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THE GLYCOPHORIN A ASSAY FOR SOMATIC CELL MUTATIONS IN HUMANS

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INTRODUCTION

The glycophorin A (GPA) assay provides a new approach for quantitating the frequency of somatic cell mutations that arise in vivo in humans. The GPA assay employs monoclonal antibody labeling and flow cytometry to enumerate rare variant erythrocytes which fail to express one of the two allelic forms (M and N) of the cell surface protein, glycophorin A, presumably due to gene-expression loss mutations in erythroid precursor cells (Langlois et al., 1986). While the assay can only be performed on MN heterozygotes, about 50% of the population are heterozygous at the GPA locus. It is hoped that the variant cell frequency (VF) will provide a measure of the level of genetic damage and ultimately cancer risk in individuals due to either exposure to genotoxic agents or unusual susceptibility to genetic damage.

The GPA assay has a number of advantages for the study mutagenesis in humans. M-loss and N-loss variants can be quantitated for each individual, and the requirement for normal expression of one allele discriminates against epigenetic events and mutations outside the GPA locus which would affect both alleles. Both allele-loss events, and events which lead to homozygosity (e.g. chromosome missegregation or recombination) can quantitated by measuring hemizygous and homozygous phenotype variant cells. Both classes of events appear to

play important roles in the development of a growing variety of human tumor types (Green, 1988). While erythrocytes have the disadvantage that molecular characterization of mutations is not possible, the ability to detect mutant phenotype cells directly without culture or selection greatly simplifies the assay and simplifies the collection and transport of blood samples from geographically distant populations. The kinetics of erythrocyte production and turnover are also well defined, so that VFs should be unaffected by clonal expansions seen with lymphocytes (Nicklas et al., 1988). Also, the time course of variant cell induction and persistence can used to determine the stages of erythropoiesis where the mutations occur.

In this report we briefly review our past experience and some new developments with the GPA assay. Particular emphasis will be placed on two areas that affect the utility of the GPA assay for human population monitoring. The first is our efforts to simplify the GPA assay to make it more generally available for large population studies. The second is to begin to understand some of the characteristics of human hemopoiesis which affect the accumulation and expression of mutant phenotype cells.

METHODS

The GPA assay utilizes pairs of fluorescently tagged monoclonal antibodies specific for the M and N allelic forms of GPA to label fixed erythrocytes from heterozygous MN donors. Flow cytometry is used to enumerate rare single-color cells that lack the expression of one allelic form of GPA. Several different antibody combinations and fixation methods have been developed to detect either M-loss variants or N-loss variants. Two variant cell types are separately measures in each assay: hemizygous phenotype variants (NØ or MØ) that lack expression of one allele and express the remaining allele at a one-copy level, and homozygous phenotype variants (NN or MM) that lack

expression of one allele and express the remaining allele at a two-copy level.

The original GPA assay utilized a dual-laser flow sorter to enumerate and purify variant cells (Langlois et al., 1986). This approach has the advantage that sorting can be used to confirm that single-color objects detected by the flow cytometer are single-color erythrocytes. One limitation of this approach, the cost and complexity of the instrumentation, has been reduced by adapting the assay to a single-laser sorter (Kyoizumi et al., 1989). We have recently completed the development of an assay method that utilizes a low-cost clinical flow cytometer and have confirmed that this method yields comparable VFs and increased precision compared with the sorting assay (Langlois et al., 1989b). This assay is well suited for large population studies because five million cells per donor can be analyzed in less than 30 minutes, with assay results available within 24 hours after the blood is received. Also, blood samples can be stored for up to two weeks before analysis, facilitating sample transport from distant populations as well as batch processing of samples.

VARIANT CELL FREQUENCIES IN NORMAL AND EXPOSED DONORS

Previous studies of normal unexposed donors have shown a mean hemizygous VF (both NØ and MØ phenotype) of 10×10^{-6}, with most donors falling in the range of 2×10^{-6} to 25×10^{-6}. Hemizygous VFs also increase significantly with age, but this increase is small (2-fold increase from age 0 to 70 yr.) (Langlois et al., 1986; Jensen et al., 1987). Normals show a mean VF of about 10×10^{-6} for homozygous variants (NN and MM phenotype), with a somewhat larger range of 3x10-6 to 34x10-6 (Langlois et al., 1989b). Given the limited precision of individual assay runs (coefficient of variation of about 30-40%), multiple assays on a few donors were used to quantitate differences among normal donors. The results in Figure 1 show donors differ significantly in both NØ and NN VFs,

and in the ratio of NØ to NN variants. Also, each donor showed a consistent pattern of NØ and NN VFs over the 1 to 5 year sampling period.

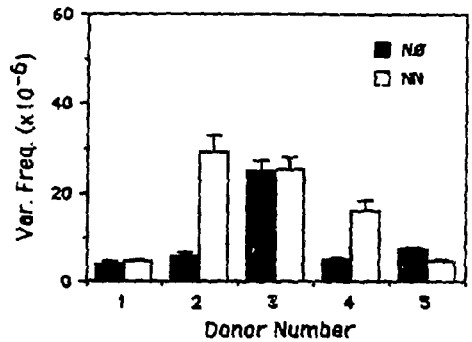

Figure 1. VFs in samples from five normal donors. Each bar represents the mean ± SEM of 7-19 samples per donor, assayed over a period of 1-5 years.

Survivors of the atomic bomb at Hiroshima were studied to determine the effect of radiation exposure on the GPA assay (Langlois et al., 1987). This study showed a significant dose-dependent increase in VF 40 years after exposure suggesting that induced mutational lesions are stably integrated in stem cells. Unexpectedly high, and low VFs were observed with high dose survivors suggesting stochastic effects in the induction of mutations in a small pool of stem cells that survived after these doses. Figure 2 shows preliminary results from GPA assays on workers involved in the reactor accident at Chernobyl, USSR that were sampled about 9 months after the accident. These results show a similar dose response as seen with the Hiroshima study, as well as an anomalous VF for the most heavily exposed worker. Thus, the frequency of radiation-induced mutations appears to remain nearly constant for the lifetime of the individual.

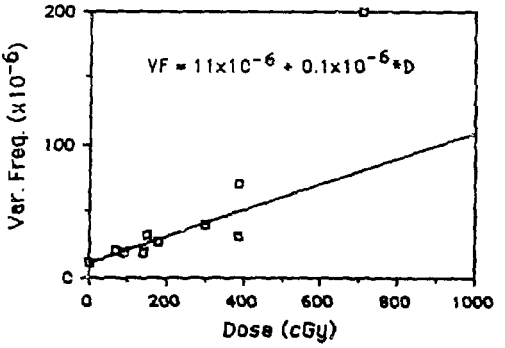

Figure 2. VFs in samples from workers at the Chernobyl accident. Points are the average of NØ and MØ VFs, with the top point corresponding to a VF of 536×10^{-6} (for methods see Langlois et al., 1987). Dose estimates are from dicentric frequencies in lymphocytes. The line is a linear regression on all points except the top point.

A longitudinal study of breast cancer patients receiving adjuvant chemotherapy provides some insight into the response of the GPA assay to exposures to mutagenic chemicals (Bigbee et al., 1989b). Patients showed a gradual increase in VF during therapy reaching a maximum of about 7-fold above background when treated with adriamycin + cyclophosphamide. VFs declined to near-normal values about 120 days after the completion of therapy, in contrast to the persistent effect seen with radiation. Thus, these two agents appear to primarily affect the rapidly dividing pool of committed erythroid precursor cells rather than stem cells. Repair of chemical lesions in stem cells which cycle infrequently may also be responsible for the lack of induced spermatogonial mutations by some chemicals in the mouse specific locus test (Russell, 1982). Patients receiving the direct-acting mutagen, adriamycin, all showed

elevated VFs, while patients that received cyclophosphamide, which requires metabolic activation, showed variable responses with some patients showing no increase in VF (Figure 3). Thus, individual differences in metabolic activation may affect the GPA response with indirect mutagens.

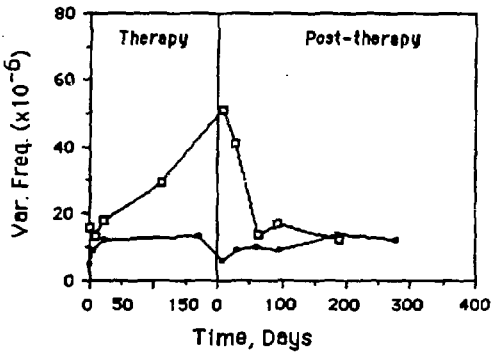

Figure 3. NØ VFs in samples from two breast cancer patients that both were treated with cyclophosphamide, methotrexate, and 5-fluorouricil.

VARIANT FREQUENCIES IN CANCER-PRONE INDIVIDUALS

Individuals could have elevated VFs due to either excessive mutagen exposure or unusual susceptibility to spontaneous or induced genetic damage. The cancer-prone syndromes provide a model for individuals with high cancer risk due to inherited defects in DNA replication or repair. The GPA assay has been performed on individuals affected with three of these syndromes, and the results of these studies are summarized in Figure 4. Individuals with xeroderma pigmentosum (XP) have a high risk for skin tumors in sun exposed areas due to a defect in excision repair. Five individuals with a severe form of XP (group A), and two individuals with a mild form of XP(XP

variant) all showed normal VFs. This result suggests that the repair defect in XP has minimal effect on the frequency of spontaneous mutations in internal organs, and helps to explain the lack of elevated risk for internal cancers in XP individuals. Individuals with ataxia telangiectasia (AT) or Bloom's syndrome (BS), in contrast, have an elevated risk for a variety of tumor types. While the defects responsible for these syndromes have not been established, high levels of spontaneous chromosome abnormalities suggest defects in DNA replication. GPA analysis shows dramatically elevated VFs for individuals with both of these syndromes (Langlois et al., 1989a; Bigbee et al., 1989a). Additionally, BS individuals show extreme elevation in the frequency of homozygous GPA variants supporting previous evidence for elevated somatic recombination in these individuals. Thus, high frequencies of spontaneous in vivo mutations in AT and BS individuals may contribute to their extreme cancer risk.

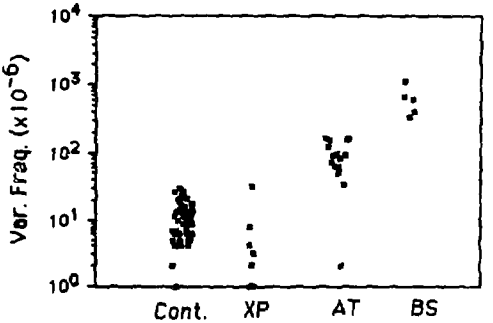

Figure 4. NØ VFs in samples from normal control donors (Cont.), and donors with xeroderma pigmentosum (XP), ataxia telangiectasia (AT), or Bloom's syndrome (BS).

SUMMARY

The GPA assay was first developed to provide a method for quantitating mutational damage arising in vivo in humans. Our experience over the past five years suggests that the assay is capable of detecting in vivo genetic damage from exposure to both ionizing radiation and mutagenic chemicals, as well as elevated levels of damage in cancer-prone individuals. It is already clear, however, that there is not a simple one-to-one correspondence between exposure dose and VF. A number of characteristics of the hemopoietic target cell populations may influence measured VFs. The transient response in the chemotherapy study may reflect differences in cell proliferation between stem cells and committed precursors. Individual differences in metabolism may influence responses to mutagens like cyclophosphamide that require metabolic activation. Anomalous VFs after high-dose radiation exposure may result from stochastic effects in a small number of surviving stem cells. Clearly, many more studies involving different mutagenic agents and different exposure schedules will be required to clarify these issues. The recent development of a simplified flow cytometric GPA assay should greatly facilitate large studies of populations with different exposure histories. These studies should help clarify the utility of the GPA assay for large scale population monitoring.

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