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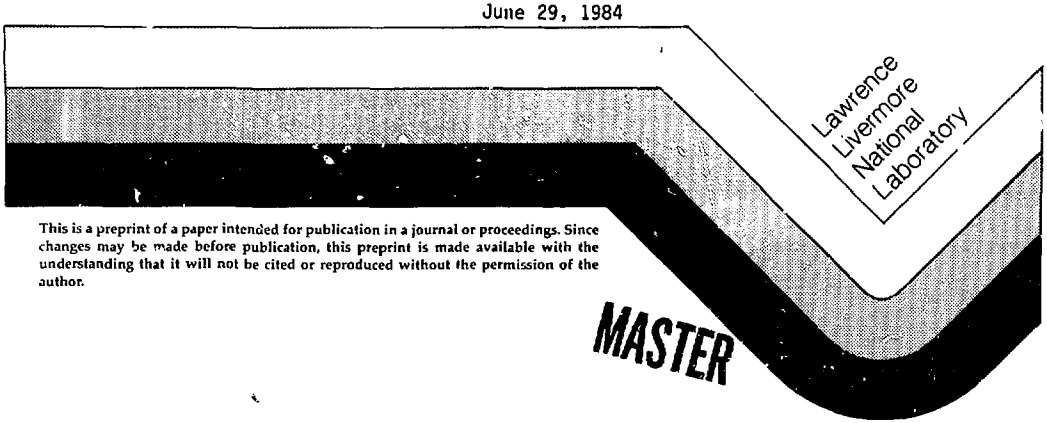

PROSPECTS FOR CELLULAR MUTATIONAL ASSAYS IN HUMAN POPULATIONS

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Practical, sensitive, effective, human, cellular assays for detecting somatic and germinal mutations would have great value in environmental mutagenesis and carcinogenesis. When available, such assays should allow us to fill the void between human mutagenicity and the data that exist from short-term tests and from mutagenicity in other species. We will be able to validate the role of somatic mutation in carcinogenesis, to identify germinally active human environmental factors, to integrate complex mixture and environmental effects in the human subject, and to identify people who are hyper-susceptible to genetic injury. Human cellular mutational assays, particularly when combined with cytogenetic and heritable mutational methods, promise to play pivotal roles in estimating the risk from low-dose radiation and chemical exposures. These combined methods avoid extrapolations of species and dose, and may be sensitive enough and credible enough to permit politically, socially and scientifically acceptible risk management.

Cytogenetic methods already have wide applicability to the human (Evans,this volume), and heritable mutational measurements are undergoing an extensive epidemiological trial in Japan (Neel, this volume). However, we presently have no validated,generally accepted methods for human somatic or germinal cellular mutational assays. Bleak as this sounds, I believe the technology is almost available and that we are on the verge of having several promising cellular mutational methods for human application.

Somatic and germinal assays have one enormous advantage over heritable assays. As is clear from the studies of the Hiroshima and Nagasaki populations (Neel, this volume), observable heritable genetic changes at any particular locus occur with great rarity. To find sufficient mutational events for a measurement of rate, one must study millions of offspring, millions of loci, or some combination of the two. The expectation is that somatic and germinal mutation rates will be similarly small, but for these measurements any one person can provide the millions or billions of cells needed to estimate a mutation rate. The subjects (ie. the cells) thus are readily and sufficiently available; the challenge is to detect the rare, one-in-a-million events at the cellular level and to have good assurance that the events represent mutational phenomena.

Detection strategies at the cellular level can be based on four types of changes:

* alteration in behavioral phenotype, such as drug resistance.

* alteration in gene product, such as a modified protein.

* alteration or loss of a messenger RNA.

* alteration or loss of a DNA sequence.

The further down the list one goes, the closer one is in principle to a verifiable mutational event; however, to my knowledge the RNA and DNA based methods are not yet available for single cell application. To identify RNA and DNA changes would require methods that detect loss of a single sequence or gain of a minimally-deviant single-copy sequence in a single cell. Assays based on changes in behavioral phenotype and in gene product seem more feasible with present technology and are discussed below.

HPRT SOMATIC CELL MUTATION BASED ON 6-THIOGUANINE RESISTANCE

R.J.Albertini has pioneered the development of assays in human peripheral blood lymphocytes based on resistance to 6-thioguanine. In the original assay (Strauss and Albertini, 1979), freshly drawn lymphocytes were exposed to phytohemagglutinin (a mitogen), 6-thioguanine (a purine analogue that is cytotoxic to cells attempting DNA synthesis in the presence of effective levels of the enzyme HPRT (hypoxanthine phosphoribosyltransferase)), and tritiated thymidine (a label to detect those cells successfully carrying out DNA synthesis).

Autoradiography was used to count both the rare, resistant, thymidine labelled cells, and the cells at risk (based on labelled cells in cultures not exposed to 6-thioguanine).

Encouraged by early results, Albertini and his colleagues collected a sizable body of data using this assay (Albertini, 1982). They observed background levels which did not change with age, increased levels in cancer patients before and after cancer therapy, and increased levels after PUVA therapy. Instability of background levels, and consistently high variant frequencies with occasional values as high as one variant per 100 cells at risk led to the suspicion that the assay was detecting phenocopies as well as mutants. One source of phenocopies seemed to be circulating cells committed to DNA synthesis before exposure to mitogen. These cells and the excessively high variant frequencies were eliminated by introducing a cryopreservation step into the assay.

At this point in the evolution of the method, sufficient doubt had been cast on the mutational validity of the autoradiographic endpoint to make Albertini refocus his efforts toward a clonogenic version of the assay (Albertini et al., 1982). He was able to clone single cells in microwell cultures at an efficiency up to 50% using a crude T-cell growth factor. This led to a dilution assay which could be used both to estimate the frequency of thioguanine resistant cells, and to support the mutational nature of the phenotype by demonstrating the corresponding HPRT enzyme deficiency in the resistant clones. Albertini is now in a position to validate the modified autoradiographic assay or to use the clonogenic assay, and to redo and continue on with his epidemiologic applications of the methods.

Several other laboratories are pursuing these approaches with mixed success. Amneus et al.(1982) at the University of Upsalla have incorporated into the original Strauss and Albertini procedure the method of flow cytometric sorting to concentrate late S and G2 cells prior to autoradiography. This facilitates counting and eliminates some sources of phenocopies. Their recent results (Amneus et al.,1984) suggest that the variant cells early after a mutagenic insult are predominantly due to DNA lesions which interfere with enzyme production. As the lesions are repaired, these variants disappear and are replaced by less frequent, presumably-stable mutants. This group feels that the short-term autoradiographic assay in human peripheral blood lymphocytes may be useful for estimating DNA damage but not mutation. On the other hand, Morley et al.(1982, 1983) have described autoradiographic and clonogenic assays which they believe successfully detect HPRT mutations in human peripheral blood lymphocytes. J.Evans and colleagues in Edinburgh (this

volume) have recently produced credible radiation dose-responses in vitro in human lymphocytes using a clonogenic version of the HPRT assay. Similarly, I. Jones et al.(1984) at Livermore have a clonogenic assay that is working well with mouse splenocytes mutagenized in vivo.

In spite of the difficulties, it seems likely that some form of HPRT assay will be available for general application in the near future. Assays for other genes involving drug resistance are also possible, although this approach is inherently limited for human application to those genes functioning effectively as single copies (ie.only to genes on the X chromosome) or to dominant selectable markers such as diptheria resistance.

HEMOGLOBIN SOMATIC CELL MUTATION ASSAY

In 1976, Papayannopoulou et al at the University of Washington developed a monospecific polyclonal antibody to Hb S (sickle hemoglobin), showed that the fluorescent antibody could be used to identify sickle trait erythrocytes in smears, and raised the possibility of using this as an assay for sickle-like somatic mutations in erythrocyte precursors. This general strategy of using known mutated gene products to generate immunologically specific reagents to detect new cellular mutants has broad potential applicability in somatic mutagenesis.

The same group developed a variety of similar immunologic probes to hemoglobins with single amino-acid substitutions (eg Hb C, Papayannopoulou et al.,1977), as well as probes to hemoglobin frame shift variants (eg Hb Wayne and Hb Cranston, Stamatoyannopoulos et al., 1980). They showed that the rate of appearance of Hb S and C bearing erythrocytes in normal adults was on the order of one in 10 million, and that this rate increased after cancer chemotherapy. However, for them to make such determinations manually by fluorescence microscopy was extremely arduous, requiring one person-month to count a single sample.

In collaboration with the Washington group, a team from the Lawrence Livermore National Laboratory attempted to automate the Hb S method using flow cytometry (Bigbee et al., 1981). They developed a way to stain erythrocytes in suspension, and to examine one million erythrocytes per second. However, direct application was prevented by false-positive events occurring at roughly one per one-hundred thousand erythrocytes. By using flow sorting to concentrate fluorescent cells, they were able to confirm the original frequencies of occurence of variants, but found the results too variable for quantitative or epidemiologic application.

Continuing independently, the Livermore group (Bigbee et al., 1983; Jensen et al., 1984) has developed a suite of monoclonal, monospecific antibodies that recognize a variety of single-base change, single-amino-acid substitutions in human, mouse and monkey hemoglobins. They have also developed improved methods of fixation that provide better antibody staining, more durable cells, and cells suitable for flow cytometric counting by scattered light. The problem of false positive signals continues to plague the method but is now reduced by a factor of 10. With the present methods, in reconstruction experiments it is possible by flow sorting to retrieve 88 antibody labelled cells (coefficient of variation, 10%) from a mixture of 100 labelled and one billion unlabelled cells. Further developments using human, mouse and perhaps monkey blood samples is underway using the partially automated sorting method, as are attempts to fully automate the procedure.

An important limitation of this method has to do with its incredible resolution. Much as one may want the flexibility to measure single, specific, base changes (ie the ultimate resolution of a genetic method), certain mutagens (such as ionizing radiation) may be very inefficient at producing such small lesions. In addition, the smaller the target size, the rarer the expected outcome. As mutant frequencies drop one or several orders of magnitude below one per million, it becomes increasingly difficult to detect the rare events or to have a large enough population of precursor cells at risk to measure the frequency of variants properly.

GLYCOPHORIN SOMATIC CELL MUTATION ASSAY

An alternative to the limitations of the hemoglobin-based assay would be an immunological assay that recognizes gene-loss mutations at the cellular level. Prior attempts at such assays are thought to have failed to detect true mutational events (Atwood and Petter, 1961), perhaps due to the high prevalence of phenocopies mimicking the desired genetic change. This problem can apparently be avoided in a setting where two alleles are codominantly expressed and can be seperately detected in the same cell. The loss of either allele can then be detected in cells in which the other allele is still functioning normally and serves as a physiological control. Glycophorin-A in erythrocytes appears to be just such a system.

Human glycophorin-A is a 131 amino acid, trans-membrane sialoglycoprotein that codes for the M and N serotypes. M individuals have a serine and glycine respectively at the 1 and 5 position, counting from the amino-terminal external end. N individuals have a leucine and glutamic acid at these same

positions. Otherwise the two molecular types are identical. Each allele on average produces roughly 250,000 copies per erythrocyte whether in a homozygous or heterozygous configuration. The principle of the somatic mutation method is to label M and N independantly with monoclonal antibodies carrying different fluorophores. In heterozygous individuals, every normal erythrocyte should show both fluorescent colors. A mutation inactivating one allele would result in erythrocytes lacking the M or N antigen. Such cells will fail to bind one of the monoclonal antibodies while expressing the second normally. Symmetry is expected. Thus the rate of variants seen for one color should be similar to that seen for the second.

Bigbee et al.(1983, 1984) have produced a series of monoclonal antibodies against the M and N glycophorins, as well as several public antibodies that recognize both forms equally. When the antibodies are fluorescently labelled they show high affinities to glycophorin in fixed erythrocytes in suspension. The flow cytometric signals of such erythrocytes typically have coefficients of variation of 12%, with peak means that vary from one person to the next by a coefficient of variation of as little as 3%. The Livermore studies have confirmed that the expression of the two alleles is quantitatively independent.

To date, putative mutants have been sought using only half of the assay. That is, the anti-M antibody has been used in conjunction with one of the public antibodies. The method in this limited form works well, finding roughly 8 variants per million erythrocytes in normal individuals. A preliminary trial on patients undergoing chemotherapy found average rates increased by 100%, with one individual increased by 800%. The full assay with both antibodies is now being tested and hopefully will provide confirmation through symmetry that the variants are indeed mutants.

One of the Livermore anti-M antibodies is able to recognize glycophorin-Mc, a rare allele which differs from N only in the presence of a serine at position 1. This antibody is thus discriminating M from N by the difference in the single terminal amino acid. It could be used to detect single amino-acid substitutions in NN homozygotes, and should be comparable in resolution and performance to the hemoglobin reagents. Thus with the set of antibodies now in hand, it may be possible to measure both point mutation and gene loss at the glycophorin locus.

LDH-X SPERM CELL MUTATION ASSAY

The same techniques described for somatic cells can in principle be applied to germinal cells, although presently it is

only mature sperm that can be readily sampled in the human. *Ansari, Baig and Malling* (1980) have described what may be the prototype for such methods. They immunized mice with rat LDH-X, a form of lactate dehydrogenase that is localized specifically to germinal cells. Highly specific polyclonal antibodies were produced. These of course recognized rat sperm, but also had the ability to recognize roughly one per million mouse sperm. The presumption is that the recognized mouse sperm were expressing an LDH-X gene which mutated to the rat genotype at a single site. The sperm were identified by staining or fluorescence of their midpiece. A dose-response curve to procarbazine treatment of the mice gave very convincing results.

Unfortunately Malling has been unable to reproduce these results and has exhausted his supply of the active antibody. Nevertheless, the potential remains to apply similar techniques to human sperm. Whether such germinal methods will or will not be predictive of heritable mutation remains to be seen, but whatever the outcome, cellularly based methods in sperm should provide an important insight into agents capable of reaching the testis, activating appropriately, and causing the expression of abnormal gene products.

DISCUSSION

No one of the methods discussed above is actually available today for application to human testing, yet I hope it is clear why I am optimistic that such methods should soon be at hand. Obviously they will need extensive elaboration before general application. Validation will be especially important since the approaches outlined for the most part do not measure mutations directly. Validation will presumably be by a variety of consistency tests in human subjects and model systems. In some situations it may be possible to clone cells and validate directly. In others, such as the methods involving anucleate erythrocytes, cloning is out of the question. For the erythrocytic methods, the best one can hope for is that isolation of the variant cells will allow biochemical confirmation of the expected electrophoretic or other mutational changes in the involved proteins.

The full extension of these methods requires a great deal more than the few isolated examples given in this presentation. Ideally one would want methods that are sensitive to all types of DNA lesions, including base substitutions in all four bases, frame shift lesions, and small and large deletions, inversions or translocations. A fully representative spectrum of genes should be included, as well as a broad and relevant sampling of tissues and cells. For somatic mutation there is an expectation that an

elevated rate would be predictive of cancer risk. But would a high rate in erythrocytes predict only polycythemia? Or would erythrocytic mutations also predict cancer in a variety of other tissues and organs?

We must understand well the kinetics of how mutants appear and disappear in cell populations. These kinetics will involve turnover of cell compartments, the maturity of the initially mutated cell, and possible selective disadvantage or advantage for the mutant phenotype. A test system with short duration (i.e. short memory or short integration time) would be best suited for studying acute exposure situations. Intermediate to long-term memory would be best for studying the effects of occupations, life styles, or geographical factors, or for searching out repair defective individuals. Finally it will be important to understand better what the time domain means to the rate calculation. Is the relevant parameter mutations per cell cycle, mutations per hour, mutations per generation, or some mixture of these? How differently is this expressed in species with different lifetimes and different body sizes?

Clearly much remains to be done to develop the methods and to gain some understanding of their application. In spite of this, direct testing in humans remains in my view the single most tractable way to approach the low-dose risk problem for carcinogenesis and mutagenesis. I hope the resources and scientific strengths will be available to make it happen quickly and effectively.

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