CHAPTER-IV : MATERIAL AND METHODS

MATERIAL AND METHODS

The cytogenetic studies were carried out in individuals with :

1. Clinical suspicion of chromosomal aberrations
2. Mental retardation (moderate to severe degree) of unexplained etiology
3. Multiple congenital defects
4. Abnormal sex phenotypes
5. Primary amenorrhoea
6. Spontaneous abortions
7. Sterility
8. Haematological malignancies.

Karyotyping was done in Cytogenetic Laboratory, Department of Anatomy, Medical College, Baroda. The cases included in the study were referred from the different clinical departments of S.S.G.Hospital, private hospitals of the city and its vicinity. Parents and siblings were investigated wherever indication arose. A separate proforma was maintained for each patient where the detailed clinical and family history was recorded.

The patients with suspected haematological malignancy were subjected to a battery of relevant investigations including complete haemogram, relevant biochemical examination, bone marrow aspiration biopsy, bone marrow trephine biopsy, lymph node biopsy to arrive at a final conclusive diagnosis of the type of haematological malignancy. All the known patients of either acute or chronic leukemia, Hodgkin's or non-Hodgkin's lymphoma and multiple myeloma were directly taken for further cytogenetic study.

Peripheral blood is very easy to obtain, small volume is required, high mitotic yield can be obtained from short term whole blood cultures and high number of exact count, quality metaphase spread can be obtained permitting a critical analysis of chromosome morphology and therefore, forms the most common source for analysis of human chromosomes.

A suitable method for karyotype study must fulfil the demands :

1. adequate scattering of chromosomes
2. no overlapping
3. minimum distortion

Methods used for mental retardation and Down's syndrome

1. Oster's diagnostic criteria (Oster, 1953) for clinical diagnosis of Down's syndrome.
2. Mental age and I.Q. were estimated by clinical psychologist, using Kamat's test for intelligence (Indian Standardized Version of Stanford Binet Intelligence Scale) and Sequin form Board Test. Clinical assessment of mental age based on monograph of motor and mental development of Indian babies from 1 to 30 months by Dr.(Smt.) P.Patak (1961).
3. Dermatoglyphic printes were obtained, wherever necessary, on drawing paper using Indian ink and a large ink pad.

The standard procedure of Hungerford (1965) was followed with slight modifications. The advantages of this technique are :

a) An easily obtainable sample that can be serially analysed (peripheral blood),
b) Predictable and high rate of mitosis obtained within 72 hours of culture,
c) Excellent photographic analysis made possible through the selection of optimal metaphase spreads.

Procedure

In brief

Five ml.of blood was collected by vein puncture in a sterile disposable plastic syringe and about 2 ml blood was transferred to the glass culture bottles containing 10 ml of media T.C. 199 immediately to avoid clotting. The rest of the blood was transferred to autoclaved sterile centrifuge tube to get the serum. The media T.C. 199 was pre-supplemented with 0.1 ml of PHA (Difco, U.S.A.) and 2 drops (by 18 number needle) of heparin were added in each culture vial. Two drops of antibiotics Streptomycin and Penicillin were added to prevent bacterial contamination. About 1.5 to 2 ml autologous serum was added to the media i.e. (about 15 to 20%). Sodium bicarbonate to adjust the pH 7.2-7.4, was added as and when required. The cultures were incubated at 37°C for 72 hours and were gently mixed twice in 24 hours to avoid clumping and to stabilise the pH of the medium. Colchicine 0.1 to 0.15 ml (2.3 ug/ml) was added to each culture at 69th hour and the cultures were harvested after 1 and 1½ hours. Centrifuge the culture for 10 minutes at 800-1000 rpm. Remove supernatant. Add 10 ml of hypotonic prewarmed potassium chloride (1.120 gm in 200 ml of distilled water), pH 7.0 and allowed to incubate for 20 minutes. Centrifuge and discard the supernatant. The cells were fixed in chilled fixative, 3:1 methanol : acetic acid, cells were kept in fixative for ½ hour. Then after, removed the fixative by centrifuging. At least three changes of fixative were given. Finally the cells were resuspended in a small volume of fixative depending upon the cell pellet.

Slides were prepared by dropping cell suspension on pre-cleaned chilled slides and dried on a slide warmer at 50-55°C for 1-2 minutes. Cell suspensions were preserved in fixative at 4°C to prepare the slides as and when required for banding techniques.

Bone Marrow

Method of karyotyping

Chromosomal changes in haematological malignancy are best reflected in the karyotypic picture of the bone marrow cells. Furthermore, direct cytogenetic examination of such cells without resort to a long term

culture reveals in-vivo status more accurately than do the long term leukocytic culture. Additional disadvantage of doing leukocytic culture from peripheral blood is the tendency of the normal cell to overgrow the abnormal ones (Sandberg and Abe, 1980) and the limitation of leukocyte culture with phytohaemagglutinin in stimulating only T-lymphocytes. This restriction makes leukocyte culture worthless in all the conditions which primarily affect granulocytic series as in chronic myeloid leukemia or red cell series or even B-lymphocytes.

Considering all these advantages and limitations, the short term bone marrow culture was mostly used during the present study.

Collection of material

In all the patients bone marrow aspiration was done for the cytogenetic analysis. It was done with Klima's Bone Marrow Aspiration Biopsy Needle from the posterior superior iliac spine. About 0.75 to 1.0 ml of marrow with bone marrow particles was collected in the media kept ready for the cytogenetic analysis. Additional marrow aspirate was also used for the haematological analysis of marrow.

Collecting media

For the direct processing of the bone marrow immediately after aspiration several investigators have recommended the use of nutrient media (Bottura and Ferrai, 1960; Kiossoglou, 1964) like T.C.199 (Difco, U.S.A.) or Ham's hapten media with colchicine treatment. The possible alternative is a balanced salt solution. Initially, a parallel study with aspirate in both nutrient media T.C. 199 and Ringer's lactate were tried. Results with both the methods were closely comparable and hence in the further analysis, Ringer's lactate was taken as collecting medium.

Throughout the study colchicine was used as an agent for blocking cell division at the metaphase.

The composition of collecting medium was as under :

Lactated Ringer's solution	10.0 ml
Colchicine solution	0.1 ug/ml
Heparin solution	500 I Units

Incubation

Owing to the great cellularity of marrow and the inherent high mitotic activity, the cells are incubated only for colchicine treatment. The use of mitotic inducers in this case is superfluous and sterile techniques, so important in leucocyte culture, totally unnecessary. The medium containing aspirate was incubated exactly for 120 minutes at 37°C with colchicine concentration of 0.1 ug/ml.

Processing of material

After incubation further processing is done as per following four main steps :

1. Collection of cells under cultured conditions
2. Swelling of cells by hypotonic solution
3. Fixation of the swelled cells
4. Spreading of the chromosomes on slides.

The exact process followed during the present study is similar as described for peripheral blood culture.

Slide preparation is one of the most important and critical step in obtaining quality of chromosome spreads. Assuming that the cells are subjected to an appropriate length of hypotonic treatment before fixing, ambient temperature and relative humidity play a significant role in chromosome spreading. The following tips can help prepare the slides in variety of conditions.

1. Cytoplasm surrounding the chromosomes can be due to insufficient hypotonic treatment. This can be dealt with longer incubation in hypotonic treatment in the remaining samples; if backup culture exists and in future samples. In addition, a few more changes in fixative may improve the spreading to a certain extent. Conversely, if many of the metaphases are incomplete, reduce the exposure of cells to hypotonic solution.

2. In a cold climate, indoor heating leads to very low humidity. The dry ambient weather causes the slides to dry quickly causing overspreading and many incomplete metaphases. This can be compensated by one or more of the following :

i) The slides may be chilled in a refrigerator before use,
ii) Keep the humidifier in the vicinity of the slide preparation area,
iii) After dropping the cell suspension on the slide, gently blow humid air orally.

3. The reverse is usually the problem during hot weather, especially when it is raining. The relative humidity may reach an extent that the slides take extremely long to dry, leading to inadequate spreading of metaphases, persistence of cytoplasmic debris and the appearance of chromosome as refractile bodies when observed using phase contrast microscopy. These problems can be overcome by lowering the ambient temperature (if cooling is efficient) in relation to that of the slides or by using slides kept on slightly warmer (not too hot) water, or by placing the slide on a slide warmer immediately after the cell suspension is dropped on the slides. One may even try to use clean dry slides to drop the cell suspension and reduce the drying time.

Finally, if the cell suspension is too important and insufficient for enough trials, it may be wise to leave the suspension in the refrigerator (2° to 5°C) overnight and try the next morning. If so, the fixative should be changed at least once before attempting to make a slide preparation.

Successful cultures were obtained in 95% cases. Failure of cultures were attributed to very poor response of PHA. Occasionally, due to contamination or other unknown causes.

The heat dried slides were stained by plain Giemsa stain. 2 to 4% Giemsa in phosphate buffer, pH 6.8 for 15 minutes and then rinsed in distilled water.

The air dried slides were subjected to following staining techniques :

a) Giemsa staining
b) Giemsa trypsin Giemsa banding
c) Centromeric banding [using $Ba(OH)_2$]
d) Qunacrine fluorescence banding using qunacrine dihydrochloride
e) NOR staining.

The details of all the above mentioned various banding techniques are as follows :

a) **Giemsa staining** : as mentioned for heat dried slides.

b) **Giemsa trypsin Giemsa banding** : The technique of Seabright was followed with slight modifications as described below :

i) Heat the one to three day old slide for overnight (17-24 hours)
ii) Incubate the slides in phosphate buffer (pH 6.8) pre-warmed at 60°C for 15-20 minutes.
iii) Immerse the slides in trypsin-EDTA solution for 5-15 seconds at room temperature.
iv) Rinse the slides in chilled normal saline water at least three changes.
v) Stain the slides in 4-6% Giemsa in phosphate buffer, pH 6.8, for 5-10 minutes.
vi) Wash in distilled water and dry at room temperature.

The banding was consistent.

c) **Centromeric banding** (Summer, 1972) :

i) Treat 4-5 days old slides with 0.2 N HCl at room temperature for 30 minutes.
ii) Wash thoroughly in distilled water and keep the slides in 5% $Ba(OH)_2$ at 56°C for 15 minutes.
iii) Wash thoroughly in distilled water to remove all the precipitates of barium hydroxide from slides.
iv) Dip the slides in 0.02 N HCl if precipitates persist
v) Wash thoroughly with distilled water
vi) Keep the slides in 2 X SSC solution for 30 minutes at 56°C

vii) Rinse slides in distilled water
Stain in 2-4% Giemsa prepared in phosphate buffer, pH 6.8 for 10-20 minutes.

viii) Rinse the slides in distilled water, dry and mount.

d) **Q-banding** (Pearson, 1972)

i) Dip the slides in McIlvaine buffer, pH 5.6 for 10 minutes
ii) Stain the slides with 0.5% Quinacrine dihydrochloride for 20 minutes.
iii) Wash thoroughly with distilled water
iv) Again dip in McIlvaine buffer, pH 5.6 for 5 minutes.
v) Mount in buffer glycerol (1:1) and seal with sealing wax.

e) **NOR banding** (Bloom and Goodpasture, 1976) : The technique of Bloom and Goodpasture (1976) was followed with slight modifications as described below :

i) Place 4 drops of filtered 50% $AgNO_3$ solution and 2 drops of 1% gelatin on 4-5 days old slides.
ii) Cover the slide with cover-glass and keep it on warmer at 60°C till the colour changes to golden brown.
iii) Wash with distilled water, dry and observe under microscope.

f) **Late replication of X-chromosome by Brdu incorporation**

i) Plant whole blood cultures according to the method of Hungerford (1965), with slight modifications.
ii) Add 5-bromodeoxyuridine at the final concentration of 60 ug/ml 6 hours before harvesting.
iii) Keep the cultures in dark and harvest by routine procedure.
iv) Stain the prepared slides with Hoechst-10 ug/ml and exposed to ultraviolet light for 3-4 hours.
v) Wash it with water and dip in 2 X SSC solution for 30 minutes at 60°C.
vi) Wash with water and stain with Giemsa for 10 minutes, blot, dry and observe under the microscope.

In each case, at least a minimum of 30 metaphase plates (WHO Standard) were screened and at least 5 plates were photographed and karyotyped as per standard nomenclature for chromosome identification. G-banding was routinely done in each case while Q, C and NOR bandings were done, whenever necessary. Cultures were repeated in case of doubtful diagnosis and when mosaicism was suspected and at least 100 metaphase plates were analysed.

The slides were screened under Reichert-Diapan Binocular Research Microscope and photographs were taken with attached photomicrographic equipment. Some of the photographs were taken as Nikon-Labophot Microscope. Metaphases were photographed under oil immersion using ORWO 35 m/Fortepan 35 mm document film with the help of green, blue and ground filter. The film was developed in IPC-76/ Fien grain developer for 15-20 minutes at 18-20°C. Photographs were printed on hard glossy paper using IPC-163 developer. ASA 125 to ASA 400 film was used to take the fluorescence photographs and developed with the same developer and printed on hard glossy paper with IPC-163 developer.

Printed metaphases were cut and arranged in pairs as per the standard nomenclature. The reports were given with 15-20 days after collecting the blood. Reports were given on the report forms to the patient.

Sex Chromatin Staining

Thionine staining for chromatin

1. Smear the buccal mucosal cells on a clean slide, semi-dry and fix in 95% alcohol for 4 hours.
2. Dip the slides in 5N HCl for 20 minutes at room temperature and wash with distilled water.
3. Stain the smears with thionine working solution for 10 minutes.
4. Finally wash the slides with distilled water, dry, mount and observe.

Nuclei which were folded, pyknotic or dappled with excess stained chromatin were not included in the examination for scanning the chromatin. A cell, with distinct nuclear membrane and the Barr Body

(about 1 microne in diameter) was situated at the periphery of the nuclear membrane to which it was closely attached and appeared as a darkly stained dot either convex, oval, circular, triangular or even sometimes rod-like in structure was considered chromatin positive. Minimum of 100 nuclei were counted and the percentage of X-chromatin positive cells was calculated.

List of Chemicals and Equipments Used

All the chemicals used in these studies were of analytical grade. The details of the chemicals and equipments and their manufacturer/suppliers are listed below :

01.	Tissue Culture Media :	
	T.C. 199	Hi-Media, Bios, Bombay
	RPMI-1640	Hi-Media, Difco, U.S.A.
	Ham F-10	Difco, U.S.A.
02.	Fetal Calf Serum	Difco, U.S.A.
03.	Antibiotics (Streptomycin and Penicillin, 5,00,000 units) liquid	Bios/Microlab, India
04.	Phytohaemagglutinin (PHA-M) (Lympholized)	Difco, U.S.A.
05.	Heparin H.P. 5,000 I.U. 5 ml.	Bios, India
06.	Colchicine	Bios, India
07.	Trypsin 1:250 lympholized	Difco, U.S.A.
08.	Giemsa stain	BDH, England Himedia, India
09.	Thionine stain	BDH, England
10.	Quinacrine dihydrochloride	Sigma Chemicals Co., U.S.A.
11.	Silver nitrate	BDH, India

12.	Barium hydroxide	BDH, India
13.	Millipore filter	Millipore Corporation, U.S.A.
14.	Photomicroscope	Reichert Diapan, Austria Nikon (Japan)
15.	Fluorescence microscope	Dialux, W.Germany.

Culture process was conducted in sterile conditions. The culture room is provided with laminar air flow system HIPA filter (Klenzeids, India) U.V. tube lights and glass hood to keep the place in an aseptic condition. The glass wares were cleaned by chromic acid, Laxbro / Labolene liquid soap, distilled water and were autoclaved at 15 lbs pressure for 20-30 minutes.

Preparation of Tissue Culture Materials

a) Media : The dehydrated media powder (T.C.199, RPMI-1640) (Ham F-10) was dissolved in 100 ml triple distilled water and was stored in deep freeze. One ml of media was taken from the stock media for each culture and to it antibiotics, Streptomycin and Penicillin (2-3 drops by 18 number needle) were added sometime. The volume of media was brought to 10 ml by adding 9 ml of triple distilled water. The whole media was stored at 4°C.

b) Phytohaemagglutinin : Each vial of Difco PHA was reconstituted by adding 5 ml of triple sterile distilled water with the help of sterile syringe. This can be stored in the freezer. 0.1 to 0.2 ml PHA was added (approximately 3 to 4 drops by 18 number needle) to 10 ml of culture medium with blood.

c) Colchicine (Bios, India) : Prepared solution of Bios, India 400 ug/ml of 5 ml vial was used. Two to three drops of this colchicine solution was added to the culture to arrest cell division.

d) Hypotonic solution : 1120 mg of KCl (0.075 M KCl) was dissolved in 200 ml of distilled water. The pH was adjusted to 7.0 by adding $NaHCO_3$. Freshly prepared KCl should be used. It has to be pre-warmed at 37°C before use for harvesting.

e) Fixative : Methanol and glacial acetic acid were used as fixative in the 3:1 ratio respectively. Freshly prepared pre-chill cooled fixative was used for harvesting every time.

f) Sorenson buffer : 5.112 gm Na_2HPO_4 was dissolved in 600 ml of distilled water and 3.264 gm of KH_2PO_4 in 400 ml of distilled water. Both the solutions were mixed with and pH was adjusted to 6.8.

g) Giemsa stain : One gram of Giemsa powder (BDH, England) was dissolved in 54 ml of glycerol at 60°C and mixed for at least 2-4 hours on a magnetic stirrer or manually. The stain was brought to the room temperature. 84 ml of methanol was added to it. This unfiltered stain was stored.

Working Giemsa solution was prepared by taking 2 ml of stain and diluting to 50 ml with Sorenson buffer. The slides were stained for 10 minutes.

h) Trypsin-EDTA solution for G-banding : 50 mg of Trypsin and 50 mg of EDTA were dissolved in 40 ml of PBS solution. This solution was properly mixed for 20 minutes.

i) Thionine stain

i) 1.0 gm of thionine was dissolved in 100 ml of 50% ethyl alcohol. The stain particles were mixed thoroughly till the stain gets completely dissolved in ethyl alcohol.

ii) **Michadis buffer**

Sodium barbiturate	14.7 gm
Sodium acetate	9.7 gm
Distilled water	500.0 ml

iii) **Thionine working solution** : To prepare thionine working solution, 28 ml of the buffer and 32 ml of 0.1 N HCl were brought to 100 ml with thionine stock solution so that the pH was maintained effectively at 5.7 ± 0.2.

j) McIlvaine buffer for quinacrine stain : 116 ml of 02 M Na_2HPO_4 was mixed with 84 ml of 01 M citric acid to get pH 5.6.

k) Q-banding - Fluorescence stain : 250 mg of quinacrine dihydrochloride was dissolved in 50 ml of McIlvaine buffer, pH 5.6. This can be stored at 4°C for 8-12 weeks.

l) Barium hydroxide solution : 5 gm of $Ba(OH)_2$ was taken and dissolved in 100 ml of distilled water, filtered through Whatman No.1 filter paper. This solution was kept at 60°C in water bath. The $Ba(OH)_2$ was prepared fresh as and when required.

m) 2 X SSC solution : 17.53 gm of sodium chloride and 8.52 gm of sodium citrate were dissolved in 1000 ml of distilled water.

n) 0.2 N HCl / 0.02 N HCl : 1.8 ml of concentrated HCl was diluted to 100 ml with distilled water to get 0.2 N HCl. This solution was further diluted 10 times to 0.02 N HCl.

o) Silver nitrate solution : Five gram of $AgNO_3$ was dissolved in 10 ml of double distilled water and filtered through Whatman No.1 filter paper and stored in bottle. $AgNO_3$ solution can be stored in the refrigerator at 4°C and can be used till the solution is clear.

p) 1% gelatin for NOR staining : One gram of gelatin was dissolved in 100 ml of distilled water at 37°C and was allowed to cool at room temperature. Few drops of formic acid were added. This reagent was stored at 4°C and remains stable for one month.

q) 5-Brdu for late replicating X-chromosomes : 5-Brdu was prepared by dissolving 1 mg of Brdu powder to 1 ml of distilled water. 0.400 ml was added to 10 ml of culture medium. This solution was stored at 4°C in dark.

r) Hoechst solution : Stock Hoechst solution prepared by adding 1 mg of powder to 10 ml of Sorenson buffer, pH 6.8. This solution was again diluted 10 times to get working solution. This solution can also be stored at 4°C in dark.