CHAPTER 1

SOMATIC CELL MUTATION ASSAY AT THE GLYCOPHORIN-A LOCUS IN HUMAN ERYTHROCYTES FOR BIODOSIMETRY – A REVIEW

1.1 ABSTRACT

For risk assessment, it is necessary to evaluate the dose of human exposure to radiation. Ionizing radiation being an environmental genotoxic factor induces dose-dependent somatic mutations. Recent studies support the somatic-mutation theory of carcinogenesis. Mutations at the Glycophorin–A (GPA) loci have been studied for establishment of somatic mutation assays. GPA assay provides a lifetime biological dosimetry technique to detect exposure to radiation as the mutations are accumulated in the long lived hematopoietic stem cells. In this review, we present the various concepts and advancements in the GPA mutational assay, and its role in biodosimetry and risk assessment.

1.2 INTRODUCTION

Dose evaluation of humans exposed to radiation becomes necessary after such exposure. Such a dose evaluation can be done by biological dosimetric methods, of which increased somatic cell mutant frequencies in exposed individuals can be a reliable index [1]. Biological dosimetry has an important role to play in assessing the cumulative radiation exposure of persons working with radiation, and also in estimating the true dose received during accidents involving external and internal exposure. Bodosimetric methods include cytogenetic, immunological and
mutational assays. There are currently advancing and new opportunities for biological dosimetry of high-risk groups in the methods for measuring somatic mutations [2]. Such an evaluation of somatic cell mutations becomes all the more important, as such mutations can be possible predictors of cancer risk [3]. Somatic mutations can now be measured in several human genes. The genes currently used include the hemoglobin (Hb) genes on chromosome 11 and 16, the glycophorin A (GPA) gene on chromosome 4, the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene on the X-chromosome, the HLA genes on the chromosome 6 and the T-cell receptor (TCR) genes on chromosomes 7 and 14. Of the various assays for somatic mutations, only GPA and HPRT have been studied sufficiently to have reliable databases.
Fig. 1.1.1 The type of damages possible by ionizing radiations to DNA
Fig. 1.1.2 Direct and Indirect effects of ionizing radiations to DNA
Table 1.1: Various mutational assays available for biodosimetry.

<table>
<thead>
<tr>
<th>Mutation Assay</th>
<th>Cells of choice</th>
<th>Sensitivity(Gy)</th>
<th>Study group</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>Lymphocytes</td>
<td>1.0</td>
<td>Morley <em>et al</em></td>
<td>1983</td>
</tr>
<tr>
<td>LDH-X</td>
<td>Spermatozoa</td>
<td>?</td>
<td>Mendelsohn</td>
<td>1984</td>
</tr>
<tr>
<td>GPA</td>
<td>Erythrocytes</td>
<td>0.5</td>
<td>Langlois <em>et al</em></td>
<td>1986</td>
</tr>
<tr>
<td>HPRT</td>
<td>Lymphocytes</td>
<td>0.5</td>
<td>Hakoda</td>
<td>1988</td>
</tr>
<tr>
<td>β-Globin</td>
<td>Erythrocytes</td>
<td>0.5</td>
<td>Tates <em>et al</em></td>
<td>1989</td>
</tr>
<tr>
<td>TCR</td>
<td>Lymphocytes</td>
<td>0.5</td>
<td>Albertini <em>et al</em></td>
<td>1990</td>
</tr>
<tr>
<td>APRT</td>
<td>Lymphocytes</td>
<td>2.0</td>
<td>Tatsumi <em>et al</em></td>
<td>1992</td>
</tr>
</tbody>
</table>

Table 1.2: Time stability of various mutational assays

<table>
<thead>
<tr>
<th>Mutation Assay</th>
<th>Cells of choice</th>
<th>Recognition Site</th>
<th>Gene locus</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>Lymphocytes</td>
<td>Receptor</td>
<td>6_p</td>
<td>Unstable (2yrs)</td>
</tr>
<tr>
<td>LDH-X</td>
<td>Spermatozoa</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>GPA</td>
<td>Erythrocytes</td>
<td>Receptor</td>
<td>4_q</td>
<td>Stable (infinite)</td>
</tr>
<tr>
<td>HPRT</td>
<td>Lymphocytes</td>
<td>Enzyme</td>
<td>X_q</td>
<td>Unstable (1-2 yrs)</td>
</tr>
<tr>
<td>β-Globin</td>
<td>Erythrocytes</td>
<td>Receptor</td>
<td>11_p</td>
<td>Stable</td>
</tr>
<tr>
<td>TCR</td>
<td>Lymphocytes</td>
<td>Receptor</td>
<td>14_q, 7_q, 7_p</td>
<td>Unstable (1-2 yrs)</td>
</tr>
<tr>
<td>APRT</td>
<td>Lymphocytes</td>
<td>Enzyme</td>
<td>16</td>
<td>Unstable (1-2 yrs)</td>
</tr>
</tbody>
</table>

*ANALYSIS OF SOMATIC CELL MUTATIONS AT THE GLYCOPHORIN-A LOCUS IN HUMAN ERYTHROCYTES.*
Fig. 1.2 The different mutational assays for biodosimetry
Fig. 1.3 Flowchart of events for detection of APRT mutation assay
CHAPTER 1

Thesis

"ANALYSIS OF SOMATIC CELL MUTATIONS AT THE GLYCOPHORIN-A LOCUS IN HUMAN ERYTHROCYTES."

---

<table>
<thead>
<tr>
<th>Cells</th>
<th>Antibody</th>
<th>Antigen</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>HLA-A2</td>
<td>HLA-A2</td>
<td>Killed</td>
</tr>
<tr>
<td></td>
<td>HLA-A3</td>
<td>HLA-A3</td>
<td>Killed</td>
</tr>
<tr>
<td>Mutant</td>
<td>HLA-A2</td>
<td>HLA-A2</td>
<td>Survives</td>
</tr>
<tr>
<td></td>
<td>HLA-A3</td>
<td>HLA-A3</td>
<td>Survives</td>
</tr>
</tbody>
</table>

[Presence of Antigen]

[Absence of Antigen]

Fig.1.4 Flowchart depicting normal and mutated cells as obtained by immunoselection

---

Fig.1.5 Events taking place for the TCR mutation assay
1.3. GLYCOPHORIN – A ORGANISATION AND MUTATIONS

Lansteiner and Levine described a blood group system in 1927 called the MN system in which two alleles M and N determine the presence of corresponding antigens on red cells. According to this theory, three possible genotypes MM, MN and NN occur. GPA genes code for the determinant molecule for the MN blood group in erythrocytes and are present on the 4q29 chromosome site. The GPA of human erythrocyte is a sialoglycoprotein with a chain of 151 amino acids and a molecular weight of 55,000 Daltons. The M and N forms vary in their amino acid composition in positions (1) and (5) [4]. The M group has Serine and Glycine in positions (1) and (5) respectively and the N group has Leucine and Glutamic acid in the respective positions. It was shown unambiguously that GPA either can be totally absent from human erythrocytes or that the protein can be altered so extensively that it can no longer be recognized [5]. This might be because of the mutations at the locus which was unexplained at that time. The aminoacid sequence of the GPA is different in positions 1 and 5, but no differences in carbohydrate structure or sites of glycosylation (*) are apparent for the two forms

\[
\begin{align*}
A^N & \quad \text{Leu-Ser}^*\text{-Thr}^*\text{-Thr}^*\text{-Glu} \\
A^M & \quad \text{Ser-Ser}^*\text{-Thr}^*\text{-Thr}^*\text{-Gly}
\end{align*}
\]
Chapter 1

Thesis

"ANALYSIS OF SOMATIC CELL MUTATIONS AT THE GLYCOPHORIN-A LOCUS IN HUMAN ERYTHROCYTES.

Fig. 1.6 The aminoacid sequence of the human Glycophorin-A
It was also suggested that [6] that the two forms of the GPA evolved from a common ancestral gene by single base substitutions at the sites in the genome coding for aminoacids in positions 1 and 5 of the sequence.

Increased mutation frequencies have been detected hitherto at the GPA locus, among high-risk groups and cancer patients [7]. Significant linear response between frequency and radiation exposure have also been observed for different cell phenotypes [8].

Normal cells are of three types, which are MN, MM and NN types. Mutations can occur which can change the allelic expression as given below.

1.4. ISOLATION OF GPA PROTEIN

It was necessary to isolate the variant forms of the GPA protein inorder to (a) study the biochemical properties of the same and (b) use it later as a pure antigenic source in the final booster immunizations for the production of suitable antibodies.

Glycophorin A from homozygous M and N individuals were purified from red cell membranes by a sequence of butanol or detergent solubilization, phenol extraction and ethanol precipitation. This was followed by chromatography on Sepharose 6B column [9]. Fresh blood samples (10 to 20 ml) were drawn from volunteers with use of acid-citrate-dextrose anticoagulant and used within one week. The erythrocytes were washed three times with 0.15M NaCl.
1.4.1 Preparation of Erythrocyte ‘ghosts’

1.4.1.1 “Dodge ghosts”

“Dodge ghosts” were prepared by washing the cells twice with 50ml of 0.103M Na$_2$HPO$_4$ adjusted to pH 7.4 with 0.155M NaH$_2$PO$_4$ (iso-osmotic buffer), and lysed by dilution into 200ml of cold iso-osmotic phosphate buffer previously diluted with 20.5vol. of cold de-ionized water. The “ghosts” were collected by centrifugation at 20,000g for 40 minutes and washed once with the same solution to yield a readily dispersed pale yellowish-pink ‘ghost’ preparation.

1.4.1.2. “Koscielak ghosts”

“Koscielak ghosts” were prepared by lysing the washed cells into 5 vol. of cold de-ionized water (which had been titrated to pH 5.5 with 1M acetic acid) and kept at 4°C for 30 minutes. The ‘ghosts’ were collected by centrifugation at 20,000g for 5 minutes and repeatedly washed in the centrifuge with the lysing solution until the supernatant was colorless. A red and sticky pellet was obtained. “Dodge ghosts” or “Koscielak ghosts” were prepared and stored at 4°C. Phase contrast microscopy showed the “Dodge ghosts” to be freely mobile, discoid, intact erythrocyte “ghosts” whereas the “Koscielak ghosts” preparations consisted of highly aggregated small vesicles [10].

Isolation of Glycoproteins from cell membranes was done using LIS-phenol, DOC-phenol-gel filtration, hot phenol, chloroform:methanol-SDS gel filtration, Aqueous pyridine, triton-SDS-phenol, triton WGA affinity chromatography and Deoxycholate
methods of which Lithium Diiodosalicylate (LIS) – phenol method and Deoxycholate methods are presented here.

1.4.2.1 Lithium Diiodosalicylate (LIS)-Phenol extraction

In the LIS method, LIS disrupts membranes and releases the glycoproteins from the membrane fragments. In this method, red cell membranes were suspended in 0.3 M LIS and 0.05M tris (hydroxymethyl) amino methane (tris) hydrochloride, pH 7.5 at a concentration of 25mg of membrane protein per milliliter and stirred at room temperature for 15 minutes. Two volumes of distilled water were added and the turbid suspension was stirred for an additional 10 minutes at 4°C. After centrifugation at 45,000 g for 90 minutes at 4°C, the supernatant, which contained most of the membrane proteins was decanted and mixed with equal volumes of freshly prepared 50% phenol in water. The mixture was stirred at 4°C for 15 minutes and centrifuged at 4000g for 1 hour at 4°C in a swing bucket rotor. The centrifuged material separates into two phases; the upper aqueous phase contained most of the soluble glycoproteins. This was removed and dialyzed against several changes of distilled water at 4°C over a period of 24 to 36 hours. The dialyzed material was freeze dried. The dry material was suspended in cold 100% ethanol and mixed for 1 to 2 hours in cold and centrifuged to collect the precipitate. The ethanol washing was repeated for three times. The washed sediment was suspended in distilled water and dialyzed against water in cold overnight. The material was then centrifuged at 10,000g for 30 minutes at 4°C, the clear supernatant containing the soluble glycoprotein. In this method of LIS-phenol extraction, approximately 35-50 mg of glycoprotein can be extracted from 450 ml of human blood [11].
1.4.2.2. Deoxycholate method

In an alternative method, Sergest et al in 1979 adopted Deoxycholate method of glycophorin isolation. This method eliminates the problem of LIS retention in glycoprotein after the separation. Here, the deoxycholate was removed by gel filtration. In this method, Sodium deoxycholate was substituted for LIS and the rest of the procedure remains unaltered. 0.25 M of sodium deoxycholate gave the best results than other concentrations. In this procedure, 25 mg glycoporphin per gram of erythrocyte ghosts was recovered with negligible amounts of lipids, pigment and detergent. There is an improved purity of the protein in this procedure when compared to the higher yields in the LIS-Phenol method [12].

The purified Glycoprotein was dialyzed extensively against distilled water, lyophilized and stored at –20°C. Purity of the Glycophorin preparations was tested by SDS PAGE and stained with periodic acid-schiff reagent. [13].

The LIS-phenol procedure gives almost twice the yield of the best of the other methods published. The hot-phenol method gives the best yields of the later methods but requires high temperatures (65 – 70°C). The remaining methods for glycophorin isolation reported have considerably less yields [14].

1.5. MONOCLONAL ANTIBODY PRODUCTION FOR THE ASSAY

Monospecific antibodies are required against the two GPA variant forms primarily to differentiate the three possible phenotypes (MM, NN and MN). Although polyclonal typing sera are commercially available, monoclonal antibodies for the
two forms of the human GPA became a necessity for the GPA mutation assay as initially developed, which employs Flowcytometric studies.

Sequence studies of the human glycophorin A purified from homozygous M and N individuals have demonstrated that immunologic discrimination is based on the amino acid sequence polymorphism at the positions one and five of the polypeptide; the M form having serine and glycine at these positions whereas the N form contains leucine and glutamic acid respectively [15].

The purified GPA variants proteins were used to immunize mice intravenously after priming the mice with whole erythrocytes. The animals with the highest serum titers were given consecutive intra venous injections for three days followed by removal of spleen. The immunization protocol was as reported earlier [16].

The myeloma cells used for fusion were grown in supplemented duubecco’s modified eagles medium (SDMEM) and were maintained at 37°C in a humidified incubator with a 5% CO₂ in the air atmosphere. For fusion, $10^8$ spleen lymphocytes and $10^7$ log phase myeloma cells were used with polyethylene glycol as the fusing agent [17]. For cloning, microtitre plates with feeder cells (peritoneal exudates cells) were used. On the $7^{th}$, $14^{th}$ and $20^{th}$ day following fusion, the wells were refed with 0.1 ml fresh media containing 0.4 μM aminopterin. On the $23^{rd}$ day, the hybridomas were refed with aminopterin free media. On the $27^{th}$ day, 50μl of medium from each well was assayed for red cell antibody production using whole cell ELISA. The cells that were most positive were used for scaling up of cultures [18]. The culture supernatants were tested on the $30^{th}$ day to determine those cultures producing high levels of antibody capable of discriminating between homozygous M
and N red cells. The most promising stocks were frozen in medium consisting of
70% SDMEM, 20% heat inactivated fetal calf serum and 10% DMSO.

Apart from in-vitro cultures, the hybridomas were also grown as ascites tumors. For
this purpose, Balb/c mice were injected twice with 0.2ml pristine at one week
intervals and irradiated with approximately 600 R of 1 MeV X-rays one day after the
second pristine injection. About $10^7$ hybridoma cells were injected intra peritoneally.
8-15 days after the injection, the animals were killed and the ascites fluids obtained
by washing the peritoneal cavities with approximately 20 ml of sterile saline. Viable,
sterile tumor cells were recovered for injection into other animals. The antibodies
thus obtained from the ascites fluid were purified by Protein-A affinity
chromatography. The protein containing fractions as monitored by the absorbance at
280 nm were pooled, dialyzed against 10 mM sodium phosphate, 0.15 M NaCl, pH
7.2 (PBS) and assayed for antibody activity.

1.6. GPA MUTATIONAL ASSAY

Mutational assays at the GPA sites use monoclonal antibodies to detect the loss of
the gene product from either of the two allelic forms [19]. Its application is limited
to MN heterozygotic individuals who constitute 50% of all human populations.
Presumptive mutant cells are of two types: those with the normal signals from one of
the alleles and none from the other (hemi zygo
tes); and those with twice the normal
signal from one allele and none from the other (homozygotes) [20].

GPA known to be the antigenic determinant of the MN blood type can be subjected
to a pair of monoclonal antibodies to facilitate detection of mutant erythrocytes
lacking either M or N products of GPA alleles among normal erythrocytes from MN
heterozygous people. The variant cell types, which can be detected, are those that lack the M form of GPA (hemizygous NØ) and those that lack the N form of GPA (MØ and MM variants) [21].

Glycophorin A (GPA) is a human blood type antigen that appears on the surface of red blood cells (erythrocytes) in two common forms, called the N and M allelic forms. Since the gene for this blood type antigen is inherited in a conventional Mendelian mode, 50% of the population expresses the heterozygous blood type NM. Essentially all the erythrocytes in individuals who are NM blood type contain both GPA\textsuperscript{N} and GPA\textsuperscript{M} on their surface. However, if a cell in the bone marrow is impacted with ionizing radiation at the glycophorin A gene, one of the two allelic forms of the GPA gene could be inactivated. As a result, all progeny of this cell would express only one of the two forms. If the GPA\textsuperscript{M} allele has been inactivated, and, since the sister chromosome has not been affected by the ionizing radiation, GPA\textsuperscript{N} is expressed normally on all the progeny of this mutant cell. When these progeny cells mature and are secreted into the peripheral blood, they appear as variant erythrocytes, called NØ or N-null cells. In order to detect the presence of such cells, a fluorescence immunolabeling technique was developed. The ideal form of this technique is to isolate two different monoclonal antibodies, each of which is specific for one of the two allelic forms of GPA. Then these antibodies are labeled with fluorescent conjugates so that immunostaining of erythrocytes from a heterozygous (GPA\textsuperscript{NM}) individual would yield primarily double-stained cells. Those cells which are variants would be single-stained, and the somatic cell mutation assay would be performed by carefully enumerating the frequency of single-stained erythrocytes in
each individual. This requirement for expression of one of the two allelic forms of GPA provides an internal control, which prevents mis-identification of degraded erythrocytes, which may have damaged membranes or cell surfaces, as being progeny of mutant bone marrow cell.

The first GPA assay was the 1W1 assay, utilized one monoclonal antibody that was specific for GPA\textsuperscript{M} (this antibody was named 6A7), but the second antibody (named 10F7) recognized both allelic forms of glycophoria A. Thus, the assay could determine the loss of GPA\textsuperscript{M} but not GPA\textsuperscript{N}.

An increased efficiency was achieved with the development of SBS assay for the GPA flow analysis so as to perform the assay on a large number of A-bomb survivors in Hiroshima [22]. The major change in this latter of the GPA assay was the use of a newly available fluorophor, phycoerythrin. This fluorophor can be excited at the same wavelength as can fluorescein (488 nm) and emits fluorescence at a long enough wavelength (580–590 nm) to be separated from fluorescein emission (520–530 nm) using dichroic mirrors, band pass filters, and electronic signal compensation. As a result a single-beam laser sorter could be used for the analysis.

The next step in the evolution of the GPA assay was an improvement in eliminating false positive events by using a new monoclonal antibody which specifically recognized GPA\textsuperscript{N}. This antibody was conjugated with fluorescein and used it in combination with the GPA\textsuperscript{M}-specific antibody, 6A7, to perform an analysis on a FACScan flow cytometer [23]. The new assay was termed BR6 as an acronym for the two antibodies (BRIC 157 & 6A7). Parallel measurements were performed using
this assay and the 1W1, dual-beam flow sorter assay on blood samples from unexposed donors to show that the assays gave comparable variant cell frequencies, and that the new assay showed improved measurement precision. For Nϕ variant cells, the group coefficient of variation for the 1W1 assay was 64%, while for BR6 the CV was 30%, a significant improvement. Another advantage of the new assay was the fact that it could be performed without cell sorting. This resulted in two improvements: (1) the new assay was less labor-intensive, since sorting is a long and tedious procedure, and microscopic enumeration is very labor-intensive; and (2) a higher precision in counting statistics could be obtained. The FACScan can perform analysis at 4,000 cells per second, while the cell sorter can be operated at a maximum of 1,000 cells per second. Using the BR6 assay, it was possible to analyze $5 \times 10^6$ cells per sample, whereas the 1W1 and SBS assay could analyze only $5 \times 10^5$ cells per sample.
Fig. 1.7 Possible mutations in a MN heterozygous individual
<table>
<thead>
<tr>
<th>Assay Version</th>
<th>Group</th>
<th>Antibodies and fluorochromes</th>
<th>Equipment</th>
<th>Year reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>1W1</td>
<td>Langlois et al</td>
<td>6A7 + 10F7</td>
<td>Sorter</td>
<td>1987</td>
</tr>
<tr>
<td>SBS</td>
<td>Kyoizumi et al</td>
<td>6A7 + 10F7</td>
<td>Sorter</td>
<td>1989</td>
</tr>
<tr>
<td>BR6</td>
<td>Langlois et al</td>
<td>6A7+BRIC 157F</td>
<td>Cytometer</td>
<td>1990</td>
</tr>
<tr>
<td>BR6 Chern</td>
<td>Jensen et al</td>
<td>6A7+BRIC 157F</td>
<td>Cytometer</td>
<td>1995</td>
</tr>
<tr>
<td>DB6</td>
<td>Jensen et al</td>
<td>6A7-PE+BRIC157F</td>
<td>Cytometer</td>
<td>1996</td>
</tr>
<tr>
<td>Magnetic cell sorting and internal standardization</td>
<td>Hempel et al</td>
<td>6A7-Biotin BRIC157-FITC</td>
<td>Sorter &amp; Cytometer</td>
<td>2003</td>
</tr>
<tr>
<td>RS-1 Assay</td>
<td>Ravi et al</td>
<td>Anti-M &amp; Anti-N</td>
<td>Heamagglutination plates</td>
<td>2002</td>
</tr>
</tbody>
</table>

Table 1.3: Chronological modifications of Human GPA mutation assay

1.7. COMPARISON TO CYTOGENETICS AND OTHER MUTATION ASSAYS

At the same time that the GPA assay was being improved, cytogenetic analysis was evolving in a collaborative fashion between Radiation Effect Research Found and Laurence Liver more National Laboratory. Historically, scientists at RERF had been performing chromosomal aberration analysis on metaphase chromosomes of lymphocytes obtained from A-bomb survivors and had acquired a large dataset indicating a significant dose response for this biological endpoint. At LLNL the human genome project had been developing a chromosome-specific human DNA
library carried by a bacterial vector. Using this library, cytogeneticists were able to apply fluorescence in situ hybridization (FISH) of chromosome-specific DNA to human metaphase chromosomes and perform chromosomal aberration analysis in a rapid and quantitative fashion with fluorescence microscopy. In a collaborative effort to compare this new technique with the already established cytogenetic analysis, the scientists at RERF performed conventional translocation frequency measurements, and those at LLNL performed FISH translocation frequency measurements on the same samples.

The results of this comparison showed that the two cytogenetic analyses agreed with each other, with $R^2$ equalling 0.92, indicating that they are indeed measuring similar phenomena [24].

Since the collaborative cytogenetic study was occurring at the same time as was the collaborative GPA comparisons, the same blood samples were used for all four assays. Thus, the results of somatic mutation assays can be compared in precision with those obtained with aberration assays. Here in the present study a comparison between the results from FISH translocation analysis and those of the BR6 assay has been performed. In order to compare these results, a best-fit function for the two datasets was derived and found to be a logarithmic translocation of the response data. After performing a log-linear regression analysis on each of the datasets, an inverse regression to determine how well each assay could be used to predict dose was performed. The results show that the two assays displayed very similar precision.
In an attempt to identify high risk cancer groups and for estimating the effects of mutagens, a comparative study employing T-Cell receptor and the GPA assay was conducted which further reinforced the usefulness of the GPA assay in such situations. The GPA assay was utilized in conjunction with the Micronucleus assay in a study which showed a significant correlation for people exposed to ionizing radiation [25]. Yet another study, showed normal T-lymphocyte *hp* mutant frequencies in individuals with stably elevated background GPA variant frequencies which suggest that the GPA is a much more sensitive locus for mutations.

Evolution of the GPA assay is continuing. It was found that the previous fluorescent labeling approach for antibody direct BRIC 157 6A7 ("6A7") would occasionally give high frequencies of variant cells in samples for which such frequencies might not be expected. Careful monitoring of such events indicated that blood samples shipped or stored under inappropriate conditions gave these variable results.

Further improvements showed that 6A7 labeled with the secondary avidin fluorescent labeling procedure was not binding to slightly damaged cells in a way that put their fluorescence intensities in the NØ variant cell window of the bivariatee histogram obtained by FACScan analysis[26]. If the same antibody was directly conjugated with phycoerythrin, the fluorophor of choice for this assay, these same samples gave cleaner histograms and lower variant cell frequencies for a number of such samples. A parallel cell sorting, fluorescence microscopic, and re-immunolabeling procedure showed that cells that would not bind the secondary complex would successfully bind the direct conjugate. This gave rise to the DB6 version of the assay.
Typically, for the assay, blood samples from normal volunteers with no known mutagen exposure are obtained with informed consent, and screened for MN type using typing sera and selected as controls. 5 – 30 ml of blood samples suffices for the assay.

Cell fixation is necessary for these studies to block antibody induced agglutination of the erythrocytes. This is achieved by producing formalin spheres by diluting whole blood in a solution containing Sodium Dodicyl Sulphate, followed by fixation with formalin. Formalin fixation should be avoided for GPA(N) – specific monoclonal antibodies. In yet another technique, the erythrocytes can be fixed with cross-linking agent dimethyl suberimidate (DMS). Such cells are compatible with the binding of all the monoclonal antibodies, but the staining is clear for only one week [27].

After the cells are fixed, they can be subjected to antibody staining. The antibody staining of the fixed erythrocytes was performed at room temperature in 10 mM Sodium phosphate, pH 7.2, 0.15 M NaCl, 5 mg/ml bovine serum albumin, 0.01% nonident P-40 and 1.5 mM NaN3 as staining buffer. The fixed cells are washed with the staining buffer by centrifugation and incubated with the monoclonal antibodies of interest, washed twice, further incubated for 1 hour with secondary antibody or avidin, and resuspended in staining buffer after washing twice. For saturation levels of antibody binding, 4 X 10⁷ erythrocytes / ml, 10μg / ml primary monoclonal antibody, 20μg / ml secondary antibody or 10μg / ml avidin was used [28]. Such stained samples can be stored for several days at 4°C with no degradation in assay sensitivity.
The assay can be performed on a single – beam flow cytometer to enumerate variant cells as evidenced by the staining \cite{29}. The assay uses two- color fluorescence labeling of fixed cells with two different monoclonal antibodies, each of which binds specifically one of the two allelic forms of GPA. These erythrocytes can be analyzed by flow-cytometry to enumerate the frequency of the two variant cell types \cite{30}.

Kyoizumi \cite{31} reported a method for measuring the frequency of variant erythrocytes at the GPA locus using a single beam cell sorter (SBS). This made the assay simpler than the previous DBS assay that used a double beam cell sorter. The fundamental difference between the two systems was in the choice of fluorochromes (fluorescein and TR for DBS and fluorescein and PE for SBS) used for labeling antibodies. In the same report, a significant correlation between GPA variant frequencies and chromosome aberration frequencies was shown thus reaffirming the GPA mutation assay to be a reliable new biological parameter indicative of radiation exposure.
Chapter 1

Thesis

"ANALYSIS OF SOMATIC CELL MUTATIONS AT THE GLYCOPHORIN-A LOCUS IN HUMAN ERYTHROCYTES.

Principle of the Glycophorin-A mutation assay by flowcytometry
1.8. SENSITIVITY OF THE GPA ASSAY

The GPA human mutational assay is now being extensively used for studies of genetic damage in human populations with exposure to potentially mutagenic agents and populations that are potentially unusually susceptible to mutational change. The assay is restricted by donor genotype, i.e., heterozygotes for MN blood type. GPA assay provides lifetime biological dosimetry of radiation exposures, presumably because the mutations are accumulated in the long-lived hematopoietic stem cells. In one analysis of the response of humans to irradiation, it was noted that the slope of the dose response for individuals exposed at high dose and dose rate (>1 Gy) was 25 X 10^{-6} variants / Gy while the slope of response at low dose and dose rate (<1 Gy) was 4.1 X 10^{-6} variants/ Gy. It was also suggested that exposure to a duration ranging from seconds to hours results in comparable increases in levels of mutation per unit dose [32].

Another analysis of the mutant frequency dose response using a descriptive model showed that the doubling dose is about 1.20Sv (95% confidence interval (CI) 0.95 – 1.56). Whereas the minimum dose for detecting a significant increase in the mutant frequency is about 0.24 Sv (95% CI :0.041 – 0.51). No significant effects of sex, city or age at the time of exposure, on the dose response could be detected [33].

Fig. 1.9 The dose response for absorbed dose in humans using the Glycophorin-A mutation assay
1.9. CURRENT DEVELOPMENTS

The GPA mutation assay is applied to the study of blood samples from patients obtained prior to, during and following chemotherapy for malignant disease in order to determine the effect of mutagenic chemical agents on the frequency of variant cells [34]. The results demonstrated that mutagenic chemotherapy agents induce elevated levels of GPA variant erythrocytes. Similar results were obtained in work carried out in germ cell tumor patients treated with Platinum based chemotherapy [35].

The most marked application for the GPA mutation assay had come from its usage as a biodosimetric technique for exposure to ionizing radiation. Elevated frequency of GPA mutations was observed from different parts of the world. Studies were conducted for the same in Chernobyl accident victims [36], atomic bomb survivors [37], Chernobyl cleanup workers from the Baltic countries [38], immigrants from the vicinity of Chernobyl [39], and Chernobyl cleanup workers from Estonia and Latvia [40]. A descriptive account on the evolution of the GPA assay for measuring biological effects of radiation on humans was given by Jensen [41] where the various developments in the assay have been dealt with.

Further, the GPA mutation assay was also used for biodosimetry of prolonged irradiation [42] where the authors suggest a limited potential for the assay, at least in dose interval up to 2.0 Gy.

The technique when applied to radiation workers in Sellafield Nuclear Facility [43] proved to be insufficiently sensitive to be used as a biological marker of low-dose chronic exposure.
In a study that compared the different GPA mutation assay methods, it was recommended that the BR6 version was the most suitable for future applications [44].

In an effort to understand the GPA in human beings, studies to characterize several GPA specific cDNA clones have been made [45]. Also, bacteriophage expressing mouse monoclonal Fab fragments against the human MN glycophorin blood group antigens were constructed [46]. Further, isolation of human anti-red blood cell antibodies by repertoire cloning was achieved [47]. With achievements such as isolation of cell surface specific human monoclonal antibodies using phage display and magnetically activated cell sorting [48], more efficient assays are possible for studies of the mutational changes at the GPA locus of the human erythrocytes.

With increasing interest in the GPA mutation assay for biodosimetry and assessment of health risks, the techniques used to detect the variant cells have been undergoing constant changes and evolution from 1986 (with the first reported version of the assay) till date. In a recently reported report, a further development in detecting GPA variant erythrocytes was high gradient magnetic cell sorting and internal standardization, which substantially improved the detection output [49]. This continued and evolving interest to maximize the human GPA mutation detection efficiency is partly due to the ease at which the cells can be analysed and also due to the fact that the GPA mutation assay remains as an effective tool which gives lifelong biological dosimetry to radiation exposures.
REFERENCES


CHAPTER 1


[32] Perera P.F; Robert J. Motzer; Deliang Tang; Eddie Reed; Ricardo Parker; Dorothy Warburton; Patrick O’ Neill; Richard Albertini; W.L. Bigbee; H. Jensen; Regina Santella; W.Y. Tsai; G. Simon-Cereijido; Colleen Randall


[37] Bigbee W.L; H. Jensen; T. Veidebaum; M. Tekkel; M.Rahu; A. Stengrevics; A. Kesminiene; J. Kurkinaitis; A. Auvinen; T. Hakulinen; K. Servomaa; T. Rytomaa; G.I. Obrams and John D. Boice jr. Glycophorin A biodosimetry in
CHAPTER 1


[42] Siebert. D, and M.Fakuda. Isolation and characterization of human glycophorin A cDNA clones and a synthetic oligonucleotide approach:
CHAPTER 1


