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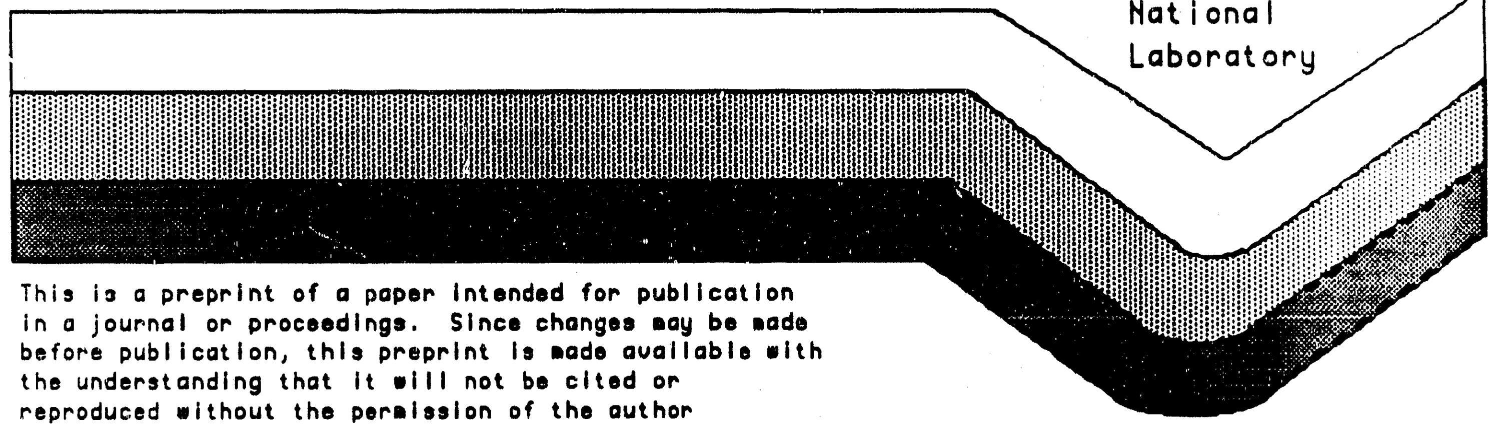

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Somatic Cell Genotoxicity at the Glycophorin A Locus in Humans

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We have developed an assay for detecting variant erythrocytes that occur as a result of *in vivo* allele loss at the glycophorin A (GPA) locus on chromosome 4 in humans. This gene codes for an erythroid-specific cell surface glycoprotein, and with our assay we are able to detect rare variant erythrocytes that have lost expression of one of the two GPA alleles. Two distinctly different variant cell types are detected with this assay. One variant cell type (called NØ) is hemizygous. Such cells might arise by mutation, deletion or inactivation of the GPA(M) allele or loss of the chromosome carrying that allele in erythroid precursor cells. Our assay also detects homozygous variant erythrocytes that have lost expression of the GPA(M) allele and express the GPA(N) allele at twice the heterozygous level. These are termed NN cells and might be generated by chromosomal loss and duplication, gene conversion or mitotic recombination in erythroid precursor cells in the bone marrow. An important feature of the assay is that it requires continued expression of one of the two allelic forms of GPA to guarantee that all variant cells are capable of normally expressing this cell surface antigen. Because of this built-in safeguard, erythrocytes that have epigenetically lost the capability to retain any cell surface GPA (e.g., by membrane defects, carbohydrate metabolism defects, or by cellular degeneration) are not included as false positives (phenocopies) in the enumeration of variant cells.

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The results of this assay are an enumeration of the frequency of NØ and NN variant cell types for each individual analyzed. These variant cell frequencies provide a measure of the amount of somatic cell genotoxicity that has occurred at the GPA locus. Such genotoxicity could be the result of 1.) reactions of toxic chemicals to which the individual has been exposed, or 2.) high energy radiation effects on erythroid precursor cells, or 3.) errors in DNA replication or repair in these cells of the bone marrow.

Thus, the GPA-based variant cell frequency can serve as a biodosimeter that indicates the amount of genotoxic exposure each individual has received. Because two very different kinds of variant cells are enumerated, different kinds of genotoxicity should be distinguishable. For example, one might expect that radiation exposure would result in DNA damage that leads to deletions of chromosomes or subchromosomal deletions affecting specific genes. These would yield hemizygous variant cell types, and our assay would show high frequency of hemizygous with near-normal frequency of homozygous cells. This is an entirely different effect than might be expected from exposure to a DNA cross-linking chemical, such as Mitomycin C (Tomasz et. al., 1987). This might very well increase somatic cell gene mutations and also somatic cell recombinations, thus leading to increased frequency of both variant cell types in the GPA genotoxicity assay.

Results of the GPA somatic genotoxicity assay may also provide valuable information for cancer-risk estimation on each individual. The kinds of genotoxic events that create variant erythrocytes at the GPA locus (e.g., mutations, deletions, and mitotic recombinations) have also been shown to induce activation of proto-oncogenes and/or inactivation of tumor suppressor genes. Thus, one might well expect that an individual who displays a high frequency of GPA variant cell types might have experienced a higher than normal amount of neoplasia-inducing DNA damage and would have a higher than normal probability of developing cancer.

Normal Individuals

The first GPA-based somatic genotoxicity assay that we developed (called the 1W1 assay) indicated that GPA variant erythrocytes occur at a very low frequency (ca. 10^{-5}) in peripheral blood (Langlois et. al., 1986). For this assay we used a dual beam cell sorter to perform the analysis. In

general we analyzed 5 x 10^5 erythrocytes and sorted cells that gave variant signals. These cells were analyzed by fluorescence microscopy to confirm their variant phenotype. This procedure proved to be rather tedious and time consuming but gave us confidence that the results were consistent with identifiable cell types.

Subsequently we developed an improved version of the assay (called BR6), which can be run much more easily and rapidly and provides higher precision (Langlois et. al., 1990a). We perform this assay on the Becton Dickinson FACScan System with no alterations using their conventional Consort 30 software package. With this system, cells can be processed at a rate of 4000 cells/second (about five-times faster than with a cell sorter), so that analysis of 5 million cells can be completed in thirty minutes. By analyzing blood samples in parallel using the BR6 assay and the 1W1 sorting assay (Langlois et. al., 1990a), we showed that the two assays measure comparable variant cell frequencies (e.g., In this small comparative study, the mean NØ frequency was 5.8 x 10^{-6} for the 1W1 assay and 6.2 x 10^{-6} for the BR6 assay.). Thus, the kinds of events detected by flow cytometry at high speed appeared to be closely correlated with the single-labeled variant cells that were sorted and identified in the 1W1 assay. Since the analytical procedure in the BR6 assay is simpler and faster than the 1W1 assay, the reproducibility of this assay is significantly better than for the sorting assay (e.g., for NØ the observed group coefficient of variation was 64% for the 1W1 assay and 30% for the BR6 assay).

Another significant improvement that was designed into the BR6 assay was an improved reliability in measuring homozygous variant cell frequencies. In the 1W1 assay we had demonstrated that frequencies of NN cells were sometimes exceedingly high. This was suspected to be due to an artifact induced by the particular specificity of one of the monoclonal antibodies that was used for labeling. In the BR6 assay we substituted a new antibody for the one that was thought to be inducing this artifact, and the comparative study distinctively showed that NN cell frequencies were being measured much more accurately with the BR6 assay (Langlois et. al., 1990a). All measurements using BR6 continue to indicate that NN cell frequencies are being measured reproducibly and accurately. This provides an important capability as an indicator of chromosome segregation errors (missegregation) or mitotic recombination in chromosome 4 of erythroid precursor cells.

Using the BR6 assay, we have undertaken a detailed study of a large normal population comprising Lawrence Livermore National Laboratory (LLNL) employees, students, retirees, and family members. Volunteers completed an extensive questionnaire detailing individual data on age, sex, smoking, socioeconomic status, and potential environmental or occupational exposures to chemical mutagens and/or ionizing radiation. We also were able to query LLNL medical and occupational exposure records, where appropriate. Individuals of MN genotype with no apparent genotoxic exposures were selected, blood samples obtained, and GPA variant cell frequencies measured using the BR6 assay. To date, we have analyzed samples from 98 individuals (53 females, 45 males), ages 8 to 76 years. The mean frequencies for the two variant cell types are NØ = 8.4×10^{-6} and NN = 13.3×10^{-6}.

These data also show a small age-dependent increase in the frequency of NØ variant cells and a somewhat greater increase in frequency of NN variant cells with age. The small increase for NØ cells agrees with our earlier measurements using the 1W1 assay (Jensen et. al, 1987). However, since the 1W1 assay contained a systematic error in analysis of NN variant cells, the age dependence of NN frequency could not be detected with that assay.

There are several reasonable hypotheses which could explain the age-dependent increase in GPA variant cells. Perhaps DNA replication occurs with less fidelity in older people, or perhaps the DNA repair system becomes less efficient with age. Either of these changes would lead to a higher frequency of genotoxic errors in older people, leading to a higher frequency of GPA variant cells in the elderly. Another explanation for the age-dependent increase is that a fraction of each individual's long-lived hematopoietic stem cells are converted to GPA variants by genotoxic effects, and they continue to produce variant cell offspring for many years. As people age, continuing genotoxicity to stem cells occurs leading to an accumulation of variant stem cells. The net result is that GPA variant cell frequency in peripheral blood increases continuously with age. The greater increase observed for NN variant cells implies that homozygous variant stem cells are more stably retained in the bone marrow than are hemizygous stem cells. This is not surprizing in that hemizygous variant cells have lost genetic information, some of which may be important for long term viability. When genotoxic events

induce the formation of homozygous variant cells, genetic information is not lost to those cells. It is merely less dispersed by missegregation events, so that cell viability is not likely to be affected.

Another interesting observation is that there appear to be 'outliers', i.e., individuals with NØ and/or NN variant cell frequencies that are significantly outside the main distribution. Two individuals with aberrantly high GPA variant cell frequencies were reported previously (Langlois et. al., 1990a), and replicate blood samples from these same individuals showed continuing expression of high frequencies in NØ and/or NN variant cells. These outliers do not appear to be expressing some confounding effect, and we believe that the measured frequencies are not clonogenic, since they remain over several years time. It may be that these individuals have experienced unknown genotoxic exposure, or alternatively they may possess high sensitivity to genotoxic effects (such as possessing poor DNA repair capacity or error-prone DNA replication enzymes). A systematic study of families of these individuals may reveal whether there is a heritable basis for these aberrantly high frequencies; alternatively a more extensive study of medical and occupational exposure data may reveal an important, previously undetected confounding factor.

Chemotherapy Patients

Several cohorts of patients undergoing chemotherapy have been analyzed to determine whether the GPA genotoxicity assay can detect effects of particular mutagenic chemicals on the bone marrow erythroid cell systems. One study was performed on breast cancer patients using the 1W1 assay (Bigbee et. al., 1990). This was a time-series study in which the patients were sampled repeatedly during and after therapy with mutagenic chemicals in their therapeutic protocol. Three different therapies were used 1.) CAF (cyclophosphamide, adriamycin, and 5-fluorouricil), 2.) CMF (cyclophosphamide, methotrexate, and 5-fluorouracil) or 3.) VMF (vinblastine, methotrexate, and 5-fluorouracil). Samples from patients treated with CAF or CMF showed an increase in the frequency of NØ variant cells during therapy. Variant frequencies gradually increased during therapy, reaching a maximum at or shortly after the end of therapy, then declined to near pre-therapy levels within 6 months. The maximum level of induced variants ranged from 2- to 7- fold over pre-therapy or normal levels depending on the combination of agents used. The cancer patients treated with the two strongly mutagenic compounds cyclophosphamide

and adriamycin showed consistent elevations in variant cell frequencies; patients treated with only one mutagenic agent (CMF) showed lower and more variable elevations. The one patient who received no mutagenic agents (VMF) showed no significant increase in variant cells during therapy.

These data demonstrate that mutagenic chemotherapy agents induce elevated levels of GPA variant erythrocytes consistent with the hypothesis that these variant cells result from somatic mutation. The increases in frequency occurred only after several weeks of therapy, in that mutational events in the bone marrow cells can only be detected after a significant number of these cells mature into the peripheral blood. Furthermore, the elevations in peripheral blood variant cells were transient, lasting only one red cell lifetime beyond cessation of therapy. Thus it appears that the mutagenic chemicals primarily affected the rapidly cycling committed erythroid cell population. Since long-lived bone marrow stem cells turn over at a much lower rate than committed erythroid precursors, any DNA adducts formed i these cells can be repaired before replication occurs.

A second, ongoing study with *cis*-platinum chemotherapy is being performed to test the persistence of genotoxicity with this compound. Since *cis*-platinum forms DNA adducts that appear to be persistent (Reed et. al. 1986), we might expect to see long-lived genotoxicity effects. To date we have seen about a three-fold increase in frequency of NØ variant cells with no increase in frequency of NN variant cells. The NØ increase appears to be persistent with time. We can detect this increase in patients up to three years post cessation of therapy.

Thus, it would appear that the GPA genotoxicity assay may provide either of two different kinds of biodosimetry for individuals who are exposed to mutagenic chemicals; one is a rapid, reversible response to exposure and the other is a long-term cumulative biodosimeter. This seems to vary with the chemical to which the individuals are exposed. One might hypothesize that the chemicals that give irreversible hematopoietic effects are potentially more dangerous. If second or third genotoxic hits are required for neoplasia to be induced, then the long term changes are ones which should give a higher probability of acquiring a second hit. Long term epidemiology studies on the cancer patients that were treated and assayed by our GPA-based assay will be carried out to test this hypothesis.

Radiation

The GPA somatic genotoxicity assay also has been used as a biodosimeter for ionizing radiation exposure of humans. The first test was using the 1W1 assay on blood samples from a cohort of 43 survivors of the atomic bomb dropped over Hiroshima in 1945 (Langlois et. al., 1987). Significant linear relations between estimated radiation dose and NØ variant cell frequencies were observed, demonstrating that ionizing radiation produces stable mutations in long-lived hemopoietic stem cells and that this damage continues to be expressed in peripheral erythrocytes more than 40 years after exposure. The measured variant cell response to dose was calculated to be 17 x 10^{-6} per Gy.

Later, a single beam sorter assay was performed on a similar cohort of the A-bomb survivors and similar results were obtained (Kyoizumi et. al., 1989). In this study, the increase in NØ frequency was 40 x 10^{-6} per Gy, about two-fold greater than the previous study. This higher slope is partially due to the fact that the dose estimation procedures have been improved such that lower dose estimates are now being assigned to many individuals who were at the A-bomb site (Roesch, 1987). A recalculation of our earlier results using the newer dose estimates yields an increased slope on those studies of 22 x 10^{-6} per Gy.

More recently, preliminary studies were performed on blood samples from victims of the Chernobyl nuclear power plant accident that occurred in 1986. During the accident, plant personnel and auxiliary staff present in the immediate vicinity of the reactor were exposed to high levels of external, relatively uniform whole-body gamma irradiation (with beta irradiation of body surfaces also). Within the first week of exposure, about 500 persons suffering from acute radiation sickness were taken to specialized treatment centers in Moscow and Kiev. The extent of radiation exposure of these individuals was estimated during the first 7 days using peripheral blood lymphocyte counts and conventional non-banded chromosome aberration analysis (Guskova et. al., 1988). Blood samples from 10 of these acutely exposed people were obtained between 6 and 9 months after the accident, and the 1W1 assay was performed on these samples. The results on these samples showed an increase in GPA variant cell frequency (Langlois et. al., 1990b) which was quite similar to the 1W1 results on the A-bomb survivors (the increase in frequency of NØ cells was 26 x 10^{-6} per Gy).

In 1989 we continued our study using the BR6 assay to monitor blood samples from Chernobyl accident victims (Jensen et. al., 1990). We analyzed blood samples from 13 accident victims approximately 3-4 years after exposure, and found that the dose-response of NØ variant cell frequency is very close to our previous measurement made in 1987 with the 1W1 assay.

$$\text{NØ frequency} = 9 \times 10^{-6} + 29 \times 10^{-6} \text{ Dose (Gy)}$$

Thus, it appears that the GPA assay is truly a cumulative biodosimeter for whole body radiation exposure. We see no change in this dose-response curve over a three year period and expect that in these individuals the GPA-based genotoxic effects of radiation exposure will continue to be expressed at the same level in the future. We are continuing this long term study to confirm this hypothesis.

The BR6 assays on the 1989 blood samples also provided good data on the frequency of homozygous NN variant cells. We observed no significant increase in NN variant cell frequency with dose using this data set (Jensen et. al., 1990). This is not surprizing in that the kinds of damage that ionizing radiation induces would not be expected to increase the frequency of chromosomal loss and duplication, gene conversion or mitotic recombination.

Cancer Risk

The fact that genotoxicity is involved in many of the molecular events that lead to neoplasia has been confirmed in recent years. One major oncogenesis process is activation of proto-oncogenes by point mutations or chromosomal translocations (Bishop, 1987). Alternatively, many tumors arise from loss or inactivation of tumor suppressor genes (Green, 1988). The spectrum of genotoxic events that can be measured by the GPA assay are identical to these neoplasia-inducing events, so that frequencies of variant erythrocytes may be useful for estimating the relative risk of each individual to develop cancer.

In our first studies on individuals with high cancer risk, we performed the 1W1 assay on samples from people with ataxia telangiectasia (Bigbee et. al., 1988) or with Bloom's syndrome (Langlois et. al., 1989). Individuals that suffer from these rare autosomal recessive genetic disorders suffer from neoplasia at a very high frequency and at young

ages. We found that both GPA variant cell types appeared at high frequencies in these blood samples. For ataxia telangiectasia, mean frequencies were NØ = 94×10^{-6} and NN = 43×10^{-6}. For Bloom's syndrome, mean frequencies were NØ = 586×10^{-6} and NN = 834×10^{-6}.

These results indicate that the GPA assay shows very large effects in individuals that are extremely susceptible to neoplasia, thus supporting our supposition that high susceptibility to somatic genotoxicity leads to high risk of developing neoplasia. Studies are in progress to determine whether modest increases in GPA-based variant cell frequencies provide an important input for determining modest increases in cancer risk. These, will require careful, long term follow up and health monitoring of individuals with high variant cell frequencies compared with individuals with low variant cell frequencies that we detect in our ongoing studies.

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