# CHAPTER 2

# MATERIALS & METHODS

The detailed Materials & Methods used in the various studies are presented in this chapter.

## 2.1 - Materials

### 2.1.1. - MN Blood group typing

The typing reagents for the MN blood groups were procured from Ortho Clinical Diagnostics, Rariton, USA. The M typing serum used was MM 557A, cat No. 606207946 MM 557A and the N typing serum used was NN 648A, Cat No. 606207956. The typing was done on plain glass slides.

### 2.1.2. - Monoclonal Antibodies against the Human Glycophorin-A

High responder Balb/c mice were chosen as the host for immunization and the spleenic donor for fusion and hybridoma generation. Sp2/o cells, which are myeloma cells of murine origin, were used as the fusion partners. Initial immunizations and the final booster immunizations were administered through the intra-peritoneal route. Erythrocytes from MM and NN homozygous Rh (-) individuals were washed thrice in cold sterile saline and were used as the immunogens. After the final boosters, the animals were checked for the immune response from the serum obtained through blood collection through the retro-orbital plexus. The immune response was checked by heamagglutination methods and the animal with the highest titre was selected as the spleenic donor. The spleen was collected aseptically and single spleenocytes were obtained by using a tissue teaser (cell dissociation kit CD1 / Sigma). Sp2/o cells which were cultured in DMEM

Culture medium (Catalogue No. Sigma D-55223) with 10% serum were used as the fusion partners. Polyethylene Glycol (Sigma - Hybrimax P 7181) was used as the fusogen. The selection medium used was prepared by HAT (GIBCO BRL 31062-03). Limiting dilutions were performed into 96 well micro culture plates (900196$^{-}$ Tarsons) which contained feeder cells. Spleen from syngenic, unimmunised mice were used as a source of the feeder cells. Further cloning were done into 24 well culture plates (980030$^{+}$ Tarsons) and12 well culture plates (980020$^{+}$ Tarsons). The positive clones were expanded serially in T-25 culture flasks with filter caps (950040$^{+}$ Tarsons), T-75 culture flasks with filter caps (950050$^{+}$ Tarsons) and 150 $cm^2$ culture flasks (950060$^{+}$ Tarsons) for the final collection of the supernatants. Further to selection, all the hybridomas were cultured in Hybridoma serum free medium (GIBCO 12045-084)

The screening of supernatants was done by Ouchterlony double diffusion and immunoelectrophoresis using Anti Mouse IgG and Anti Mouse whole serum (Bangalore Genie, AS-6S and AS-23S respectively). Anti erythrocytic activity and final screening was done employing Heamagglutination plates (Tarsons 934396). The positive clones were cryopreserved using Freezing medium (Sigma C6295) and Cryovials (523050 Tarsons). The storage was in Cryocans (IBP- BA11, BA35 & TA 26 cryocans). Water jacketed $CO_2$ incubator (Thermo Forma, Model No. 311 was used for the culture. The cultured cells were observed using and inverted microscope (Model No.3/1/ Olympus Ck2 ). Surgical spirit was used constantly as the disinfectant.

### 2.1.3 - Flow Cytometric analysis of variant erythrocytes

Flow cytometric analysis of variant erythrocytes from control and exposed samples was performed on a Beckton Dikenson (Facscalibur) flowcytometer. The results were analysed useing "cell quest" software. The blood samples from the donors were collected heparinised (Heparin, Gland pharma ltd, India, HEP25). Further to blood typing, the samples from MN heterozygous individuals were processed using Erythrocyte fixing solutions and the final dual staining of the erythrocytes was done by Anti M – PE conjugate (6A7-PE-9412PE) and Anti-N – FITC conjugate (BRIC 157-FITC, 9401F) obtained from International Blood Group Reference Laboratory, Bristol, UK. The results obtained from the flowcytometry were analyzed using the MS office Excel software.

### 2.1.4 - RS-1 Assay

RS-1 assay was done in heamagglutination plates already mentioned and the typing sera (Anti - M & Anti - N) as used for the blood group typing. Cold normal saline was used for the washing of erythrocytes and the agglutination patterns recorded. The analysis was done using the RS1 ratio formula and the results tabulated.

**THE RS-1 RATIO FORMULA**

$$\frac{n}{m} = x$$

**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**

### 2.1.5. RS-1 in Cancer samples

The peripheral blood samples were collected from 50 cancer patients exposed to various doses of radiotherapy and were processed in the same manner as that for the RS-1 Assay (given in the previous section 2.1.4) The final results were analyzed using the software – SPSS for the graphical representation of the over-all results.

### 2.1.6 T-Cell Receptor Mutation Assay

The peripheral blood samples were collected from 50 cancer patients exposed to various doses of radiotherapy and were processed in the same manner as that for the RS-1 Assay (given in the previous section 2.1.4) The density gradiant separation method was adopted to yield mononucleate cells (lymphocytes). Phosphate Buffered Saline was used for washing the lymphocytes. Dual staining was achieved by Anti CD3-Phycoerythrin, Anti CD4-FITC dual conjugates. 12% formaldehyde was used for fixing the cells. The doubly stained lymphocytes were analyzed as given in section 2.1.4.

## 2.2 - Methods

### 2.2.1. - MN Blood group typing

Blood samples were collected from 413 South Indian volunteers who were chosen randomly for the MN blood groupings. Blood typing was done using commercial anti-M and anti-N antisera from Ortho Clinical Diagnostics, USA using standard agglutination methods on glass slides. The results were recorded and the gene frequencies were calculated using gene counting method.

### 2.2.2. - Monoclonal Antibodies against the Human Glycophorin-A

Venous blood was collected from persons homozygous for M and the N blood groups. The same was washed thrice with large volumes of cold saline and the erythrocytes were collected after careful removal of the buffy coat. These erythrocytes were pooled and a final suspension of cells prepared in saline under aseptic conditions, which were used for immunization. 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.3 intra-peritoneal injections of washed human erythrocytes in 1 ml saline were administered at two-week intervals. After three such antigenic administrations, the immunized animals were checked for antibody response and the magnitude of the same. The animals were hyperimmunised and four days further to the last intra-peritoneal antigenic administration, the animals were checked for the response and the magnitude of the antibody generation.

The following two routes of blood collection were employed to obtain small samples of blood from the hyper-immunized animals. **i. Retro-orbital plexus:** In this method blood samples of 0.3 ml volumes were collected and, **ii. Tail vein:** It was possible to obtain samples up to 1 ml volumes in this method. The blood samples were collected in glass test tubes and were allowed to clot at room temperature for about four hours. The test tubes were centrifuged resulting in clear serum separation. The serum supernatant was carefully collected taking care to avoid erythrocyte

contamination. Further to the separation of the serum, the tubes with the blood clots were refrigerated for overnight. This results in the further contraction of the clot and is possible to obtain maximal serum retrieval from the collected blood samples. The sera samples from the hyperimmunised animals were tested for the antibody response by looking for the anti-erythrocyte activity. A small quantity of the sera was subjected individually to a suspension of erythrocytes of M, N and the MN types on glass slides. The antibody response was ascertained by the agglutinating phenomenon of the erythrocytes.

The magnitude of response was judged by the extent of agglutination and also from the time taken for a clear reaction. 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. Spleen was aseptically removed from a healthy unimmunised Balb/c mouse and the spleenocytes were collected as single cell suspension in HAT medium. The cells were distributed into the 96 well micro culture plates as 100 micro liters per well. The micro culture plates with the feeder cells were incubated at $37^0$C with 6% $CO_2$ in a humid atmosphere. The feeder cells immediately on plating appeared spherical and uniform in size. As the days progressed, at about the fifth day, they showed a spindle shape and were well

attached to the culture-plate substratum. On the seventh and eight day, the medium turned slightly yellow with a well spread spindle shaped cells fully covering the culture area.

The hyperimmunised animal with the highest titer for anti erythrocytic activity was selected for further booster doses and upon administering three booster doses on consecutive days, the animal was chosen as the splenic donor. The chosen animal was etherized and was pinned with the ventral side facing upwards on to a dissection board. The animal was applied with liberal quantities of surgical spirit and was positioned in such a way that the spleen was on the right side of the flank. An incision was made through the skin and the body wall so as to expose only the spleen. The exposed spleen was carefully pulled out and dissected with minimal extraneous tissue and was placed in a sterile petridish. The retrieved spleen from the hyperimmunised mouse collected in a sterile petridish was soaked in about 5 ml of plain medium. The spleenocytes were separated by gentle grinding of the spleen using a syringe barrel and collected in a sterile centrifuge tube. 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. Further to the final washing and pooling of the fusion partners, the supernatant was aspirated out and the cell pellet obtained. The pellet was gently dislodged and the cells fused. Polyethylene Glycol was used as the fusogen. Immediately after subjecting the cells to Polyethylene Glycol, the cell mixture was diluted in large quantities of plain medium. The protocol followed for fusion is as given below:

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| 0.0 minute | 1 ml of Polyethylene Glycol was added |
| 2.0 minutes | 1 ml medium |
| 2-4 minutes | 2 ml medium |
| 4-6 minutes | 3 ml medium |
| 6-8 minutes | 10 ml medium |
| 8-10 minutes | 10 ml medium |
| 10-12 minutes | 10 ml medium |

Further to the dilution as above, the mixture was centrifuged and the cell pellet obtained. The entire pellet was suspended in about 25 ml medium with 10% serum. The suspended cells further to fusion were plated into 5 micro culture plates previously layered with the feeder cells. About 5 ml of the cell suspension was taken into a pipette and was added drop wise into all the wells having the feeder cells. The culture supernatants from wells with the clones were used for screening. 100 micro liters of the culture supernatants were collected from each of these wells. The supernatants thus obtained were stored at $4^0$C till further screening, which was done within four days of collection of the supernatants. The supernatants were subjected to a series of tests to ascertain the nature of the clones. Initially, the supernatants were tested for finding out if the clones were positive (secretory) or negative (non-secretory). This was achieved by subjecting the supernatants to Ouchterlony Double Diffusion studies with Anti-Mouse Whole serum and Anti-Mouse IgG. This not only enabled us to know about the usefulness of the clones, but also a fair indication of

the secretory nature of the clones was ascertained. The supernatants from the positive clones were subjected to further screening to check for the true monoclonality of the antibodies and their specificity to specific epitopes of interest. Subjecting washed erythrocytes of different blood groups to the culture supernatants and the reactions obtained as agglutination were observed and recorded. Expansion of the positive clones with the desired secretory properties was done. Transferring the cells from developing clones to 24 well culture plates did the same. Some clones with high proliferative properties were directly transferred to T-25 culture flasks and their progress monitored.

### 2.2.3 - Flow Cytometric analysis of variant erythrocytes

Blood samples were collected heparinised from 20 control and 50 cancer patients undergoing radiotherapy. The samples were obtained from MN heterozygous individuals after prior informed concent: 0.1 ml of blood was processed for flowcytometric analysis. The samples were coded and erythrocytes were converted into spherocytes and formalin fixed.

Formalin-fixed spherical erythrocytes were prepared by adding 0.1 ml of whole blood to 1.0ml containing 50µg/ml sodium dodecyl sulfate (SDS) and 1 mg/ml bovine serum albumin (BSA). After a 1 minute incubation, the sphered cell mixture was added to a fixative solution containing 9.7ml of Saline, 0.3ml of formalin (37% formaldehyde), and 10µg/ml SDS. After 1.5h, an additional 0.8ml of formalin was added to the mixture, and the suspension was allowed to fix overnight. All fixation steps were performed at room temperature. The fluid cells were washed twice with staining buffer, and resuspended and stored in 1 ml of sustaining buffer, giving a

final cell concentration of about 5 x $10^8$ cells ml. Stating buffer (SB) contains the following containing; 10 mM sodium phosphate (pH 7.2), 0.15 M NaCl. 5mg/ml BSA, 0.01% Nonidet P-40, and 100µg ml Na $N_3$. Fixed cells were stored refrigerated for up to two weeks with minimal effects on antibody binding.

The fixed cells were doubly stained using the antibody conjugates as mentioned in section 2.1.3. 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). A rectangular gate in the forward scatter vs log side scatter distribution was used to limit the analysis to erythrocyte singlets.

Cells were analyzed at a rate of 3,000 – 4,000 cells s, with 40,000 events collected in each FL1 vs. FL2 bivariate distribution. VFs were calculated as the number of events in the variant cell windows divided by the total number of scatter-gated events in the flow distribution. A rectangular window was gated for the scored cells and final events were recorded as histograms and dot-plots. The % gated events and differentially stained cells were obtained using the cell-quest software. Final analysis of the results obtained was done using Microsoft Excel software for graphical representations.

### 2.2.4 - RS-1 Assay

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 2, were prepared. 96 well Heamagglutination 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.

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| Antiserum(µl) | 50 | 40 | 30 | 20 | 10 | 9 | 8 | 7 | 6 |
| Saline (µl) | 50 | 60 | 70 | 80 | 90 | 91 | 92 | 93 | 94 |

Table 2: Differential dilutions of the antisera as used for the RS-1 Assay

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.

### 2.2.5 T-Cell Receptor Mutation Assay

Heparinised blood samples were collected from Radiotherapy exposed donors as mentioned in the section 2.2.3. Three ml of the blood samples were subjected to density gradient separation of lymphocytes by established procedures. The mononuclear layer was aspirated and the cells were washed twice in PBS. Dual antibody conjugate (20 microlitres) was administered to each of the samples and incubated for 30 minutes in dark. The samples were then suspended in 0.5 ml 12% formaldehyde in PBS. Such dually stained lymphocytes were analyzed by flowcytometry as described in the section 2.2.3.