Materials and Methods
3. MATERIALS AND METHODS

Patients suffering from 'preleukemic conditions' namely Down syndrome (DS), Fanconi anemia (FA) and myelodysplastic syndrome (MDS) constituted the sample for the study.

3.1 MATERIAL

Blood (5 ml) or bone marrow (0.5 - 1 ml) was collected employing heparinized syringe under aseptic conditions. Samples collected from the hospitals were transferred to the laboratory for further processing and analysis. Patients referred to the following organizations provided the samples.

a. DS: 1. Department of Genetics - Out patient clinic, Institute of Obstetrics and Gynaecology, Egmore, Chennai-600 008,

2. Pathway - Centre for mentally retarded, Thiruvanmiyur, Chennai - 600 041, and

3. Department of Genetics, Dr.ALM.P.G. Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai - 600 113.

b. FA: Department of Haematology, Institute of Child Health, Egmore, Chennai - 600 008.
3.2 METHODOLOGY

3.2.1 Cytogenetic evaluation

3.2.1.1 Requirements

Culture medium

McCoy's 5A medium without folic acid (HI-MEDIA) was used for the culture of lymphocytes. 11.9g of the medium obtainable in the form of powder was dissolved in a litre of sterile distilled water. The pH was adjusted to 7.4 with sodium bicarbonate. The medium was filtered through seitz filter and was transferred to sterile bottles. 2 ml of streptopenicillin (1 g Dicrysticin-S, dissolved in 5 ml distilled water) was added to the medium and stored below 4°C.

AB serum

Non-citrated blood was collected from a donor with AB group and was stored in a refrigerator for 2-3 days. The serum was separated out and centrifuged at 2000 rpm for 20 min. The clear serum obtained was then subjected to heat inactivation at 56°C for 15 - 20 min. It was filtered using a seitz filter and stored in small aliquots at below 4°C.
Phytohaemagglutinin (PHA)

PHA, the mitogen was prepared as follows: 50 g of the seeds of red kidney bean, *Phaseolus vulgaris* were surface sterilized with 70% ethyl alcohol and soaked overnight in 250 ml of sterile Ringer solution. The seeds were then ground well for 15 min. and the paste thus obtained was centrifuged at 1000 rpm for 15-20 min. Of the four layers, the top two layers were transferred to centrifuge tubes (15 ml) and centrifuged at 2500 rpm for 15 min. To 100 ml of the clear solution, 15-20 mg of fungizone (Mycostatin) was added. This constituted the stock PHA solution and was stored at -20°C. The working solution was prepared by adding 1 ml of the stock solution to 9 ml of sterile distilled water.

Bromodeoxyuridine (BrdU)

1.25 mg of BrdU (Sigma) was dissolved in 5 ml of distilled water. 0.1 ml of this solution was added to each culture vial. The vials were kept completely covered with black paper to prevent exposure to light.

2-deoxy-D-glucose (2-DG)

2-DG (Sigma) was dissolved in sterile distilled water to obtain 2 mM solution. 2-DG was added to irradiated (3 Gy) lymphocyte cultures to study its modulatory effect on chromosomal damage.
Giems stain

1 g of Giems stain (E.Merck) obtained in powder form was dissolved in 54 ml of glycerol in a conical flask at 56°C for 2 h on a magnetic stirrer. The solution was then allowed to cool to the room temperature. To this, 84 ml of methyl alcohol was added and left on the magnetic stirrer for 1 hour. The staining solution was filtered using Whatman filter paper (No.1) and the filtrate which constituted the stock solution was stored at 4°C. The working solution was prepared by mixing 2 ml of the stock solution, 2 ml of the buffer (10% disodium hydrogen phosphate) and 46 ml of double distilled water (Marimuthu et al., 1974).

3.2.1.2 Irradiation

Cultures of lymphocytes from DS individuals were irradiated at G₀ stage, that is, just before the initiation of culture by the addition of PHA. They were exposed to either 1 Gy or 3 Gy of gamma rays employing Caessea Gammatron unit, a facility available at the Mechanical Engineering, Research and Development Organization, CSIR, Taramani, Chennai-600 113.

3.2.1.3 Culture of lymphocytes and harvest

Whole blood lymphocyte cultures were set up following the modified procedure of Hungerford (1965).

Of the 5 ml of blood collected from each patient, 0.5 ml was inoculated under aseptic conditions into a sterile culture vial containing 5 ml of the
medium, 1 ml of AB serum and 0.2 ml of PHA. Cultures were incubated at 37°C for 69 h. Carbon dioxide was released every day by loosening the cap of the vials. The cultures were shaken periodically.

The following procedure was adopted to harvest the cultures:

0.05 ml of colchicine (0.01%, Loba) was added to each culture vial at the end of the period of incubation, to arrest the dividing cells at metaphase, and the cultures were allowed to incubate further for a period of 40 min. The contents of the vial were transferred to a centrifuge tube and were centrifuged at 800 rpm for 5 min. The supernatant was discarded and the cell button obtained was gently tapped. 6 ml of prewarmed (37°C) KCl solution (0.075 M), which constituted the hypotonic solution, was added to the cell button, mixed and gently aspirated with a pasteur pipette. After 5 min. of hypotonic treatment at 37°C, the contents of the vial were centrifuged at 800 rpm for 5 min. The supernatant was discarded and the cell button was gently tapped. The cells were then fixed employing freshly prepared fixative (methyl alcohol : glacial acetic acid, 3:1) and were left at room temperature for 1 h. Cells were then subjected to at least three changes of the fixative, and stored at 4°C till further processing to obtain slides.

3.2.1.4 Direct chromosome preparations from bone marrow samples

10 ml of the medium was taken into a 15 ml centrifuge tube. To this, 5-10 drops of the bone marrow were added and the cells were suspended. The suspension was centrifuged at 800 rpm for 8 min. The cells were resuspended
in fresh medium and centrifuged at 800 rpm for 8 min. The supernatant was
discarded. The cells were exposed to 10 ml of fresh medium supplemented with
antibiotics and AB serum. Colchicine (0.05 ml of 0.01% solution) was added
and the cells were incubated at 37°C for 90 min. The contents were centrifuged
at 800 rpm for 8 min. and the supernatant was discarded. The pellet was
disturbed by thorough tapping and the cells were suspended in 5 ml of 0.075 M
KCl solution. The cells were exposed to this hypotonic treatment for 5 min. at
37°C, spun at 800 rpm for 5 min. and fixed in methanol-acetic acid fixative and
allowed to stand for 10 min. Fixation was repeated three times and cells were
finally suspended in a small volume of fixative and stored at 4°C till processed
for preparation of slides.

3.2.1.5 Slide preparation

Grease-free microslides, previously cleaned and chilled were
employed. A drop of the cell suspension of the cultured lymphocytes or bone
marrow cells was placed over a slide warmer maintained at 40°C. The test
slide was examined through the microscope to check the density of cell
suspension for the presence of enough number of metaphases as well as the
spread of chromosomes at metaphase and accordingly the density of the cells
in the suspension was modified.

3.2.1.6 Staining

Chromosomal preparations obtained from cultured lymphocytes
(untreated, irradiated and irradiated plus 2-DG treated) of DS patients and
from cultured lymphocytes of FA patients were aged for 4-5 days and then
stained in 4% buffered Giemsa solution for 5 min. The slides were rinsed in
distilled water and air dried.

3.2.1.7 GTG-banding

Application of GTG-banding technique enabled detection of exact
break points involved in the chromosomal aberrations. The procedure followed
to obtain G-bands was that described by Seabright (1971). Chromosomal
preparations obtained from cultures of irradiated lymphocytes from DS
individuals and from peripheral lymphocytes and/or bone marrow samples of
MDS patients were processed to obtain GTG-bands. The slides were treated
with 0.05% trypsin solution for 30 sec., rinsed well with distilled water and
stained employing 4% buffered Giemsa solution for 5 min. The slides were
washed in running tap water and air dried.

3.2.1.8 Differential staining of sister chromatids

The procedure followed to obtain differential staining of sister
chromatids in chromosomal preparations was a modified version of the
technique described by Goto et al. (1975). The frequency of sister chromatid
exchanges was studied in cultured lymphocytes from DS and FA patients.
5-bromodeoxyuridine (5 μg/ml of the medium) was added to the culture vial 24
h after the initiation of culture and these cultures were harvested as usual at
the end of 69 h. Slides were prepared (as described in 3.2.1.3 and 3.2.1.5) and
were treated with Hoechst 33258 (10 μg/ml) for 15 min. in the dark. They were
washed in distilled water and were exposed to 2xSSC (1.7532 g sodium chloride and 0.8823g of trisodium citrate in 100 ml of distilled water) for one hour in sunlight. At every 15 min. interval 2xSSC was added to prevent the slides from drying. The slides were briefly rinsed in distilled water and stained in 4% buffered Giemsa solution for 5 min. They were washed in distilled water again and air dried.

3.2.1.9 Chromosomal analysis

The chromosomal preparations were analysed for aberrations under oil immersion objective using a NEOVAR microscope. Fifty well spread, Giemsa-stained metaphases (100 metaphases in lymphocytes irradiated with 3 Gy of gamma rays) were scored for chromatid- and chromosome- type of aberrations. These aberrations were classified according to the guidelines of Buckton and Evans (1982).

Twenty-five well banded metaphases from chromosomal preparations of MDS patients were analysed for the presence of any chromosomal abnormality. Fifty banded metaphases from lymphocyte cultures of DS patients irradiated with 1 Gy of gamma rays and 100 metaphases in the case of cells irradiated with 3 Gy of gamma rays were scored to detect non-random breakpoints involved in the different chromosomal aberrations. Chromosomal breakpoints were designated according to the standard nomenclature (ISCN, 1995).
Twenty-five well spread metaphases showing differential staining of sister chromatids were analysed to record the frequency of sister chromatid exchanges (SCEs).

3.2.1.10 Microphotography

Giemsa-stained metaphases showing different chromatid-and chromosome-types of aberrations as well as normal metaphases from each patient were photographed. Photographs were taken under an oil immersion lens using NP 22 NOVA (black and white) film in NIKON photomicroscope. Well banded metaphases and metaphases showing SCEs were also photographed in a similar manner.

3.2.2 Molecular Study

3.2.2.1 DNA isolation

a. Materials Required

5 ml of peripheral blood, RBC lysis buffer (0.155 M ammonium chloride, 0.17 M Tris in the ratio of 9:1), WBC lysis buffer (10 mM Tris chloride, 400 mM sodium chloride and 2 mM trisodium EDTA), 10% SDS, proteinase K (1 mg/ml), 6 M NaCl, 100% ethanol, 70% ethanol.

b. Protocol

DNA was isolated according to Miller's method (Miller et al., 1988). Five milli litre of peripheral blood was collected in a disposable Falcon tube and centrifuged at 3,500 rpm (1500 g) for 25-30 min. at room temperature. The
supernatant was removed and the buffy coat was transferred to another centrifuge tube. Double the volume of RBC lysis buffer was added to the buffy coat and aspirated well before incubating the same at 37°C in a water bath for 0 min. It was then centrifuged at 3,500 rpm for 20-30 min. The supernatant was removed and double the volume of RBC lysis buffer was added and the entire step was once again repeated. Three millilitre of WBC lysis buffer was then added to the pellet along with 0.2 ml of 10% SDS and 0.5 ml of proteinase. This was left overnight at 37°C. To this, 1 ml of 6 M sodium chloride was added and shaken vigourously till there was foaming. Centrifugation was then tried out at 3,500 rpm for 25-30 min. The supernatant was taken out and unsferred to another disposable centrifuge tube and double the volume of added 95% ethanol was added. The centrifuge tube was gently tilted and the lA was allowed to precipitate. The DNA was spooled on to a sterile glass rod and then transferred into an eppendorf tube containing 70% ethanol and ed.

2.2 Amplification of p53 using PCR

Materials Required

10 X PCR buffer (500 mM KCl; 100 mM Tris HCl, pH 8.3; 15 mM MgCl2; 0.1% gelatin), oligonucleotide sense and antisense primers, 4X dNTP (200 μM), α-32p dCTP (10 μCi; sp.act. 3000 Ci/mmol), Taq polymerase (0.25 μl), double distilled water, and sample (template) DNA.

The four sets of primers that were used to amplify the exons 5 to 8 of p53 gene are given below (Sakai and Tsuchida, 1992).
E5 (S) : 5' - TGTTCACTTGCCCTGCTT-3' (sense)
E5 (A) : 5' - CAGCCTGTCGTCTCTCCAG-3' (anti-sense)
This primer explores the entire exon 5 (269 bp)
E6 (S) : 5' - GCCTCTGATTCTCCTCAGT - 3' (sense)
E6 (A) : 5' - TTAACCCCTCCTCCAGAGA - 3' (anti-sense)
This primer explores the entire exon 6 (181 bp)
E7 (S) : 5' - ACTGGCCCTCATCTTCGGGCCT - 3' (sense)
E7 (A) : 5' - TGTGCAGGGTGCAAGGTGGC - 3' (anti-sense)
This primer explores the entire exon 7 (171 bp)
E8 (S) : 5' - TAAATGGGACAGGTAGGACC - 3' (sense)
E8 (A) : 5' - TCCACCGCTTCTTGCTGTCGCC - 3' (anti-sense)
This primer explores the entire exon 8 (229 bp).

Protocol

A 10 µl reaction containing 1 µl of 10 X buffer, 0.2 µl of 4 x dNTPs, 2 µl of sense and antisense primers (10 µM each), 0.05 µl of Taq polymerase, µl of sample DNA (0.1 µg/µl) and double distilled water making up the rest as set up in 0.2 ml thin walled PCR tubes (Sigma Chemical Co., St.Louis, SA). Temperature and time for the reaction cycles of exons 5, 7 and 8 were 95°C (1 min.), 60°C (1 min.) and 72°C (30 sec.), and for exon 6 it was 95°C min.), 55°C (1 min.) and 72°C (30 sec.) with 35 cycles of amplification.
3.2.2.3 Agarose gel electrophoresis

a. Materials Required

6 x loading buffer (0.025% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water), 1 X TAE (40 mM Tris-Acetate, pH 8.0; 1 mM EDTA) containing 0.5 μg/ml of ethidium bromide.

b) Protocol

The labelled products were checked for amplification in a 3% agarose gel by electrophoresis. The gel platform and comb were cleaned and the comb was placed in proper position. Tris-acetate-EDTA buffer containing ethidium bromide was used to prepare agarose gel. Molten agarose was poured on the gel platform and it was allowed to solidify. The comb was carefully removed. Care was exercised to see that the wells were not damaged. The platform with the gel was placed in a gel electrophoresis box. The box was filled with TAE buffer up to the point of covering the gel and completely immersing the sample wells. The PCR products were mixed with one third volume of 6 X loading buffer and were loaded in the wells. Electrophoresis was carried out at 5 v/cm. The gel was removed from the plate and the DNA bands were visualized on a UV transilluminator.

3.2.4 SSCP analysis

Materials required

Loading and denaturation buffer (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol, 10 mM EDTA, 0.1 N NaOH) (pH 8), running buffer
(5% glycerol and 0.5 X TBE (45 mM Tris-Borate (pH 8.3); 1 mM EDTA), thermostatic electrophoresis apparatus, Whatman No.3 filter paper, X-ray film.

b. Protocol

The PCR and SSCP techniques were employed to analyse cellular DNA from patients with MDS. The PCR reaction was carried out in the presence of $\alpha-^{32}$P dCTP (10 µCi) in a reaction mixture of 10 µl (Feinberg and Vogelstein, 1983; Koch et al., 1986). They were then diluted to 10-100 fold in loading and denaturation buffer and boiled for 5 min. at 85°C and the tube was immediately plunged into ice. They were then subjected to SSCP analysis by using a 6% polyacrylamide gel with 5% glycerol and 0.5 x TBE as running buffer. Electrophoresis was carried out in a thermostatic electrophoresis apparatus (Pharmacia LKB, Uppasala, Sweden) at a constant power of 35 Watts for 3.5 h at 25°C. Whatman No.3 filter paper was used to dry the gel after which it was exposed to X-ray film at -80°C with intensifying screens. After 24 h of exposure, it was processed to obtain the autoradiograph (radioautograph).