# MATERIALS AND METHODS

The Out Patients Department (O.P.D.) of the Gynaecology and Obstetrics of North Bengal Medical College and Hospital was attainded in a routine way to contact the pregnant women of different categories. Main intension was to study the HLA antigens (human leucocytes antigen) frequency and to asses the immune status of different unsuccessful groups as well as the normal patients. But as the number of patients in each unsuccessful group, as mentioned below, were not sufficient even after attending the O.P.D. for more than 3 years, all the three groups of the unsuccessful pregnant women have been amulgamated into one group as unsuccessful pregnancy in broder sense. Though the unsuccessful group is not that uncommon in the North Bengal region, but due to the reluctancy among the patients to give blood from time to time and to come for the followup treatment, in a number of cases the repeated experiments could not be done and therefore have been excluded from the data presented in the thesis.

## Patients :

The four groups of the patients including the normal category were identified by considering the following criteria :

The unsuccessful groups were classified into three categories : Spontaneous or repeated abortions, Pregnancy induced hypertension (Pre-eclampsia) and women with congenital fetal anomalies. Pregnant women without any uncommon complications were considered as normal.

Aborters group :

3 miscarriages spontaneously, normal karyotypes of both parents (data have not been presented in this thesis), normal clinical investigations, (morphological and functional) and absence of hypertension, diabetes mellitus, infections with toxoplasma, hysteria etc.

Pre-eclamptic group :

Systolic blood pressure is more than 150 mm Hg and diastolic blood pressure is more than 100 mm Hg, a rise in systolic blood pressure of 30 mm Hg or more and a rise in diastolic blood pressure of 15 mm Hg or more compared with a booking blood pressure recorded before 20 weeks gestation, persistent proteinuria ($2^+$ on more on albustix) appearing in association with the rise in blood pressure.

Normal Pregnancy :

Singleton, normotensive ( <85 mm Hg, diastolic), absence of detectable anti-Rhesus antibodies in the maternal circulation, absence of vaginal bleeding, absence of clinically significant maternal glycosuria or proteinuria, absence of maternal anaemia (defined as HB <10.5 g/100 ml on two or more occasions during the third trimester or below 10 g/100 ml at any time), and absence of maternal history of heart disease, diabetes or any other chronic disease except mild atopic disorders.

The study was performed on 337 patients including the normal group. 282 patients were normal and 55 patients were from 3 unsuccessful groups. Though it was desired to study 25 patients each from all groups of unsuccessful patients, but as mentioned earlier that in most of the cases follow up study could not be carried out due to several problems and hence excluded them from the present study. The study includes both primigravidae and multigravidae. All groups of patients, considered in this study, were came from the same geographical area.

Collection of Blood :

Approximately 20 ml of blood samples were collected from each patients with the help of disposable syringes from

time to time till delivery by veinpuncture. Placental blood samples were collected at the time of delivery. Collected blood was allowed to stand at room temperature for an hour, After clotting of the blood, the serum was collected by centrifugation at 2000 RPM for 10 minutes, aliquoted and preserved at -20°C freezer for complement dependent anti-HLA screening. 2% sodium azide was added as preservative in each case.

Source of complement :

Rabbit complement for HLA-A, -B, -C and -DR typing was purchased from one Lambda (One Lambda Inc. Canago Park, U.S.A.). Rabbit complement was also prepared in our laboratory from time to time by collecting blood from at least 20 rabbits at a time from ear vein. Sera were collected from each tube after centrifugation and pooled together. After titration, non toxic complement was aliquoted and kept at -20°C freezer until use.

Separation of lymphocytes :

Blood samples were collected with heparine (25 U/ml blood) and then diluted with phosphate buffered saline (PBS) in a sterile clean tube. Diluted blood samples were then layered

on to Ficoll-Hypaque (Pharmacia, Uppasala, Sweden) carefully with the help of a pasteur pipette and then centrifuged at 2000 RPM for 20 mins. at room temperature (22 to 24°C). The white foggy layers of the mononuclear cells were aspirated from the interface with a clean pasteur pipette in clean centrifuge tubes. Cells were then washed with PBS for 10 minutes at 1000 RPM for 2-3 times and then resuspended in the medium RPMI 1640 with 25 mM - Hepes and L-glutamine supplimented with 10% heat inactivated goat serum (Choudhuri and Chakravarty, 1983).

Nylon wool column was used for separating the T and B cells from the cell suspension. Columns were prepared by using locally available straws. The straw was cut to approximately 14 cm in length and heat sealed at one end at an angle of 45°C. Approximately 0.1 gm teased nylon wool, (Robbins Scientific Corporation, U.S.A.) soaked in RPMI-1640, was packed gently up to a height of 5-6 cm. The column was filled in with the medium and kept at 37°C incubator for some time. Immediately before use the straw, the sealed tip at the bottom was cut and the nylon wool column was washed with 37°C medium 1640 supplimented with 10% goat serum. Cell suspension was added to the top of the column and the cells were allowed to move all the way into the nylon wool. The column was kept at 37°C for 45 minutes. Non-adherent cells or the T cells were collected by washing the column with 10-15 ml of prewarmed (37°C) medium and the B cells were collected by washing the column with chilled (4°C) medium as well as by gentle physical agitation.

Separation of $CD4^+$ and $CD8^+$ T cells from peripheral blood lymphocytes with the help of Dynabeads :

Peripheral mononuclear cells were isolated by density gradient centrifugation as mentioned earlier. The cells were washed twice at $4^oC$ in RPMI 1640 with 25 mM-Hepes and L-glutamine supplimented with 5% heat-inactivated fetal calf serum. Cells were resuspended in the medium and adjusted the number at $2 \times 10^6$ cells/ml.

0.5 ml cell suspension was mixed with 0.02 ml Dynabeads (R) M-450 CD4 (number of Dynabeads $3 \times 10^6$) and incubated for 60 minutes at $4^oC$ on an apparatus that provides both gentle tilting and rotation in case of separating the $CD4^+$ cells. In case of separating the $CD8^+$ cells, Dynabeads TM M-450 CD8 was used and incubated for 10 to 15 minutes in the same way. Rosetted cells (both $CD4^+$ and $CD8^+$) were isolated by placing the tubes in the Dynal$^R$ MPC-E-1 for 2 to 3 minutes. Cells were resuspended in the medium and counted with the help of haemocytometer under the microscope.

HLA typing and detection of HLA - alloantibodies

60 well HLA Terasaki trays (NUNC, Denmark) were used for HLA typing. 1 µl antisera of different HLA specificities

were poured in the trays and prior to that wells were filled in with light liquid paraffin oil to avoid the evaporation of such a little quantity of antiserum. 1 µl cell suspension from 2 x $10^6$ cells/ml was added to each well and incubated at 22°C for 30 minutes in case of HLA-A, -B, -C, and 60 minutes in case of HLA-DR typing. At the end of the incubation period for respective hours, 5 µl rabbit complement was added to each well and again incubated. The incubation period was 60 and 120 minutes in case of HLA-A, -B, -C and -DR typing respectively. 5 µl of 5% water soluble yellow shade eosin (Qualigens Fine Chemicals, India) was used for 5 minutes for staining the dead cells in each well. 5 µl of 40% formalin (S.d. fine-chem Ltd., India) of pH - 7.0 was added at the end to fix the cells. A cover glass (50 mm x 76 mm) was layered on the wells. Trays were read under the inverted phase contrast microscope (Leitz, Labovert FS) with 10x objective and 10x eye piece. Results were recorded as follows :

| Code | Increase in cell death over the negative control % | Interpretation |
|---|---|---|
| 1 | 0 - 19% Kill | Negative (-) |
| 2 | 20 - 29% Kill | Weak positive (+) |
| 4 | 30 - 39% Kill | Positive (+) |
| 6 | 50 - 79% Kill | Strong positive (+++) |
| 8 | 80 -100% Kill | Very strong positive (++++) |
| 0 | (Not readable) | Invalid. |

The detection of HLA alloantibodies from pregnant women were performed by using the above said technique. 1 µl of sera obtained from parous women were poured instead of known anti-HLA sera as above. Microlymphocytotoxicity (Terasaki and McClelland, 1964) assay were performed against cells of known HLA specificities, taken from a well known unrelated donar panel.

Quantitative estimation of serum proteins (IgM & IgG) by Ammonium Sulphate Precipitation technique :

Serum proteins were isolated from the serum by precipitating with the help of saturated ammonium sulfate (Kendall, 1937) and for purification sephadex G-200, with the help of column chromatography (2 x 45 cm), was used which has the fractionation range of 5,000 to 8,00,000 daltons for globular proteins (Flodin and Killander, 1962). The concentration of IgM and IgG were measured in an UV spectrophotometer (SHIMADZY UV-Visible Recording spectrophotometer, UV-160) at 280 nm wave length. The largest globulin, the IgM (MW = 900,000), was appeared in the effluent as the first component. If the elution is continued with physiologic saline, the other smaller glabulins (IgG etc.) will also leave the column, and finally the albumin, which has the smallest molecular weight among the major protein components of the serum will appear.

Quantitative estimation of serum proteins (IgM and IgG) by immunodiffusion technique :

Quantitation (mg/dl) of IgM and IgG were also accomplished by means of single radial immunodiffusion technique (Mancini, Carbonara and Hermans 1965) which utilized 5 µl undiluted sera, immuno-plates (Hc-Partigen plate for IgG HOECHEST and Tri pantigen plate for IgM- HOECHST) and pure reference serum of each immunoglobulin. Ring diameters of precipitation (D) were allowed to developed for 80 hours in case of IgM and 50 hours in case of IgG in a moist atmosphere at room temperature and then read on a millimeter scale through magnifying viewer against on obliquely illuminated background.

Mitogen :

Phytohaemagglutinin-P (PHA-P) was obtained from SIGMA (Sigma Chemical Company, U.S.A.) and the total amount of a vial (5 mg) was dissolved in 5 ml sterilized distilled water. The stock solution of PHA-P was stored in -20°C freezer. 5 µg dose was used in 2 x $10^6$ cells/ml suspension for activating the lymphocytes.

Cell viability test :

The percentage of viable cells was counted by haemocytometer in presence of trypan blue (Sigma Chemical Company, U.S.A.). Viability of the cells in *in vitro* culture was determined at different hours of culture like 24, 48 and 72 hours and the percentage of viable cells at different hours was calculated by considering the number of viable cells at the begining as hundred percent.

*In vitro* cell culture :

Cell numbers were adjusted at $2 \times 10^6$ cells/ml and resuspended in Minimum Essential Medium (Hi-Media, Bombay) supplimented with penicillin - streptomycin (50 U/ml) and nystatin (50 U/ml) as an antifungal agent for *in vitro* culture. 5% autologous serum was used instead of fetal calf serum for our purpose. 5% AB serum was used instead of autologous serum in case of control. Culture tubes were incubated in humidified atmosphere of 7.5% $CO_2$ in air at $37^oC$ for different hours like 24, 48 and 72.

Measure of blastogenesis :

After binding with the mitogen or antigen, metabolic

activities of small lymphocytes get augmented and they gradually transform into bigger cells, known as blast cells. Thus blastogenesis is considered as one of the indicator of activation of lymphocytes and the percentage of blast cells in a lymphocyte population stimulated with mitogen or antigen can be a measure of the degree of activation of lymphocytes. Blast cells were counted by haemocytometer in presence of trypan blue under the microscope fitted with an occulometer. Cells with diameters greater than approximately 7 µm were scored as medium sized, and cells with diameter greater than 10-11 µm were scored as large. The proportion of transformed or "blast" cells is determined from the sum of viable medium plus large lymphocytes divided by the total viable lymphocytes counted (Choudhuri and Chakravarty, 1983). Blast cells were counted at the end of 24 hours, 48 hours and 72 hours culture.

Statistical analysis :

The results were analyzed in WIPRO S-386 mini computer using programme developed in Fortran Language. The phenotype frequency, gene frequency, haplotype frequency, Coefficient of linkage disequilibrium (delta value) were calculated according to Mattiuz *et al* (1970).