mutations with gross structural alterations increased after ionizing radiation exposures, the percentage of lesions with large deletions is increased. In individuals from whom 10 or more independent *in vivo hprt* mutations could be analyzed, the correlation between fraction of mutations with gross structural alterations and cumulative radiation dose was 0.79 ($r = 0.82$, $r^2 = 0.67$ for the linear regression). Therefore, in our studies, 67% of the variability in fraction of *in vivo* mutations with gross structual alterations could be explained by ionizing radiation exposure.

Taken *in toto*, the data for *in vivo hprt* T-cell mutations indicate that ionizing radiation produces deletions and that deletions, particularly large deletions, characterize the *in vivo* mutational spectrum for this sort of human mutagen exposure. There may also be a characteristic point mutational spectrum for *in vivo hprt* mutations resulting from ionizing radiation exposure. Sequence studies are in progress.

Occupational or Residential Mutagen Exposures

There are several published reports of human monitoring, using the *hprt* cloning assay, for *in vivo* effects of occupational or residential chemical or radiation mutagen exposures (*18,19,37–41*). For ionizing radiation, mutation induction appears to be more efficient for low-dose irradiations, being in the range of 10^{-5} induced mutants/cGy in some studies also (*39–41*). Occupational exposures to chemical mutagens (e.g., chemotherapy nurses) resulted in elevations in Mf (*18,37*), but not when special precautions were taken (*19*).

A recent, well-controlled study of the association between *hprt* Mf in peripheral blood T-cells and household radon concentrations deserves special mention (*38*). A significant association was found between the log Mf and radon concentration. The doubling dose for Mf, estimated from two separate measurements of household radon concentrations, was 330 Bq/m^3 and 210 Bq/m^3, respectively. The authors caution that these data must be regarded as preliminary and that further, more extensive studies should be done. Molecular studies of these *in vivo hprt* mutations are clearly indicated.

Relevance of Mutation in Somatic Recorder Genes to Cancer

Several features of somatic cell gene mutation discovered thus far suggest that the recorder genes being studied serve as surrogates for cancer-causing mutations. First, considered in detail, is the finding that exposures to genotoxic carcinogens result in elevations in Mf (Vf) and, for ionizing radiation, result in a characteristic mutational spectrum. Also, two of the recorder genes being studied, GPA and HLA, permit recognition of somatic cell recombination and/or gene conversion. These mutational events are known to be important in the genesis of cancer. Two additional features indicating relevance to cancer are demonstrated by studies of *hprt*.

Analysis of background *hprt* mutations in normal individuals indicates that cell division *in vivo* is an important mutagenic influence. Several individuals with *in vivo* clonal amplifications of T-cells, detected by concomitant studies of TcR genes, have shown that the *hprt* mutations are occurring within the expanding clone (*1,2*). One individual with a massive clonal expansion has shown four different mutational events in this single clone. This suggests that unremitting cell division can create space for sequential gene mutations to accumulate in descendants of the same cell. Perhaps the spontaneous mutations resulting from copy error in cells stimulated to continual division have an important

role in the genesis of spontaneous human cancers, with the role of environmental agents being to induce such cell division.

A more recent observation comes from studies of fetal *in vivo hprt* mutations, as reflected by studies of placental cord blood. Background Mf values for newborns, determined in this way, are approximately 10-fold lower than for adults, i.e., 5×10^{-7} (*16,42,43*). Molecular analyses of these fetal mutations show that they frequently arise in prethymic cells and that their mutational spectrum is characterized by specific deletions of *hprt* exons 2 and 3, with deletion breakpoints giving rise to these deletions ocurring at a single site in intron 1 and at several sites in intron 3 (*45*). The sites have in common all or an important portion of the consensus heptamer [CAC(T/A)GTG] for the VDJ recombinase that mediates cleavage of the TcR genes during thymic ontogeny (*46*). Other hallmarks of a VDJ recombinase-mediated cleavage event characterize the junctional regions of the *hprt* deletions. Therefore, an illegitimate recombinase-mediated event can occur in the *hprt* gene which, when operative, accounts for 30% or more of the background mutations. A similar event has been shown to occur in a growth factor gene in 25% of acute T-cell leukemias (*47,48*), where it may be pathogenic, and as an intermediate to several of the chromosomal translocations seen in B-cell lymphomas (*49*). Thus, a mutagenic event that may be termed developmental mutagenesis has been uncovered by studies of *in vivo hprt* mutations in humans. Further studies should reveal its relevance to human cancer.

Conclusions

The status of *in vivo* somatic cell mutations as biomarkers for human studies has changed a good deal in recent years. Several markers of mutation are now available, and more are certain to come. Also, insights have been obtained that give a somewhat different picture than that derived from studies of *in vitro* mutations. These insights have provided an understanding of the biology of the different *in vivo* mutagenicity assays in use. Results of human monitoring studies can now be interpreted in light of this biological understanding. The use of these assays in basic investigations of somatic cell mutation, and its role in human disease, is great.

The role of human *in vivo* somatic cell mutations as biomarkers for genotoxic carcinogen exposures is also being developed. Studies reviewed here indicate that mutations are induced by exposures to these agents and may be quite sensitive indicators of such exposures. The markers certainly are useful as population indicators of genotoxicity. Also, the ongoing molecular studies of *in vivo*-induced mutants may reveal characteristic mutational spectra that will allow "diagnosis" of specific deleterious agents. It may even be possible eventually to make individual health risk assessments following genotoxic exposures, although the potential for this is currently unexplored. A major goal in continuing studies of *in vivo* somatic cell mutation in humans remains the development of methods for mutagenicity monitoring that will result in health benefits to the individuals studied.

This manuscript was presented at the Conference on Biomonitoring and Susceptibility Markers in Human Cancer: Applications in Molecular Epidemiology and Risk Assessment that was held in Kailua-Kona, Hawaii, 26 October–1 November 1991.

We thank Linda M. Sullivan, Michael T. Falta, Timothy C. Hunter, and Lisa Zimmerman for technical assistance and V. Maher, J. Cole, H. Vrieling, and A. Van Zeeland for sequencing information. This study was supported in part by U.S. EPA Contract no. 68-02-4456, EPA Cooperative Agreement CR-183565, NCI ROI CA 30688, and DOE FG028760502. This article has not been subjected to U.S. EPA review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred. Mention of tradenames or commercial products does not constitute U.S. EPA endorsement or recommendation for use. The contents of this article are solely the responsibility of the authors and are not endorsed as the official views of the Department of Energy or the National Cancer Institute.

REFERENCES

1. Nicklas, J. A., Hunter, T. C., O'Neill, J. P., and Albertini, R. J. Molecular analyses of *in vivo hprt* mutations in human T-lymphocytes: III. Longitudinal study of *hprt* gene structural alterations and T-cell clonal origins. Mutat. Res. 215: 147–160 (1989).
2. Albertini, R. J., Nicklas, J. A., O'Neill, J. P., and Robison, S. J. *In vivo* somatic mutations in humans: measurement and analysis. Annu. Rev. Genet. 24: 305–326 (1990).
3. Langlois, R. G., Bigbee, W. L., and Jensen, R. H. The glycophorin A assay for somatic cell mutations in humans. Prog. Clin. Biol. Res. 340: 47–56 (1990).
4. O'Neill, J. P., Nicklas, J. A., and Albertini, R. J. Age related increase in *in vivo hprt* mutations in human T-lymphocytes. Environ. Mol. Mutagen. 17(19): 75 (1991).
5. Cole, J., Green, M. H. L., Stephens, G., Waugh, A. P. W., Beare, D., Steingrimsdottir, J., and Bridges, B. A. HPRT somatic mutation data. Prog. Clin. Biol. Res. 340: 25–36 (1990).
6. Turner, D. R., and Morley, A. A. Human somatic mutation at the autosomal HLA-A locus. Prog. Clin. Biol. Res. 340: 37–46 (1990).
7. Trainor, K. J., Wigmore, D. J., Chroysostomou, A., Dempsey, J. L., Seshadri, R., and Morley, A. A. Mutation frequency in human lymphocytes increases with age. Mech. Age Dev. 27: 83–86 (1984).
8. Bradley, W. E. C., Garearu, J. L. P., Seifert, A. M., and Messing, K. Molecular characterization of 15 rearrangements among 90 human *in vivo* somatic mutants shows that deletions predominate. Mol. Cell. Biol. 7: 956–960 (1987).
9. Hakoda, M., Hirai, Y., Kyoizumi, S., and Akiyama, M. Molecular analyses of *in vivo hprt* mutant T cells from atomic bomb survivors. Environ. Mol. Mutagen. 13: 25–33 (1989).
10. Turner, D. R., Morley, A. A., Haliandros, M., Kutlaca, R., and Sanderson, B. J. *In vivo* somatic mutations in human lymphocytes frequently result from major gene alterations. Nature 315: 343–345 (1985).
11. Nicklas, J. A., Hunter, T. C., Sullivan, L. M., Berman, J. K., O'Neill, J. P., and Albertini, R. J. Molecular analyses of *in vivo hprt* mutations in human T-lymphocytes I. Studies of low frequency "spontaneous" mutants by Southern blots. Mutagenesis 2: 341–347 (1987).
12. Nicklas, J. A., Hunter, T. C., O'Neill, J. P., and Albertini, R. J. Fine structure mapping of the *hprt* region of the human X chromosome (Xq26). Am. J. Hum. Genet. 49: 267–278 (1991).
13. Craft, T. R., Cariello, N. F., and Skopek, T. R. Mutational profile of the human *hprt* gene: summary of mutations observed to date. Environ. Mol. Mutagen. 17(19): 18 (1991).
14. Turner, D. R., Grist, S. A., Janatipour, M., and Morley, A. A. Mutations in human lymphocytes commonly involve gene duplication and resemble those seen in cancer cells. Proc. Natl. Acad. Sci. U.S.A. 85: 3189–3193 (1988).
15. Morley, A. A., Grist, S. A., Turner, D. R., Kutlaca, A., and Bennett, G. Molecular nature of *in vivo* mutations in human cells at the autosomal HLA-A locus. Cancer Res. 50: 4584–4587 (1990).
16. Cole, J., Green, M. J. L., James, S. E., Henderson, L., and Cole, H. A further assessment of factors influencing measurements of thioguanine-resistant mutant frequency in circulating T-lymphocytes. Mutat. Res. 204: 493–507 (1988).
17. Tates, A. D., Bernini, L. F., Natajaran, A. T., Ploem, J. S., Verwoed, N. P., Cole, J., Green, M. H. L., Arlett, C. F., and Norris, P. N. Detection of somatic mutations in man: HPRT mutations in lymphocytes and hemoglobin mutations in erythrocytes. Mutat. Res. 213: 73–82 (1989).
18. Huttner, E., Mergner, U., Braun, R., and Schoneich, J. Increased frequency of 6-thioguanine-resistant lymphocytes in peripheral blood of workers employed in cyclophosphamide production. Mutat. Res. 243: 101–107 (1990).
19. Albertini, R. J., Sullivan, L. S., Berman, J. K., Greene, C. J., Stewart, J. A.,

Silveira, J. M., and O'Neill, J. P. Mutagenicity monitoring in humans by autoradiographic assay for mutant T-lymphocytes. Mutat. Res. 204: 481–492 (1988).

20. Branda, R. F., O'Neill, J. P., Jacobson-Kram, D., and Albertini, R. J. Factors influencing mutation at the *hprt* locus in T-lymphocytes: studies in normal women and women with benign and malignant breast masses. Environ. Mol. Mutagen. 19: 274–281 (1992).
21. Bernini, L. F., Natarajan, A. T., Schreuder-Rotteveel, A. H. M., Giordano, P. C., Ploem, J. S., and Tates, A. Assay for somatic mutation of human hemoglobins. Prog. Clin. Biol. Res. 340: 57–68 (1990).
22. Dempsey, J. L., Seshadri, R. S., and Morley, A. A. Increased mutation frequency following treatment with cancer chemotherapy. Cancer Res. 45: 2873–2877 (1985).
23. Ammenheuser, M. M., Au, W. W., Whorton, E. B., Jr., Belli, J. A., and Ward, J. B., Jr. Comparision of *hprt* variant frequencies and chromosome aberration frequencies in lymphocytes from radiotherapy and chemotherapy patients: a prospective study. Environ. Mol. Mutagen. 18: 126–135 (1991).
24. Albertini, R. J. Somatic gene mutations *in vivo* as indicated by the 6-thioguanine-resistant T-lymphocytes in human blood. Mutat. Res. 150: 411–422 (1985).
25. Bigbee, W. L., Wryobek, R. G., Langlois, R. G., Jensen, R. H., and Everson, R. B. The effect of chemotherapy on the *in vivo* frequency of glycophorin A "null" variant erythrocytes. Mutat. Res. 240: 165–175 (1990).
26. Sala-Trepat, M., Cole, J., Green, M. H. L., Rigaud, O. L., Vilcog, J. R., and Moustacchi, E. Genotoxic effects of radiotherapy and chemotherapy on the circulating lymphocytes of breast cancer. III: Measurement of mutant frequency to 6-thioguanine resistance. Mutagenesis 5: 593–598 (1990).
27. Ammenheuser, M. M., Ward, J. B., Jr., Whorton, E. B., Jr., Killian, J. M., and Legator, M. S. Elevated frequencies of 6-thioguanine resistant lymphocytes in multiple sclerosis patients treated with cyclophosphamide: a prospective study. Mutat. Res. 204: 509–520 (1988).
28. Branda, R. F., O'Neill, J. P., Sullivan, L. M., and Albertini, R. J. Factors influencing mutation at the *hprt* locus in T-lymphocytes: Women treated for breast cancer, in press.
29. Messing, K., and Bradley, W. E. C. *In vivo* mutant frequency rises among breast cancer patients after exposure to high doses of gamma radiation. Mutat. Res. 152: 107–112 (1985).
30. Seifert, A. M., Bradley, W. C., and Messing, K. Exposure of nuclear medicine patients to ionizing radiation is associated with rises in HPRT- mutant frequency in peripheral T-lymphocytes. Mutat. Res. 191: 57–63 (1987).
31. Ostrosky-Wegman, P., Montero, R., Palao, A., Cortinas de Nava, C., Hurtado, F., and Albertini, R. J. 6-thioguanine-resistant T-lymphocyte autoradiographic assay. Determination of variation frequencies in individuals suspected of radiation exposure. Mutat. Res. 232: 49–56 (1990).
32. Hakoda, M., Akiyama, M., Kyoizumi, S., Awa, A. A., Yamakido, M., and Otake, M. Increased somatic cell frequency in atomic bomb survivors. Mutat. Res. 201: 39–48 (1988).
33. Langlois, R. G., Bigbee, W. L., Kyoizumi, S., Nakamura, N., Bean, M. A., Akiyama, M., and Jensen, R. J. Evidence for increased somatic cell mutations at the glycophorin A locus in atomic bomb survivors. Science 236: 445–448 (1987).
34. Kyoizumi, S., Nakamura, N., Hakoda, M., Awa, A. A., Bean, M. A., Jensen, R. H., and Akiyama, M. Detection of somatic mutations at the glycophorin A locus in erythrocytes of atomic bomb survivors using a single beam flow sorter. Cancer Res. 49: 581–588 (1989).
35. Nicklas, J. A., Falta, M. T., Hunter, T. C., O'Neill, J. P., Jacobson-Kram, D., Williams, J., and Albertini, R. J. Molecular analysis of *in vivo hprt* mutations in human lymphocytes V. Effects of total body irradiation secondary to radioimmunoglobulin therapy (RIT). Mutagenesis 5: 461–468 (1990).
36. Nicklas, J. A., O'Neill, J. P., Hunter, T. C., Falta, M. T., Lippert, M. J., Jacobson-Kram, D., Williams, J. R., and Albertini, R. J. *In vivo* ionizing irradiations produce deletions in the *hprt* gene of human T-lymphocytes. Mutat. Res. 250: 383–396 (1991).
37. Chrysostomou, A., Seshadri, R. S., and Morley, A. A. Mutation frequency in nurses and pharmacists exposed to cytotoxic drugs. Aust. N.Z.J. Med. 14: 831–834 (1984).
38. Bridges, B., Cole, J., Arlett, C. F., Green, M. H. L., Waugh, A. P. W., Beare, D., Henshaw, D. L., and Last, R. D. Possible association between mutant frequency in peripheral lymphocytes and domestic radon concentrations. Lancet 337: 1187–1189 (1991).
39. Messing, K., Seifert, A. M., and Bradley, W. E. C. *In vivo* mutant frequency of technicians professionally exposed to ionizing radiation. In: Monitoring of Occupational Genotoxicants (M. Sorsa, and J. Norppa, Eds.), Alan R. Liss, New York, 1986, pp. 87–97.
40. Messing, K., Ferraris, J., Bradley, W. E. C., Swartz, J., and Seifert, A. M. Mutant frequency of radiotherapy technicians appears to be associated with recent dose of ionizing radiation. Health Phys. 57: 537–544 (1989).
41. Messing, K., Seifert, A. M., and Lemaire, J. *In vivo* mutant frequencies of factory workers exposed to very low doses of ionizing radiation. Environ. Mole. Mutagen. 15(17): 41 (1990).
42. McGinniss, M. J., Falta, M. T., Sullivan, L. M., and Albertini, R. J. *In vivo hprt* mutant frequencies in T-cells of normal human newsborns. Mutat. Res. 240: 117–126 (1990).
43. Lippert, M. J., Manchester, D. K., Hirsch, B., Nicklas, J. A., O'Neill, J. P., Sweet, L. M., and Albertini, R. J. Patterns of *hprt* mutations detected in newborns and elderly by cloning assay. Environ. Mol. Mutagen. 15(17): 35 (1990).
44. McGinniss, M. J., Nicklas, J. A., and Albertini, R. J. Molecular analyses of *in vivo hprt* mutations in human T-lymphocytes IV. Studies in newborns. Environ. Mole. Mutagen. 14: 229–237 (1989).
45. Fuscoe, J. C., Zimmerman, L. J., Lippert, M. L., Nicklas, J. A., O'Neill, J. P., and Albertini, R. J. V(D)J recombinase-like activity mediates *hprt* gene deletion in human fetal T-cells. Cancer Res. 51: 6001–6005 (1991).
46. Tonegawa, S. Somatic generation of antibody diversity. Nature 302: 575–581 (1983).
47. Aplan, P. D., Lombardi, D. P., Ginsberg, A. M., Cossman, J., Bertness, V. L., and Kirsch, I. R. Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. Science 250: 1426–1429 (1990).
48. Brown, L., Cheng, J. -T., Chen, Q., Siciliano, M. J., Crist, W., Buchanan, G., and Baer, R. Site-specific recomination of the *tal*-1 gene is a common occurrence in human T cell leukemia. EMBO J. 9: 3343–3351 (1990).
49. Tsujimoto, Y., Jaffe, E., Cossman, J., Gorham, J., Nowell, P. C., and Croce, C. M. Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11;14) chromosome translocation. Nature 315: 340–343 (1985).
50. Antonarakis, S. E., Kazazian, H. H., Jr., and Orkin, S. H. DNA polymorphism and molecular pathology of the human globin gene clusters. Hum. Genet. 69: 1–14 (1985).
51. Human Gene Mapping 9. Ninth International Workshop on Human Gene Mapping. Cytogenet. Cell Genet. 46(1–4) (1987).
52. Verwoerd, N. P., Bernini, L. F., Bonnet, J., Tanke, J. J., Natarajan, A. T., Tates, A. D., Sobels, F. H., and Ploem, J. S. Somatic cell mutations in humans detected by image analysis of immunofluorescently stained erythrocytes. In: Clinical Cytometry and Histomety, 1st ed. (G. Burger, J. S. Ploem, and K. Goerittler, Eds.) San Diego Academic Press, San Diego, CA, 1987, pp. 465–469.
53. Kudo, S., and Fukuda, M. Structural organization of glycophorin A and B genes: glycophorin B gene evolved by homologous recombination at *Alu* repeat sequences. Proc. Natl. Acad. Sci. U. S. A. 86: 4619–4623 (1989).
54. Langlois, R. G., Bigbee, W. L., and Jensen, R. H. Measurements of the frequency of human erthrocytes with gene expression loss phenotypes in the glycophorin A locus. Hum. Genet. 74: 353–362(1987).
55. Patel, P. I., Framson, P. E., Caskey, C. T., and Chinault, A. C. Fine structure of the human hypoxanthine phosphoribosyltransferase gene. Mole. Cell. Biol. 6: 393–403 (1986).
56. Albertini, R. J., Castle, K. L., and Borcherding, W. R. T-cell cloning to detect the mutant 6-thioguanine-resistant lymphocytes present in human peripheral blood. Proc. Natl. Acad. Sci. U. S. A. 79: 6617–6621 (1982).
57. Morley, A. A., Trainor, K. J., Seshadri, R., and Ryall, R. G. Measurement of *in vivo* mutations in human lymphocytes. Nature 302: 155–156 (1983).
58. O'Neill, J. P., Sullivan, L. M., Booker, J. K., Pornelos, B. S., Falta, M. T., Greene, C. J., and Albertini, R. J. Longitudinal study of the *in vivo hprt* mutant frequency in human T-lymphocytes as determined by a cell cloning assay. Environ. Mole. Mutagen. 13: 289–193 (1989).
59. Malissen, M., Malissen, B., and Jordan, B. R. Exon/intron organization and complete nucleotide sequence of an HLA gene. Proc. Natl. Acad. Sci. U. S. A. 79: 893–897 (1981).
60. Janatipour, M., Trainor, K. J., Kutlaca, R., Bennett, G., Hay, J., Turner, D. R., and Morley, A. A. Mutations in human lymphocytes studied by an HLA selection system. Mutat. Res. 198: 221–226 (1988).