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ABSTRACT

An analytical technique has been developed that allows laser-based flow cytometric measurement of the frequency of red blood cells that have lost allele-specific expression of a cell surface antigen due to genetic toxicity in bone marrow precursor cells. Previous studies demonstrated a correlation of such effects with the exposure of each individual to mutagenic phenomena, such as ionizing radiation, and the effects can persist for the lifetime of each individual. During the emergency response to the nuclear power plant accident at Chernobyl, Ukraine, USSR, a number of people were exposed to whole body doses of ionizing radiation. Some of these individuals were tested with this laser-based assay and found to express a dose-dependent increase in the frequency of variant red blood cells that appears to be a persistent biological effect. This effect is similar to that which was previously observed in individuals who were exposed to ionizing radiation at Hiroshima in 1945 because of the A-bomb explosion. All data indicate that this assay might well be used as a biodosimeter to estimate radiation dose and also as an element to be used for estimating the risk of each individual to develop cancer due to radiation exposure.

2. INTRODUCTION

When a person is exposed to significant amounts of ionizing radiation, many cells in his/her body can be damaged. Damage to the DNA in these cells often is long lasting and leads to pathological health effects, such as the induction of carcinogenesis leading to neoplasia. The traditional method for assessing this genotoxicity has been microscopic analysis of chromosomes that are contained in lymphocytes circulating in the peripheral blood. This cytogenetic screening is used to detect the number and the nature of chromosomal aberrations, which can indicate the extent of exposure of the host (biodosimetry) and also can give an indication of the risk of developing carcinoma[1]. Most commonly, this cytogenetic dosimetry has been performed by measuring the frequency of readily recognizable dicentric chromosomes and/or ring chromosomes and comparing this frequency against dose-response curves obtained by

analysis of human lymphocytes exposed to similar radiation *in vitro*. However, because this type of chromosomal change ultimately results in cell death, the frequency of cells containing such chromosomes in circulating blood decreases with time after exposure[2]. Thus these unstable chromosomal aberrations cannot be used for precise biodosimetry of chronic exposure or long after acute exposure.

This limitation of cytogenetic dosimetry can be avoided by scoring for balanced chromosomal translocations, since several types of these aberrations are usually not cytotoxic. Using conventional chromosomal staining techniques, balanced chromosomal translocations have been scored[3]. However, a more accurate determination can be performed by staining chromosomes by either of two techniques, called G-banding[4] or R-banding[5], for definitive chromosomal identification by microscopic analysis. Analysis of such banded chromosomal preparations is very difficult because of the complex staining pattern which must be recognized. Thus, the analysis of a sufficient number of metaphase chromosome preparations to reliably detect small increases in the frequency of cells that carry balanced translocations is extremely slow and labor intensive. Therefore, this type of cytogenetic analysis is usually not performed unless a critical radiation accident has occurred.

Another effect of ionizing radiation on cells in exposed individuals is the induction of mutational or chromosomal segregation changes, which result in unusual functional characteristics of the effected cells. We developed a system for analyzing human peripheral blood and detecting rare red cells, designated as variant erythrocytes, that appear to be the progeny of mutated or abnormally segregated erythroid precursor cells[6]. This system uses fluorescently labeled monoclonal antibodies to label red cells for the presence of the cell surface antigen, glycophorin A (GPA), and laser-based flow cytometric analysis to enumerate the frequency of variant cells. Glycophorin A is the major cell surface glycoprotein of human erythrocytes and is expressed at about one half million copies per cell[7]. It occurs in two allelic forms that are very similar to each other, differing in only two amino acids of the 131 amino acids and 16 tetrasacharrides that compose the molecules. These two allelic forms are the basis for the MN blood group and are co-dominantly expressed so that individuals who are blood type MN express both forms on their erythrocytes. In blood samples from an MN heterozygote, gene loss variant cells would express only one of the two allelic forms of glycophorin A.

Our analytic procedure can detect two different types of gene loss variant cells. The first is denoted as "null" cells MØ or NØ to signify which of the allelic forms is missing from the cells. The cause of such a gene loss can be any of several different mechanisms, including missense mutations, substitution mutations, or deletions. The other type of cells which we can detect are denoted homozygous variants MM or NN. These would result when chromosomal events, such as non-dysjunction, mis-segregation or mitotic recombination occur. The assay requires expression of one of the two allelic forms of GPA so as to guarantee that the variant cells are capable of normally expressing this cell surface antigen. This precaution prevents misclassifying cells that are damaged by non-genetic changes (phenocopies) such as cellular degradation or metabolic alterations affecting GPA expression.

The development of this GPA-based assay for measuring the frequency of rare variant erythrocytes in human peripheral blood has provided a new approach for measuring genetic damage to somatic cells in people exposed to potentially genotoxic agents[8]. Because erythrocytes do not carry DNA, we cannot confirm the DNA toxicity of the events that cause GPA-variant cells. However, we have observed elevated frequencies in individuals exposed to high energy radiation[9,13]. The magnitude of these increases is similar to that of the increases observed for another *in vivo* somatic cell genotoxicity assay, HPRT[10,11] and also that of cultured cells in the HPRT, TK, DHFR, and HLA loci[12] when the cells are exposed to ionizing radiation. Thus, since these other assays have been confirmed to be due to DNA effects of radiation exposure, we can presume that the GPA assay is measuring similar geneticphenomena. To confirm such an explanation, analysis must be performed on erythroid precursor cells in human bone marrow after the host is exposed to ionizing radiation.

In 1988 a modified single beam sorter GPA assay technique was developed, and measurements on samples from atomic bomb survivors were compared with chromosome aberration frequencies on the same individuals showing a significant linear correlation between the two measurements[13]. These results indicate that our analytical technique should serve as a biodosimeter and a risk analysis tool in much the same way that cytogenetic chromosomal aberration analysis does.

3. FLOW CYTOMETRIC ASSAY

During the last few years, our GPA-based somatic mutation analysis has been adapted for use on a commercially available single beam, laser excitation flow cytometer, the Becton Dickinson FACScan, so that monitoring of variant cell frequencies can be performed rapidly and precisely in clinical laboratories[14]. A typical two-parameter histogram derived from such an analysis is shown in Figure 1. The assay as devised can measure only one type of loss, that of the M allele. Thus, as previously described[14] we can detect two populations of variant cells; NØ cells that have lost the M-allelic form and NN cells that have lost the M-allelic form and gained one extra copy of the N allelic form of GPA. In typical analysis of blood samples, we simultaneously enumerate the frequency of each of these two types of variant erythrocytes.

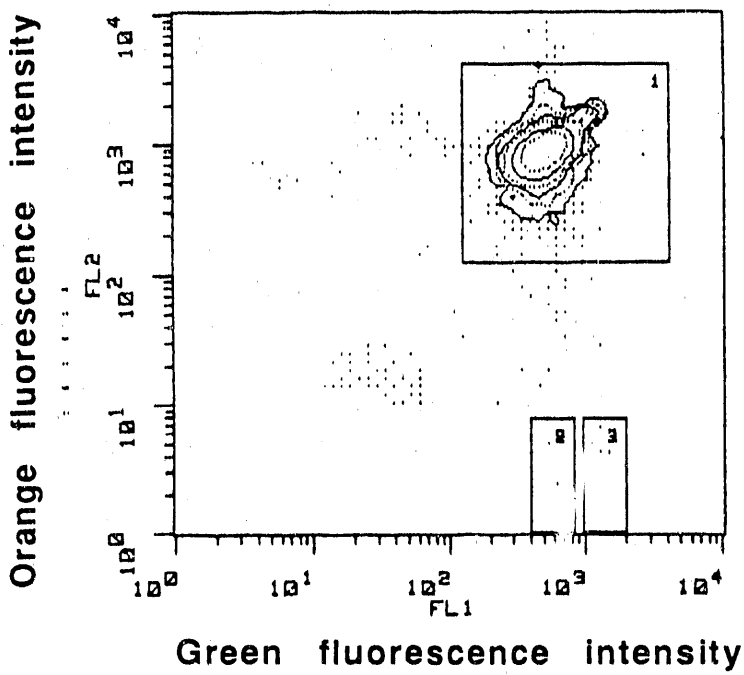

FIGURE 1. Typical two parameter histogram from GPA assay of peripheral blood sample from unexposed individual. One million cells were analyzed on the laser-based flow cytometer using our published protocol[14]. The values on the two axes are the intensities of fluorescence emitted by each cell as a result of excitation of fluorophor-conjugated monoclonal antibodies that bind to the GPA (M) or GPA (N) cell surface antigens. Green fluorescence is emitted from fluoresceinated anti-GPA(N) antibody and orange fluorescence is emitted from phycoerythrin-conjugated anti-GPA(M) antibody. Dots in the plot indicate one cell that displays the intensity values displayed; contour lines in this plot are at 3, 10, 30, 100, 1000, 10,000 signals per channel. The windows inscribed as rectangular areas indicate the areas for signals that are generated by 1- normal cells, 2- NØ variant cells, and 3- NN variant cells.

Figure 2 shows a summary of the frequencies of each variant cell type in control populations who have not been knowingly exposed to significant amounts of mutagenic chemicals or ionizing radiation.

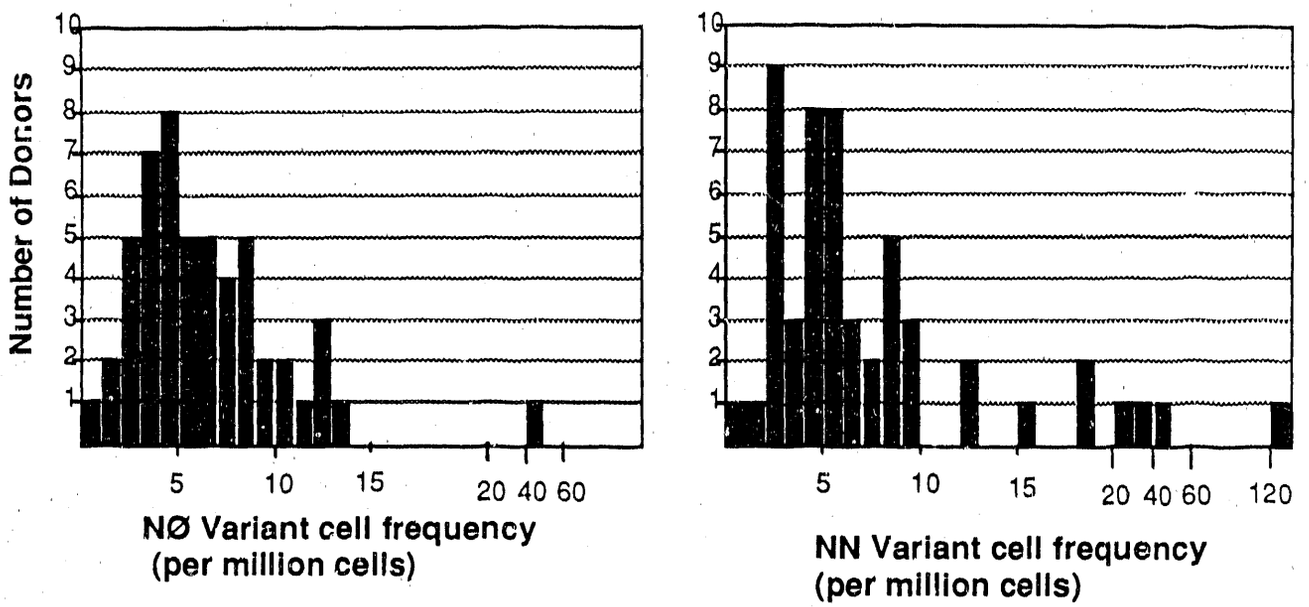

FIGURE 2 Histograms of NØ and NN variant cell frequencies for normal population. Samples from 53 middle aged (mean age = 36 yrs.) volunteers from the Lawrence Livermore National Laboratory work force were analyzed by the GPA-based assay and the frequency of variant cells was determined for each individual. Mean frequencies are NØ = 7.1 x 10^{-6} and NN = 10 x 10^{-6}.

The spread in variant cell frequency among individuals in this control population is about 5 times as large as would be expected from Poisson variation on the 5 x 10^6 cells analyzed. Thus, there appears to be inter-individual biological variation that is responsible for these differences. Part of this variation may be explained by a general increase in frequency that appears to occur as individuals age[8]. This effect could be caused by accumulated exposure to small amounts of genotoxic agents with real differences in this accumulated exposure among these individuals. Another possibility is that each individual possesses a different capacity for DNA processing and repair in the bone marrow stem cell population, and the same amount of exposure may lead to a different size of effect on different people.

3.2 Chernobyl accident victims

To confirm our previous results on dose-response of the GPA-based assay for human exposure to ionizing radiation, we performed the analysis on individuals that were involved in the accident at the nuclear power plant in Chernobyl, the Ukraine, USSR. A number of the victims of exposure during the May 1986 accident were sent to Hospital # 6, Institute of Biophysics in Moscow, to be treated for acute radiation syndrome[15]. Cytogenetic analyses, hematologic analyses and acute radiation symptoms were obtained for many of these individuals. These observations were converted into estimated doses of exposure of these individuals.

During 1989, we obtained blood samples from 11 such individuals who were exposed to significant amounts of radiation according to the early dose estimates. These blood samples were analyzed using the GPA-based flow cytometric somatic cell mutation assay. As a control, 5 individuals from each site, the Moscow Hospital #6 and the Kiev Institute population, were selected as unexposed individuals and analyzed using the same technique. Plots of variant cell frequencies versus the estimated dose for all of these individuals are shown in Figures 3a and 3b.

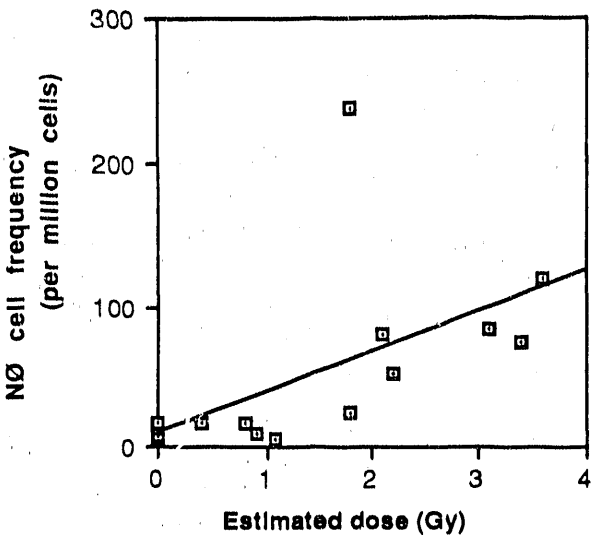

FIGURE 3a. GPA-based null variant erythrocyte frequency in peripheral blood. Each data point represents the flow cytometric analysis of a single blood sample obtained in 1989 from an individual who was exposed to an acute dose of ionizing radiation during the accident in May 1986. Estimated dose is based on the cytogenetic analysis, hematologic analysis and acute radiation symptoms obtained in 1986.

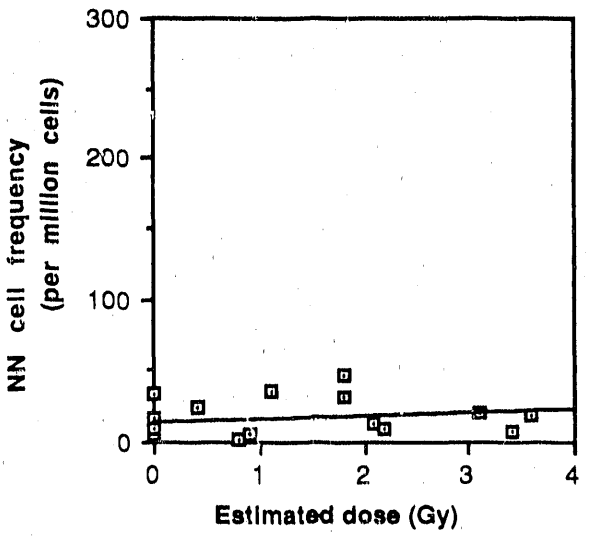

FIGURE 3b. GPA-based homozygous variant erythrocyte frequency in peripheral blood obtained in 1989. Results as shown in legend for FIGURE 3a.

Figure 3a shows that there is a significant dose-response of the NØ frequency. A linear regression was performed on these data resulting in a relation described by:

Equation 1: NØ frequency = $9 \times 10^{-6} + 29 \times 10^{-6}$ Dose (Gy)

The results fit this regression with a correlation coefficient of $r^2 = 0.42$. Note that one individual (who received an estimated 2 Gy) shows a very high NØ frequency (238×10^{-6}). Perhaps this individual is more sensitive to mutagenic effects of ionizing radiation than the other exposed individuals. If this outlier is dropped from the data set, the dose-response does not change dramatically;

Equation 2: NØ frequency = $5 \times 10^{-6} + 24 \times 10^{-6}$ Dose (Gy).

However, the linear correlation of the data points increases to $r^2 = 0.84$.

Fig 3b indicates no dose-response for the NN frequency with a line described by:

Equation 3: NN frequency = $12 \times 10^{-6} + 2.7 \times 10^{-6}$ Dose(Gy).

This result implies that radiation exposure does not cause a significant amount of damage that would result in mis-segregation or recombination. This is not particularly surprizing in that the high energy damage that occurs to DNA when cells are exposed to ionizing radiation typically causes chromosomal deletions or translocations, which do not usually result in changes that give rise to homozygous variant cells.

An aliquot of the blood samples obtained from the exposed individuals also was prepared for cytogenetic analysis to determine the frequency of cells that contain dicentric or fragmented chromosomes. Figure 4 shows the dose-response of this chromosomal aberration frequency versus estimated dose.

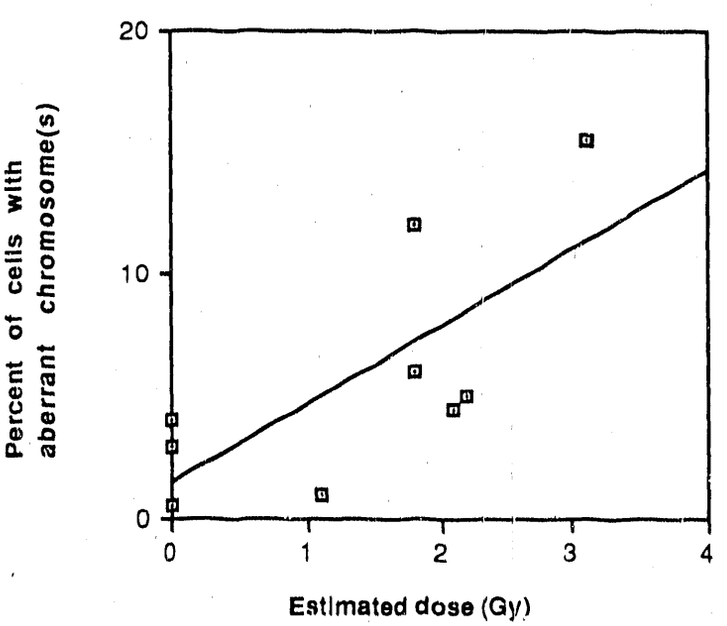

FIGURE 4. Chromosomal aberration frequency in lymphocytes from peripheral blood samples obtained in 1989. Estimated dose is based on the cytogenetic analysis, hematologic analysis, and acute radiation symptoms obtained in 1986.

The figure also shows a linear regression fit of the data, which indicates a rather similar correlation to that obtained for the NØ frequency versus estimated dose (r^2 = 0.53).

The relationship between NØ frequency and chromosome aberration frequency measured on these same samples is shown in Fig. 5.

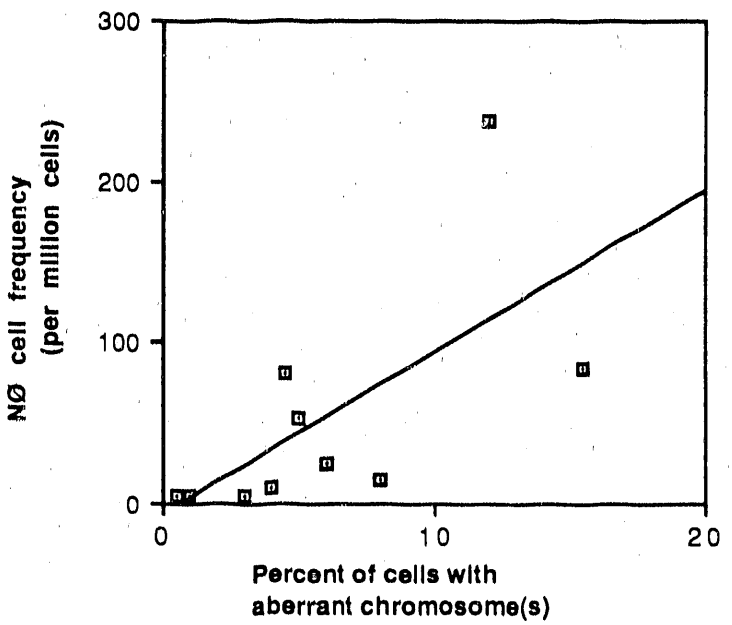

FIGURE 5. Comparison of GPA-based somatic cell mutation frequency in erythrocytes with frequency of lymphocytes with chromosome aberrations.

Linear correlation of these data indicate a reasonable agreement between these two biodosimetric end points (r^2 = 0.43), although the same individual as described previously shows much higher mutation frequency than aberration frequency.

3.3 Persistence of effects

Our previous results, which showed similar frequencies of NØ variant cells in blood samples of exposed survivors of the A-bomb, indicated that these biological effects are very long-lived (those effects were found forty years after exposure)[9]. An analysis of data obtained on victims of the Chernobyl accident concur with this implication. During 1987, we were able to obtain and analyze blood samples from 10 of the victims that were transported to Hospital #6, Institute of Biophysics in Moscow. Two of the individuals that were analyzed in 1987 also were included in the cohort that was analyzed in 1989. Table 1 shows the NØ frequencies measured on the two blood samples obtained from each of these individuals with a two year interval between collection times.

TABLE 1
COMPARATIVE NØ VARIANT CELL FREQUENCY IN SAMPLES
OBTAINED AT TWO DIFFERENT TIMES AFTER RADIATION EXPOSURE

	NØ variant cell frequency	
Donor	1987	1989
K4	24×10^{-6}	27×10^{-6}
K8	52×10^{-6}	39×10^{-6}

The two frequencies measured on each of the donors are very similar in size, with that of donor K4 appearing to increase slightly and that of K8 appearing to decrease slightly. These preliminary data indicate that the effect detected within six months of exposure may well be a lifetime effect. A continued longitudinal study on these donors as well as a larger cohort of exposed donors is in progress.

4. CONCLUSION

These results all indicate that the GPA-based gene loss "null" cell variant assay can be used as a biodosimeter for ionizing radiation exposure. The frequency of null mutant type of variant cells shows a reasonable response with estimated doses, and this dose-response was unchanged over a 3 year period after exposure.

If the persistence of this effect proves to be life long, as is indicated by the results on A-bomb survivors, it would make a very powerful lifetime accumulative biodosimeter. As each individual is exposed, the GPA-based somatic mutation frequency in the peripheral blood of that individual would increase to a new level depending of the amount of exposure he received and his sensitivity to radiation mutagenesis. That new level of variant cell frequency would remain at the new level until further exposure occurred.

Since it is probable that an increase in frequency of mutations in bone marrow cells is an indicator of the potential for acquisition of carcinogenic DNA damage[16,17], the assay should also be a lifetime estimator of the risk of each individual to develop cancer. It would thus be an important monitoring assay for health services to provide for individuals that are at risk of being exposed to mutagenic phenomena; such as nuclear reactor workers, radiation therapy technologists, cosmonauts, and individuals that live in areas with high radiation background.

5. ACKNOWLEDGEMENTS

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