6.1 ABSTRACT

Dose evaluation of exposed individuals to radiation becomes necessary after exposure. GPA mutational assay is a biodosimetric technique for such assessments. There are two forms of the protein, the M and the N type. The GPA assay can be performed on the heterozygous MN individuals. This mutational assay provides for population studies and gives a life-long dosimetry in such individuals. Essentially, the GPA assay uses monoclonal antibodies against the two forms of the protein. Flowcytometric analysis of the stained erythrocytes was done to evaluate the dose received.

Here, we present a novel assay the RS-1 assay. This assay employs the agglutinating antibodies against the two forms of the GPA protein. In cases of loss of the protein as in the case of radiation-induced mutations, the antibodies fail to react with the variant erythrocytes. Thus, the agglutination patterns achieved will give the extent of erythrocytes with protein loss. The preliminary results using the RS-1 assay on heterozygous individuals have given us the indication of GPA distribution pattern. There seems to be a slight but distinct shift towards the variant types with. This assay provides us with a rapid and simple technique with blood samples as low as one milliliter.
6.2 INTRODUCTION

Ionising radiations induce dose-dependent somatic mutations. In recent times mutations at the somatic cell level have been gaining ground for their effective use in biodosimetry. Assessment of the somatic cell mutations at the human GPA locus is one such detection method for biological dosimetry.

The human GPA is a sialoglycoprotein of 151 amino acid and spans the erythrocyte membrane in about $5 \times 10^5$ copies per cell. The protein is present as two forms and three possible genotypes MM, NN or heterozygous MN can occur in a given population. It is regarded that about 50% of the population will express the MN heterozygous genotype as this is based on the Mendalian inheritance. The M and the N forms of the protein vary in their amino acid composition in positions (1) and (5) [1]. The M type has Serine and Glycine in positions (1) and (5) respectively and the N type has Leucine and Glutamic acid in the respective positions.

Increased mutation frequencies were detected hitherto at the human GPA locus among high risk groups and cancer patients [2]. Significant linear response between frequency and radiation exposure were observed for different cell phenotypes [3].

Mutational assays at the GPA locus developed till date use both the forms of monoclonal antibodies to detect the loss of the gene products from either of the two allelic forms [4]. GPA known to be an antigenic determinant of the MN blood type. Monoclonal antibodies facilitate the detection of mutant erythrocytes lacking either the N or the M products of the alleles among normal erythrocytes from the MN heterozygous individuals. The variant cell types, which can be detected, are those that lack the M form of the protein and those that lack the N form of the protein [5].
Typically 5 – 30 ml blood is necessary for the conventional assay. The erythrocytes are fixed and stained using monoclonal antibody- fluorochrome conjugates [6]. The erythrocytes are therefore doubly stained and the enumeration of the variant cells can be done by flow-cytometric analysis [7].

The GPA mutational assay is now being extensively used for studies of genetic damages to human populations exposed to potentially Mutagenic agents and also to populations that are potentially, unusually susceptible to mutagenic change. This assay provides a life-long biological dosimetry to radiation exposures, presumably because the mutations are accumulated in the long-lived hematopoietic stem cells. The minimum dose for detecting a significant increase in the mutation frequency with the technique as available till now is 0.24 Sv (95% CI : 0.041 – 0.51).

In the present work GPA mutational assay has been developed to enumerate the variant erythrocytes. The understanding of the essential mechanisms of the GPA distribution and certain immuno technique has led us to the development of an assay, which is rapid as well as sensitive.

Here, we present a novel technique, the RS-1 assay which detects the variant cells at the human GPA locus. Apart from mere detection of such mutations, the technique has also the potential to quantify the variant cells, thus proving to be an invaluable tool for biological dosimetry. The RS-1 assay can be performed on the MN heterozygous individuals and is essentially an immunotechnique, which benefits from the haemagglutination mechanisms.
6.3 MATERIALS AND METHODS

Blood samples were collected from 153 volunteers who were chosen randomly for the blood grouping of MM, NN or MN types. Blood typing was done using commercial antisera for M and N types from Ortho Clinical Diagnostics, USA using standard agglutination methods on glass slides. One milliliter of blood was collected in heparinised condition by standard vein puncture method from MN heterozygous individuals with informed consent for studies with RS-1 assay. Care was taken to obtain blood samples from individuals who had no known previous exposure to mutagens or transfusions. Cold saline was used to wash the erythrocytes thrice and a 10% suspension was used uniformly for the RS-1 assay. 50μl of the 10% erythrocyte suspension, which yielded about 1 x 10^4, was used uniformly.

Agglutinating antibodies as obtained from Ortho Clinical Diagnostics, USA, specific for M and N blood types were used for the assay. For the assay, antibody solutions in different concentrations as given in Table 1, were prepared. 96 well Hemagglutination plates were used for the assay and 50μl of the agglutinating antibodies for M and N types were added to two rows of the microtitre wells. Further, 50μl of the erythrocyte suspension was added to the antibody mixtures and incubated at room temperature for 1 hour. The results were observed from the plates as either button formation or mat formation and were interpreted accordingly. In
case of a reaction between the antibodies and the GPA protein on the erythrocyte surface results in the erythrocytes taking the form of a mat, covering the bottom of the well and in dilutions where there is no reaction, a button form occurs by simple settling of the erythrocytes due to gravity in the U button wells. Based on the distribution of the GPA molecules of the two types, different patterns of agglutination take place. By comparing the M and the N rows and the extent of dilutions where agglutination occurs, a reasonable conclusion can be obtained as to the variant erythrocytes for a given MN heterozygous sample.

\[ \frac{n}{m} = x \]

- \( n \) = Dilution of Anti-N
- \( m \) = Dilution of Anti-M
- \( x \) = the RS-1 ratio

Normal Base-line ratio range = 1.5-2.2
- Ratio > 2.2 = mutation shift towards the N allele
- Ratio < 1.5 = mutation shift towards the M allele

*Thesis*

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

The results as obtained by the RS-1 assay for heterozygous individuals are represented in Table 1. The end point is taken as a dilution factor where the button formation starts after the culmination of mat formation. The results obtained from the RS-1 assay shows a pattern of the GPA distribution and further suggest a shift towards the wells subjected to the Anti N antibody reagents. This suggests the presence of detectable base-line mutations/deletions at the GPA locus and therefore, the presence of the variant cell types.
CHAPTER 6

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Table 1: The results obtained as Haemagglutination patterns. Agglutination occurs up to the dilution of the antisera where they react with erythrocytes with intact protein molecules. The cells take a button formation in the dilutions of the antisera where there is no reaction.

6.5 DISCUSSION

The GPA mutational assay for biological dosimetry was first developed in 1986. Since the two allelic forms of the protein differ only by two amino acids, it was necessary to have antibodies specific for each of the two forms, which proved to be a difficult task. Initially, only one monoclonal antibody specific for the M form of the protein was developed. Based on this the first GPA mutational assay [8] was...
developed. The assay was named as 1W1. This assay could determine the loss of the M form but not the variant erythrocytes with loss of the N form of the protein. With the development of another monoclonal antibody specific for the N form of the protein, the BR6 version of the assay was developed. This led to the possibility of scoring a large number of erythrocytes, thus improving the sensitivity of the assay. Recently, another development was made by direct labeling of fluorophores to the antibodies, which resulted in the development of the DB6 assay. Therefore, the GPA mutational assay has undergone significant changes in design that has improved the ease of analysis and precision of measurements.

The assay technique that we have developed was adopted for heterozygous MN individuals with no known previous mutagen exposure, as a preliminary study. As the assay is based on Hemagglutination, it is quantitative and gives indications of detecting the variant erythrocytes present in the samples. When the two specific antibodies are suitably diluted and are presented in different concentrations to a sample erythrocyte suspension, they react with cells with normal protein expression. The agglutinations of such reactions ceases with the cells that lack the expression of the protein as in the case of radiation induced mutations. Therefore, the dilution factor up to which agglutination occur in wells containing the antibody types will give a suitable pattern of cells with intact native protein and also the erythrocytes with a loss of the protein expression as the case may be.

6.6 CONCLUSION
Our understanding of the basic mechanisms of the GPA distribution on the erythrocyte membrane and in conjunction with time tested immunotechniques led us to the development of a simple, rapid and sensitive RS-1 assay. This assay does not require techniques such as antibody conjugation to flurophores and the need for the equipments such as fluorescent microscope and flowcytometer. The result can be obtained within two hours by using a blood sample less than one milliliter. The results obtained in these experiments suggest the possibility of using of this assay as a simple one, for biodosimetry. With further refinement, the RS-1 assay can be evolved as a screening method for exposed population.

REFERENCES

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


glycophorin A mutations in Erythrocytes from Chernobyl accident victims.
