ABSTRACT

Introduction: Dose evaluation of exposed humans to radiation becomes necessary after 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. Biodosimetric methods include cytogenetic, immunological and mutational assays [2].

The need for biodosimetry is essential for calibrating the actual dose received to a biological system where other conventional forms of dosimetry are unavailable. Even in the presence of other dosimetric tools such as Thermo Luminescent Devices (TLDs) etc, biological dosimetry gives actual doses apart from an indication of the extent of damage caused by such damages. This becomes all the more important with the current situations such as space programmes and dirty bombs.

There are currently many advancing methods for measuring somatic mutations and new opportunities for biological dosimetry of high-risk groups. An evaluation of such 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.

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 [4]. This continued and evolving interest to maximize efficiency with
which the human GPA mutation can be detected is partly due to the ease with which the cells can be analysed and also due to the fact that the GPA mutation assay remains an effective tool which gives life-long biological dosimetry to radiation exposures.

The thesis begins with an overall abstract of the entire research work carried out and the thesis.

The first chapter gives an elaborate review of the work done earlier from published literature. The review covers the work done in biodosimetry in brief, and utilization of the GPA mutation assay as a biodosimetric tool [5,6,7] in detail. The review encompasses relevant work done in this area from 1960s till date.

Biological indicators of various types are being used to assess mutagenic insult to human systems and include biochemical, biophysical, physiological, prodormal, cellular, haematological, immunological, molecular, cytogenetical and molecular cytogenetic approaches [8]. Of these, the human GPA mutation assay is thus far the only known tool for assessing cumulative and life-long exposure to mutagenic insults [9], which forms the basis of the present research work and therefore reviewed in detail.

The second chapter gives details of the materials and methods that were used in all the experimental protocols used in the present investigation.

The third chapter deals with the frequency of M and N allelic expression in terms of phenotypic blood groups in South India. This is a population study, which gives us an indication of MN blood group distribution in our population apart from the genetic inheritance patterns. The GPA mutational assay is an effective biodosimetric tool for population studies. As the assay can be performed only on MN heterozygous individuals, a representative population study with regard to the MN blood group distribution becomes necessary. A randomly collected cross sectional study was performed for the same. The MN blood group system in humans is determined by two alleles M and N, and these determine the presence of corresponding antigens on red cells. Accordingly, 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 erythrocytes is a
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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). The M group has Serine and Glycine in positions (1) and (5) respectively while the N group has Leucine and Glutamic acid in the respective positions [10].

The results obtained from the population study with respect to the frequency of MM, MN and NN blood groups are discussed. The differences between the expected and the observed frequencies of these blood groups are not significant. The genotype frequency for MM blood group was 0.3267 (p^2) and for MN blood type was 0.5555 (2pq). The same for NN blood type was 0.1176 (q^2). The allelic frequency for M and N types was 0.6045 and 0.3955 respectively. The expected genotype frequencies for MM, MN and NN types were 0.3654, 0.4781 and 0.1564 respectively. This suggests a Mendelian mode of inheritance of this allele and also that approximately 50% of the population is heterozygous for MN and are the suitable subjects for the GPA assay [11].

The fourth chapter discusses the production of Monoclonal Antibodies for human erythrocyte surface proteins [12]. Whole erythrocytes of homozygous MM and the NN blood groups were used as Antigens and pooled samples were used aseptically to immunize Balb/c strain of high responder mice. The immunizations were performed by the intra-peritoneal route, which was considered the most preferred route for this type of experiments. The sera samples from the hyperimmunised animals were tested for the antibody response by looking for the anti-erythrocyte activity.

The animal whose serum showed the maximum response as identified by its hemagglutinating property and the rapidness of the same, was selected for further booster doses of the antigen and earmarked as the splenic donor. The animal with the maximum response was given final booster doses of pooled erythrocytes as previously described for three consecutive days. On the second day after the last booster, the animal was used for spleenectomy and as a source of primed spleenocytes. Spleenocytes from syngenic, unimmunised mice were used as feeder cells.
The retrieved spleenocytes and the cultured Sp2/0 cells were washed twice in plain medium. Further to individual washing, the cells were pooled into a single centrifuge tube and were washed once with plain medium and were ready for fusion. Polyethylene glycol was used as the fusogen [13]. Immediately after subjecting the cells to polyethylene glycol, the cell mixture was diluted in large quantities (40 ml) of plain medium and the resultant cell mixture was distributed into 96 well microtitre plates by limiting dilution.

The developing clones were identified, indicating the plate number, and the well number, thus facilitating easy identification of individual clones. The data of all the clones obtained were recorded. Apart from this type of record maintenance, the clones were also documented by microphotography. Feeder cells, young clones and clones at various stages of development were photographed and documented.

The culture supernatants from wells with the clones (100 microliters/well) were used for screening. The supernatants thus obtained were stored at 4°C till further screening; this was done within four days of their collecting. The supernatants were subjected to a series of tests to ascertain the nature of the clones.

Expansion of the positive clones with the desired secretory properties was done by transferring the cells from developing clones to 24 well culture plates. Some clones with high proliferative properties were directly transferred to T-25 culture flasks and their progress monitored.

The fifth chapter of the thesis discusses the flow-cytometric analysis of variant erythrocytes at the human GPA locus. 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 [14].

Mutational assays at the GPA sites use monoclonal antibodies to detect the loss of the gene product from either of the two allelic forms. Its application is limited to MN heterozygotic individuals who constitute 50% of all human populations.
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Presumptive mutant cells are of two types: those with the normal signals from one of the alleles and none from the other (hemi zygotes); and those with twice the normal signal from one allele and none from the other (homozygotes). As controls, for the assay, blood samples from normal volunteers with no known mutagen exposure were obtained with informed consent and screened for MN type using typing sera. 5 – 30 ml of blood samples was processed for the assay [15]. 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) [16].

The sixth chapter presents a novel technique, The RS-1 Assay, developed by the candidate. Assays developed to date to assess mutations at the GPA locus use monoclonal antibodies to the two forms of the protein to detect the loss of the gene products from either of the two allelic forms. Application of the assays is limited to the MN heterozygous individuals. GPA known to be an antigenic determinant of the MN blood type can be subjected to a pair of monoclonal antibodies to 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. Typically 5 – 30 ml blood is necessary for the conventional assay. The erythrocytes are fixed and stained using monoclonal antibody- fluorochrome conjugates. The erythrocytes are therefore doubly stained and the enumeration of the variant cells can be done by flow-cytometric analysis.

We were able to develop a new (RS-1) GPA mutational assay, which can enumerate the variant erythrocytes. The understanding of the essential mechanisms of the GPA distribution and certain immunotechniques led to the development of an assay technique, which is both rapid, and sensitive. 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.

**The seventh chapter** summarizes the results obtained in a study of human Carcinogenesis and health risk assessment by use of the RS-1 assay. One millilitre of venous blood was collected heparinised with prior informed consent from previously determined MN heterozygous individuals with no known exposure to mutagens and were considered as control samples. Such samples were also collected from ten persons with an advanced stage of prostrate cancer. The mutations at the Glycophorin-A locus were observed through the RS-1 assay as previously described. Briefly, blood samples were washed thrice with cold saline and a final 10% erythrocyte suspension was prepared in saline. Fifty micro liters of this suspension was used uniformly for the assay and were subjected to a series of antibody dilutions. The plates were observed after one hour for the pattern of haemagglutination and button formations. The assay was performed in haemagglutination plates and the results were interpreted as a factor of the antibody dilutions where agglutination ceases and button formation starts.

High molecular weight DNA was prepared from the peripheral blood samples using high salting out method and purified further by PCI (Phenol:Chloroform:Isomethyl alcohol) extraction method. The quality and quantity was checked by Agarose Gel Electrophoresis and spectrophotometry. The ratio of 1.8 (A260 / A 280) quality DNA samples was subjected further to PCR DNA amplification of p53 gene at exons 6 and 7. The amplicons were subjected to Agarose Gel Electrophoresis (2% Agarose) and analysed further to staining with Ethidium bromide. The band patterns were analysed by comparing with a known molecular weight 100 base pair DNA ladder.

The results as obtained in the RS-1 assay for the control samples show a minor shift in the agglutination patterns, thus indicating base line mutations. A significant shift in the patterns was observed in the cancer samples. This indicates an increased level of mutations at the GPA locus in cancer samples. Intact amplified bands at the exons 6 and 7 of the p53 locus were observed for the control samples in the amplicons of
control samples subjected to Agarose Gel Electrophoresis stained with Ethidium Bromide. The bands were absent in cancer samples subjected to similar procedure indicating mutations at the p53 locus at exons 6 and 7.

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.

The eighth chapter of the thesis is based on the work carried out for RS-1 Assay in comparison to Flow-cytometric analysis of human Glycophorin-A mutations. This was done for enumeration of variant cells in the methods as being followed in the quoted literature and also to assess the comparative effectiveness of the RS-1 Assay.

The ninth chapter presents the RS-1 Assay in conjunction with other Biodosimetric Genetic Markers. Samples from normal and cancer patients were chosen randomly and RS-1 assay performed. The same samples were subjected to T Cell receptor mutation assay by flowcytometry. The dose responses obtained by both the methods were compared and discussed.