End Points for Biomonitoring: Assay Sensitivity/Selectivity

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Estimation of population exposure and biological impact of potential hazards are central reasons for performing biomonitoring. The sensitivity of the biomonitoring methods and the linkage of the measured phenomenon to human disease are also important, but often overlooked, considerations. We are conducting experiments to evaluate the sensitivity of hprt mutation measurement in the nonhuman primate, the cynomolgus monkey. Our findings demonstrate in the monkey that hypoxanthine guanine phosphoribosyltransferase (hprt) mutations produced in vivo can be detected using techniques originally used on human cells; cynomolgus monkeys were chosen to avoid many of the complications encountered in studying humans. Sequencing of mutants from the monkey using reverse transcriptase polymerase chain reaction methods has led us to conclude that there is similarity of the spectra observed between the spontaneous mutations detected in the two species. However, more recent data suggest that due to low sensitivity, the method is probably not appropriate for routine biomonitoring of randomly selected populations. For example, the inability of the hprt mutation assay to detect some very potent mutagens in the monkey and the effects of the time-dependent pattern of mutant occurrence serve to urge caution in interpretation of elevation or lack of elevation in mutant frequency. Mechanisms for splitting and archiving samples of human tissues/blood from populations at risk may prove valuable as methods improve. — Environ Health Perspect 104(Suppl 3):521–525 (1996)

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Introduction

In the area of genetic toxicology, the direct link between genotoxins and human disease has been elusive (1). This is true for most human cancer as well as for heritable effects (2). The resulting approach has therefore been to select surrogate markers that respond to known genotoxic influences in laboratory animals and tissue culture and to use these surrogates to estimate the human exposure to genotoxins. The most comprehensive paradigm for extrapolation of this laboratory data to the human condition, the parallellogram approach (3,4), depends on perceived links between the surrogate markers and genetic ill health. Long ago the use of cytogenetic damage was established as a suitable surro-gate dosimeter (5) for estimation of exposure to ionizing radiation; however, many chemicals do not efficiently induce chromosomal damage observable in the microscope. Faced with this challenge, other methods are proposed (see Table 1) for use in biomonitoring when genotoxic hazards are suspected. In particular, methods have been proposed and used for monitoring of workers (6) and other populations (19), based on screening for gene mutations such as the absence of hprt gene activity in lymphocytes. We will discuss some of our data which suggests that adoption of these methods may give a sense of false security and should therefore be approached cautiously.

In large measure, reliance on human epidemiology to supply the evidence of linkage between genetic disease (or cancer) and exposure to risk factors suffers from the intrinsic insensitivity of the methods and the amount of time that must transpire between exposure and end point. Molecular epidemiology (19), i.e., use of molecular surrogates of exposure such as mutant frequencies or DNA adduct levels, may represent improvements in the methodology and decrease the time for carrying out such studies. Discovery of a probable linkage between hazard and end point (often using data from animal studies) results in public health efforts to reduce the exposure of the affected population. Such discovery processes are particularly compromised in human populations because significant portions of the population participate in activities that are known to have some component of genotoxic potential, i.e., smoking, sunbathing, etc.

Several years ago we began a series of studies designed to determine whether the hprt method might provide a suitable surrogate marker for in vivo genotoxic activity. These studies suggest that the low sensitivity observed in the peripheral lymphocytes of the cynomolgus monkey, i.e., inability to detect genotoxic activity of known potent mutagens, complicates the interpretation of negative results. Some practical aspects of the deployment of available gene mutation biomarkers for human monitoring in environmental mutagenesis will also be discussed.

Methods

Chemicals

All chemicals were reagent grade. Ethylnitrosourea (ENU) was obtained from Pfaltz and Bauer, Inc., Waterbury, CT. Cyclophosphamide (CP) was obtained from Sigma Chemical Co., St. Louis, MO. Ethylmethanesulfonate (EMS) was obtained from the Eastman Kodak Company, Rochester, NY. Chloroethylmethanesulfonate (Cl-EMS) was obtained from Aldrich Chemical Co., Milwaukee, WI. Azolesin (U-73975) and CC-1065 were manufactured by The Upjohn Company, Kalamazoo, MI.

Isolation of Mutants from the Cynomolgus Monkey

The methods for isolation and cloning of the hprt mutations from the (wild caught)

### Table 1. Candidate methods for biomonitoring.

| Method                      | Reference(s) |
|-----------------------------|--------------|
| Chromosome aberrations      | (6–9)        |
| Sister chromatid exchanges  | (10,11)      |
| Protein adducts, Th-postlabeling | (12)   |
| Concurrent multiple end points | (6,13,14) |
| DNA repair enzymes          | (1,15)       |
| Gene mutation               | (6,9,16–18)  |
The weekly where the cynomolgus monkey are described elsewhere (20). Blood samples were collected weekly or at somewhat more separated intervals over the course of the studies described here.

**DNA Sequencing**

Detailed information concerning the determination of DNA sequence using conventional methods is described elsewhere (21,22).

**Dosing**

Doses were administered either by intraperitoneal injection or by intravenous infusion. ENU (77 mg/kg), EMS (300 mg/kg), and CI-EMS (35–50 mg/kg) were administered by intraperitoneal injection. CC-1065 (6 μM/M²), adozelesin (6 μM/M²), and cyclophosphamide (75 mg/kg) were administered intravenously.

**Results**

The spontaneous frequency of mutations at the hprt locus in the cynomolgus monkey is similar to that observed in the human, in the rat, and in the mouse (Table 2) (16,20). Following treatment of the monkey with ENU, the mutant frequency increases significantly in a time-dependent fashion, reaching levels as high as a 35-fold increase over the pretest spontaneous frequency during a period of 80 days. A reproducible reduction or falloff in mutant frequency follows the peak; however, in repeated experiments the mutant frequency does not decrease to the levels of mutant incidence established prior to exposure. The induced mutant fraction stabilizes (Table 3); this elevated plateau level has been observed even several years after exposure to ENU. Sequencing of mutants at long periods after exposure reveals them to be predominately of independent origin and typical of an ENU-induced mutation spectrum (21). Sequencing of a large group of spontaneously occurring mutations (22) suggests similarity of the spectrum of observable mutations to that of humans. Therefore the experiments described here were carried out to evaluate the capability of the in vivo hprt mutation assay to reflect exposure to a variety of mutagens (Table 4). No evidence of induction of mutations was seen following single dose exposure to high levels of EMS (a direct acting ethylating agent), cyclophosphamide (a mutagen that requires metabolic activation), CI-EMS (a potent cross-linking agent), or adozelesin [produces a large adduct in the minor groove of the DNA (1,26)] or its parent compound CC-1065. While the experiment with cyclophosphamide was limited in extent (data from a single animal), strong evidence of mutation induction was not seen. The only suggestion of mutation induction (2-fold increase), i.e., elevation of the calculated frequency of mutants, occurred at very low cloning efficiencies; the variability in cloning efficiency significantly affects the calculated mutation frequency. All other compounds listed except ENU were more extensively tested than CP without observed induction of mutation.

In addition to single-dose experiments with ENU, we carried out two kinds of ENU split-dose experiments. In the first experiment (Table 4), we treated monkeys with ENU a second time more than 2 years after the initial dose. This interval was chosen to ensure that the mutant fraction had achieved stabilization following the first dose. The second experiment (Table 4) was carried out by the more traditional split-dose regimen, namely, giving five equally spaced doses with the total equivalent to the initial dose (77 mg/kg) used to induce mutations. The result (Tables 4 and 5) of readministration of ENU after 830 days was a repeat of the earlier observed mutation induction kinetics, namely, a rise in mutation frequency to a peak at about 80 days after treatment and a slow but steady decrease in mutation for the next 3 to 4 months until a higher plateau was reached at about double the plateau level after the first exposure. This result strongly suggests additivity of the effect of multiple doses of ENU. In the second experiment, in which five daily doses were administered (each dose was 1/5 the initial dose in the single dose experiment), the data also supports a simple additivity of dose (Table 5). The mutants we have sequenced are consistent with the expectation from the ENU spectrum.

**Table 3.** Mean mutation frequencies observed in cynomolgus monkey T lymphocytes treated twice with ENU (77/ kg).

| Regimen       | Time after first treatment (days) | Mean mutation frequency | Number of animals | Number of observations (each animal) |
|---------------|-----------------------------------|-------------------------|-------------------|-------------------------------------|
| First treatment | -160 to 830                      | 20–25 × 10⁻⁶            | 2                 | 16                                  |
| Second treatment | -1806-2163                       | 40 × 10⁻⁶               | 2                 | 6                                   |

The mean mutation frequency is the arithmetic mean calculated based on all observations made during the time after first treatment. These monkeys were treated at day zero and again at approximately day 830. Not shown are the peak mutation frequencies that occurred at approximately 80 days after each treatment (see Table 5). The pretest mean spontaneous frequencies observed in these two monkeys were 1.7 and 6.3 × 10⁻⁶ respectively. Note the time interval is given relative to the first treatment in both lines of the table.

**Table 4.** Compounds evaluated for the induction of mutations in the T lymphocytes of cynomolgus monkeys.

| Compound                  | Dose (mg/kg) | Type agent | Number of animals | Result |
|---------------------------|--------------|------------|-------------------|--------|
| Ethylnitrosourea          | 77           | Ethylating | 6                 | Positive |
| Cyclophosphamide         | 75           | Metabolically activated | 1 | Likely negative |
| CC-1065                  | 6 μg/M²      | Bulky DNA adduct | 3 | Negative |
| Azolesin                 | 6 μg/M²      | Bulky DNA adduct | 5 | Negative |
| Ethylmethanesulfonate     | 300 mg/kg    | Ethylating | 2                 | Negative |
| CI-ethylmethanesulfonate  | 35–50 mg/kg  | Crosslinker | 3                 | Negative |

**Table 5.** Comparison of hprt mutation frequencies observed in cynomolgus monkey peripheral T lymphocytes following multiple doses of ethylnitrosourea.

| Regimen/treatment number | Dose (mg/kg) | Peak mutation frequency (10⁻⁶) | Number of animals |
|--------------------------|--------------|-------------------------------|-------------------|
| Single treatment         | 77           | 33–77.9                       | 4                 |
| Second treatment         | 77           | 159–190                       | 2                 |
| Five daily doses         | 15.4 mg/kg/day | 24–33                        | 2                 |

Spontaneous mutation frequency of all animals (33 animals and 103 determinations) in our colony is 2.9 ± 2.9 × 10⁻⁶.
Discussion

Biomonitoring is carried out to assess whether individuals or populations have been exposed to toxicants. The application of biological indicators of exposure is frequently viewed as more relevant than purely chemical or physical measures because biomonitoring provides an impression of the likely effects on man to be expected from exposure. The avoidance of mutational disease (1) is the end goal of the activity. There are techniques that might be appropriate for monitoring for evidence of exposure to mutagenic hazards. Perera and Whyatt (19) have reviewed the available biomarkers and their uses in molecular epidemiology; these authors point out that validation of the available biomarkers is still needed. As part of the validation described by Perera and Whyatt (19), there is a need to obtain confirmation of the assay results by other methods. Essentially the results in this paper constitute part of the kind of validation needed to understand the limitation of the hprt gene mutation assay, which was originally developed using human cells.

Cynomolgus Monkey

hprt Mutation Induction

We have shown (20) that measurement of mutation frequencies, as well as sequencing of the mutants (22), at the hprt locus in the cynomolgus monkey is feasible using the techniques developed for use with human cells. We have also shown (21) the ability to detect increases in mutant frequency following exposure to ENU, a very potent mutagen. However, several features of the time course of occurrence of the increase in mutant frequency were unexpected. For example, the peak in mutant yield is seen only at approximately 80 days following treatment, with a gradual increase slowly occurring before this time. This is surprising with a direct acting alkylating agent such as ENU because the damage occurs very rapidly; thus, lymphocytes that are stimulated to divide shortly after treatment should experience mutagenesis as they pass through S-phase. The lag before mutation is observed could be due to mutation in a stem cell population, but this speculation is far from proven; however, sequencing of mutants detected at long periods after treatment fails to confirm clonal origin of these mutants (Harbach and Aaron, unpublished data). The second feature of the time course experiments, which is somewhat difficult to reconcile with the expectation of clonality, is that after approximately 150 days the observed mutant frequency stabilizes (Table 3) at an elevated level compared to predose levels. This observation raises the question about whether observed mutation frequency increases can be used to establish the source of mutagenic insult in a randomly selected population.

The induction of mutations in cynomolgus monkey T lymphocytes was not observed using compounds (Table 2) that are representative of several types of agents including CP, EMS, Cl-EMS, CC-1065, and adozelesin. Of these compounds, the result with adozelesin is the most disturbing because this is the most mutagenic compound tested in this laboratory in a variety of standard genetic toxicity tests. Adozelesin has been tested in assays for gene mutation (CHO/Hprt as well as AS52/gpt) and chromosomal aberration assays (27,28) and has systematically been observed to be a particularly potent mutagen and clastogen. Adozelesin (26) reacts directly with DNA and does not require metabolic activation. Interestingly, the binding is due to alkylation via ring opening of a cyclopropyl group and the binding appears to be reversible (29), one of very few examples of such an alkylation or reversible alkylation. Furthermore, several lines of evidence reveal the capability of the compound and others of the class to produce mutations at the site of the consensus binding domain in DNA (the binding target is a run of five A residues [AAAAA] or an ATT site). Consistent with this expectation, the only strain of the standard Ames tester set that is positive under the influence of this drug is TA102 (unpublished data), the only strain in the Ames tester set to contain an ATT genetic target for mutagenesis. Also, the spectrum of mutations recovered in the AS52/gpt gene is exactly what would be predicted from the known locations of putative binding sites (Tindall and Aaron, unpublished data). The doses at which mutagenesis is detected are extremely low in both TA102 and AS52. In the in vivo experiments with monkeys at doses that give complex chromosomal aberrations in approximately 32% of the lymphocytes (data not shown), we could find no evidence of gene mutagenesis at hprt up to 6 months after exposure. The failure to detect mutant frequency increases with this diverse array of compounds even at very high (toxic) doses suggests that the assay is particularly insensitive to many mutagens. The logical conclusion that follows from these observations is that there is a significant likelihood of false negative results, i.e., one would be very likely to incorrectly conclude that no exposure had taken place even though very significant exposure could have occurred. Furthermore, we found little evidence in our studies using monkeys that techniques such as use of mutational spectra as a means of fingerprinting the compound and retrospectively identifying the toxicant would prove feasible. However, the spectra of the spontaneous mutants analyzed to date (22) suggest good agreement between the types of mutants currently seen in cynomolgus monkey and human hprt databases.

Conclusions

One concern about the available methods for detecting mutagen exposure through use of biomonitoring is the lack of sensitivity of the hprt gene mutation assay. Methods such as multiplex PCR-based techniques could provide methods for addressing this concern. In addition, the ad hoc nature of the current process for obtaining and processing samples needs to be revamped. Our data from experiments with the cynomolgus monkey indicate that mutation frequency measurements require great care in interpretation, even when data are gathered under the controlled laboratory conditions established for these primates.

In the field, i.e., at the sites of accidental or other exposures, these methods for sample procurement need to be very carefully worked out and the strength of the overall protocols needs to be further validated and improved. A more systematic approach to the collection and handling of samples is important. Perhaps a central international repository for collection and storage of biological samples might be established with the aid of an international organization. Such a repository should ensure timely sample acquisition following an accident, as well as ensure appropriate storage and sample integrity. Recently, data have been published showing that the profile of lymphocyte subpopulations is not affected by storing frozen cells for periods up to 8 days (30) or by stimulation of G0 lymphocytes (31). Such a repository approach would be similar to approaches used for monitoring human diseases. The advantage of the repository approach is obvious, namely, a more systematic and thorough evaluation of samples could be planned. In addition, stored samples in a repository could be analyzed retrospectively when either better techniques become available or greater insight leads to more
informative questions. Such a system ought to offer the opportunity to carry out the necessary technology transfer to laboratories in less developed areas so that local laboratories could be trained to do the evaluation and interpretation.

When evidence of exposure is detected, appropriate public health steps should be taken to protect the individuals (3, 4, 7, 8); lack of evidence of exposure is usually not accompanied by any necessary public health measures. However, the state of the art for biomonitoring with respect to identifying mutagen exposure is relatively new compared to that for infectious diseases, for example, and thus the assays available are in varying states of validation (19). Some serious drawbacks, i.e., low sensitivity (32), to the widespread deployment of the available tools of biomonitoring are apparent. Furthermore, the availability of equipment and technical skill is limited in many parts of the world where biomonitoring must be practiced. Systematic collection and archiving of some of these samples would provide biological material that could significantly contribute to the retrospective identification of causative factors.

Our interest in the mechanisms of in vivo mutagenesis has led us to evaluate the induction of hppt mutations as well as a large number of other kinds of mutations. In those experiments, the question of sensitivity has often come up. In the case of hppt in the monkey, the series of experiments described here leads us to urge caution in too heavy reliance on single end point methods, particularly the hppt assay, as the basis of decision making. In the context of these findings, it is pertinent to ask "What question does biomonitoring address?" Conference such as this provide a forum for addressing this question.

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