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**DETERMINATION OF SOMATIC MUTATIONS IN HUMAN ERYTHROCYTES
BY FLOW CYTOMETRY**

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INTRODUCTION

The predominant cell surface glycoprotein on human red blood cells is glycophorin A (GPA). It is the product of a pair of co-dominantly expressed alleles of the GPA gene, located on the long arm of chromosome 4 (Tippet, 1981). The allelic forms define the MN blood group antigens and are inherited by classical Mendelian genetics to give blood groups MM, NN or MN (Issitt, 1981). Biochemical analysis of the MN antigens reveals that the two forms differ by polymorphisms in the amino acids at position 1 and 5 from the amino-terminus. GPA(M) has serine and glycine while GPA(N) has leucine and glutamic acid in positions 1 and 5 respectively. All other characteristics (eg., amino acid sequence, glycosylation pattern, and sialic acid substitution) are identical in both forms of GPA (Anstee, 1981).

We are developing a flow cytometric assay for human peripheral blood to detect rare variant cells that may arise from gene loss mutations in erythroid precursor cells (Jensen et al., 1984). This assay can be performed on blood from heterozygous GPA(MN) donors, and is designed to detect cells expressing one of the two allelic forms but not the other. To label cells for flow analysis, we isolated monoclonal antibodies that bind specifically to the M or the N form of GPA (Bigbee et al., 1984). Antibodies were then conjugated with a green or a red fluorescent dye or labeled with a specific secondary reagent in order to simultaneously mark red cells for

the presence of either or both M and N antigens. Flow cytometry was performed using a dual beam excitation system on blood samples doubly labeled with fluorescein-conjugated antibody for one allelic form of GPA and Texas Red-labeled antibody for the other allelic form (Langlois et al., 1985). Most cells in a sample from a heterozygote would thus fluoresce both red and green, while functionally hemizygous cells (NO or MO) would fluoresce with only one color. Enumeration of the frequency of singly-labeled peripheral red blood cells should be a measure of the mutagenic burden on erythroid precursor cells of the donor, thus affording an individual's biological response to genotoxic exposure.

FLOW CYTOMETRY WITH MONOCLONAL ANTIBODIES

Of our eight different monoclonal antibodies that specifically bind human GPA, seven proved useful for flow cytometric analysis of rbc's. These antibodies are; GPA(M)-specific, 9A3, 6A7; GPA(N)-specific, NN3, NN4, NN5; GPA(M,N)-specific (ie., bind specifically to human GPA, but do not discriminate between N and M.), 10F7, MN1 (Bigbee et al., 1984). To allow precise flow analysis, cells must be fixed by either of two procedures (Langlois et al., 1985). One procedure produces formalin fixed spherocytes, while the second produces discoid shaped cells fixed with dimethylsuberimidate (DMS).

A typical histogram of red cells labeled with one of our GPA(M)-specific antibodies, 9A3, (Figure 1) shows that the fluorescence intensity of MM cells is twice that of MN cells, which fluoresce 100 times more brightly than NN cells. In addition, the distribution of each cell type is tight with a coefficient of variation of ca. 10% in both fluorescence intensity and light scatter intensity. Thus, formalin-spherocytes emit constant scatter signals, and precise antibody staining reveals that the number of GPA(M) molecules per cell is closely controlled.

Labeling with each of our monoclonal antibodies was performed in a similar manner, and flow cytometric analyses were performed on mixtures of blood types to quantitate the specificity of each antibody. A summary of those analyses is shown in Table 1.

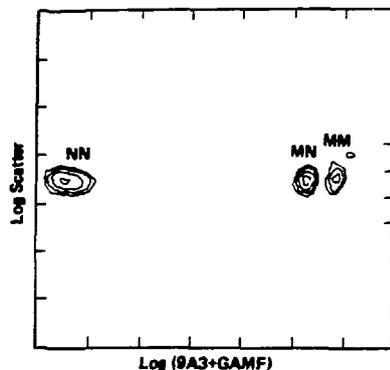


Figure 1. Fluorescence distribution of a mixture of human red cell types labeled with 9A3. Erythrocytes from individuals of blood types MM, MN and NN were formalin-fixed, mixed at 0.33×10^7 cells/ml of each type and stained according to our standard protocol (Langlois et al., 1985). Flow cytometry was performed on the LLNL Dual Beam Sorter (Dean and Pinkel, 1978) using an argon ion laser at 488 nm for excitation. Right angle light scatter was measured through a 488 nm narrow band emission filter, and fluorescein fluorescence was measured simultaneously through a 514 nm narrow band emission filter. Logarithmic amplification (with a range of approximately three decades of intensity) was used to allow display of cells that differ dramatically in fluorescence intensity.

TABLE 1. Relative Binding of GPA-Specific Monoclonal Antibodies to Red Cells from Donors of Different MN Types

Antibody	Relative Fluorescence Intensity of Cells*		
	MM	MN	NN
6A7	1.01	0.50	<0.01
9A3	0.99	0.50	<0.01
10F7	1.00	1.00	1.00
MN1	1.00	1.00	1.00
NN3	<0.01	0.47	1.00
NN4	0.04	0.49	1.01
NN5	<0.01	0.51	1.06

* Footnote for Table 1 appears on following page.

* Mean of measurements made on formalin-fixed rbc's from 10 different donors for each blood type with antibodies 6A7, 9A3, 10F7, and MN1; or on DMS-fixed rbc's from 5 different donors for each blood type with antibodies NN3, NN4, and NN5. For each antibody fluorescent intensities were normalized to 1.00 for rbc's from a single homozygous donor (MM or NN).

These results illustrate the specificity of our antibodies against GPA. Homozygous cells stain exactly twice as brightly as do heterozygous cells using any of the GPA(N) or GPA(M) specific markers. The "public" antibodies, 10F7 and MN1, confer equal intensities on cells from all three blood types. Thus, the expression of GPA on the surface of red cells is rigidly controlled, and our antibodies possess all the properties necessary for them to be used in a flow cytometric somatic cell mutation assay.

SOMATIC CELL MUTATION ASSAYS

Two versions of flow cytometric analyses were developed; one (two-way assay) that uses GPA(M)-specific antibody simultaneously with GPA(N)-specific antibody, and the other (one-way assay) that uses GPA(M)-specific antibody simultaneously with "public" antibody (GPA(M,N)). Histograms obtained on mixtures of different blood types labeled for each different version (Figure 2) show that there are large differences between cells that are double, single or unstained. The rectangular boxes encompass regions where we expect to find signals from gene-loss variant cells. In a two-way assay on blood from a heterozygote, we expect to detect signals from MO and NO in the two boxes shown in Figure 2A, while in a one-way assay we would detect signals from NO variants in the box shown in Figure 2B. Null cells, which would give signals at the origin in either assay, would not be scored as variants. This precaution should protect against misscoring "phenocopies" as mutant cells. Phenocopies might arise by epigenetic phenomena, such as metabolic insufficiencies or pathogenic cellular degradation. Our requirement for normal expression of one allelic form of GPA guarantees that the cellular apparatus for expression of GPA is intact.

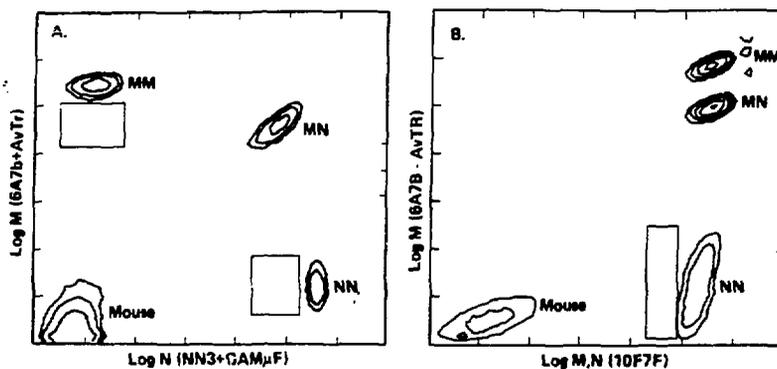


Figure 2. Bivariate flow distributions of mixtures of MM, NN, MN and mouse cells. Each histogram was generated by dual beam flow analysis using the argon ion laser at 488 nm to excite fluorescein and the argon ion pumped-dye laser at 590 nm with a 630 nm narrow band emission filter to detect Texas Red fluorescence. Narrow angle light scatter from the 488 nm beam was also measured and used to gate signals to discriminate between debris and erythrocytes.

A. Two-way assay on DMS-fixed cells incubated with biotinylated 6A7 and secondary labeling with avidin conjugated with Texas Red (6A7b+AvTr) for GPA(M), as well as NN3 and fluoresceinated goat anti-mouse γ -chain specific IgG (NN3+GAM F). Since NN3 is IgM, γ -chain specific secondary antibody labels specifically for GPA(N).

B. One-way assay on formalin-spherocytes labeled with biotinylated 6A7 and avidin-Texas Red (as in A.) for GPA(M) as well as 10F7 conjugated with fluorescein for total GPA on each cell.

SOMATIC MUTATION ASSAYS ON BLOOD SAMPLES

Both versions of our somatic mutation assay were performed on blood from 10 different volunteers who received no known exposure to mutagenic compounds or high energy radiation. In order to be certain that the events enumerated were from cells that exhibited fluorescence expected of NO or MO variants, we sorted all particles that produced signals in the variant cell

boxes. Such sorted samples were analyzed by fluorescence and phase microscopy to confirm that the signals were produced by singly-labeled cells. To obtain precise enumeration, two corrections were applied to the sorted cell frequency. One corrects for sorting inefficiencies which occur because of anti-coincidence logic in the sort routine. The other corrects for aggregation artifacts, which affect enumeration of cells. Both these corrections were measured by adding 100 homozygous NN cells to a suspension of 10^6 MN cells and sorting from a window for NN cells. In general these correction factors differed for different antibody combinations, but were quite constant for different samples stained with the same antibody combination.

Results obtained from assays on bloods from untreated volunteers (Figure 3A) indicate that the two versions of assay are comparable. The mean frequency of M0 or N0 phenotype are similar (ca. 10 variants in 10^6 cells) with a standard error of 2 in 10^6 cells when measured by either one-way or two-way assay. The variability seen may be due to measurement precision but may be due to variations among blood samples or among individuals' baseline frequencies. Variant cell frequencies were measured on multiple samples taken from each of several individuals over a 9 month period. While there occurred a standard error of 2 in 10^6 among the samples from each individual, two of the individuals showed frequencies that differed significantly from the population mean (one high and one low). Further assays on normal individuals are being performed to carefully determine measurement, sample, and person-to-person variability.

The one-way assay was performed on 10 oncology patients who had received therapy for their illness (Figure 3B). Each patient had received at least one treatment from radiation or mutagenic chemotherapy at least 3 weeks before being assayed. The patients were suffering from many different malignancies (e.g., breast, renal, bone, colon and lung), and were treated with several different mutagenic therapeutics (e.g., cisplatinum, adriamycin, daunomycin, and cyclophosphamide each were used on one or more patients).

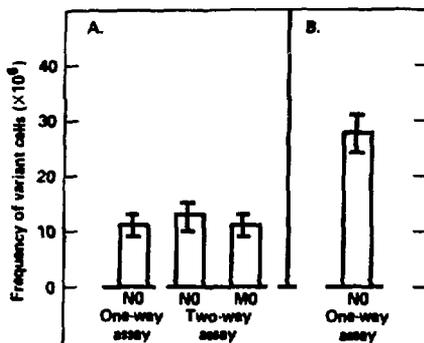


Figure 3. Variant cell frequency in samples from A.)untreated volunteers and B.)cancer patients treated with radiation and/or mutagenic chemotherapy. Each bar represents the mean frequency of variant cells measured, and error bars represent SEM for each data set.

These results indicate that the one-way assay measures a property correlated with mutagenic exposure. The mean frequency of variant cells measured on treated patients is three times that measured on untreated volunteers, using either one-way or two-way assays. A two-sample t-test comparing the one-way assay results between untreated and treated cohorts, shows these two populations to be clearly distinguished ($p < 0.001$). To determine whether cancer victims display high frequencies of variant cells prior to treatment, we are analyzing samples from newly diagnosed patients before therapy. Preliminary results on four such patients, indicate they display normal frequencies (mean variant cell frequencies of 7.0 in 10^6 cells).

CONCLUSIONS

Our results strongly argue that these flow cytometric assays of human erythrocytes labeled with monoclonal antibodies specific for GPA can be used to enumerate variant cells that appear in peripheral blood as a result of somatic gene-loss mutations in erythrocyte precursor cells. The frequency of these variant cells is an

indication of the amount of mutagenic damage accumulated in the individual's erythropoietic cell population. Comparing these results to HPRT clonogenic assays, we find similar baseline frequencies of somatic mutation as well as similar correlation with mutagenic exposures (Albertini et al., 1982; Morley et al., 1983). Both these assays should serve as biotests of the genotoxic burden an individual is carrying. Their successful application may allow us to perform risk estimations for cancer or passing on of heritable birth defects.

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