CHAPTER 5

FLOW CYTOMETRIC ANALYSIS OF VARIANT ERYTHROCYTES AT THE GLYCOPHORIN A LOCUS IN HUMANS

5.1. INTRODUCTION

The GPA mutagenesis assay was developed to quantitate the frequency of somatic cell mutations that occur in vivo in humans [1]. This assay system utilizes fluorescently tagged monoclonal antibodies specific for the M and N allelic forms of erythrocyte cell-surface protein GPA to label cells from heterozygous MN individuals. Rare variant cells lack binding of one antibody, presumably due to gene expression loss mutations in erythorid precursor cells. Variant cells are detected at a frequency of about ten per million erythrocytes in normal individuals [2]. Significantly elevated variant frequencies (VF) have been observed in individuals exposed to mutagenic drugs [3] or high energy radiation [4], as well as in individuals with hereditary cancer-prone syndromes [5]. Thus, the GPA assay has the potential to identify individuals at increased risk for cancer due to either mutagen exposure or elevated susceptibility to mutations.

5.2. MATERIALS AND METHODS

The assay was performed on formalin-fixed erythrocytes which were dually labeled with fluorescent conjugates. A single-beam flow sorter was used to sort labeled cells and the enumeration of erythrocytes with labeling characteristics expected for variant cells. VFs were determined from the number of visually identified variant...
cells per 40,000 cells analyzed. The detailed materials and procedures adopted are given in section 2.1.3 and 2.2.3.

Blood samples were obtained with informed consent form randomly selected volunteers with no known mutagen exposures. Twenty donors of MN blood type (“normal donors”) were identified using rabbit typing sera (Ortho Diagnostics, Raritan, NJ). This group of donors was composed of males and females, all non-smokers, from 22 to 35 years of age. All samples were collected in sodium heparin anticoagulant and were stored refrigerated for various periods of time up to 3 weeks before fixation and analysis. Samples from cancer patients undergoing radiotherapy were collected and processed similarly. The detailed fixation and staining protocols are given in section 2.2.3.

Flow cytometric analysis was performed on a Beckto-Dikinson FACSCalibur single-laser flow cytometer using the analysis software package “Cell sort” (Becton Dickinson Immunocytometry Systems, CA). The analysis protocol is as given in section 2.2.3

5.3. RESULTS

Formalin fixation was selected because this method yields spherical erythrocytes with very uniform light scatter properties. Thus, light scatter can be used to differentiate erythrocytes from fluorescent debris and leukocytes. To minimize possible artifacts resulting from loss of antibody binding due to alterations in GPA conformation or glycosylation during sample preparation, M- and N-specific
antibodies were selected that both require normal GPA glycosylation and bind to the amino-terminal region of the GPA molecule. Fluorescein and phycoerythrin were selected for immunofluorescence because both dyes are efficiently excited by the 488 nm argon-ion laser line, and their emitted light can be separated using commercially available flow cytometers [13].

The flow distribution from normal samples is shown in Figure 1.1 – 1.4
Figure 2.1 – 2.14 shows the flow distribution obtained from the analysis of cells from the radiation-exposed samples.
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Fig 2.3

Fig 2.4

Fig 2.5

Fig 2.6

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Figure 2: gives the dose response curve as obtained by the flowcytometric analysis of erythrocytes by the Glycophorin-A mutational assay.

5.4. DISCUSSION

The GPA assay provides a new approach for studies of in vivo somatic cell mutation in humans. This assay for variant phenotype erythrocytes complements other mutagenesis methods, such as the HPRT [14] and HLA [15] assays for mutant peripheral lymphocytes, because each of the methods provides information on different loci and different target cell populations. While each assay has an important advantage for large scale screening studies of genotoxicity in humans. The GPA assay does not require cell culture and selective growth of mutant

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phenotype cells. Assay runs can be performed in less than an hour compared with days to weeks required for the cell culture-based assays. Also assays can be performed on geographically distant populations where the special sample handling required to maintain lymphocyte viability is often not possible.

The assay was performed on a commercially available, relatively inexpensive, single-beam flow cytometer. We also find that the speed, simplicity, and reliability of this instrument greatly increase the number of assays that can be performed because reduce set-up time compared with a sorter. The ability to process samples at a rate of 4,000 cells/s reduces the time required for an assay run to about 30 min and contributes to increased statistical precision of the assay because definite numbers of cells can be analyzed in the same time period.
REFERENCES


