MATERIALS AND METHODS

The study was conducted at the Department of Pathology, King George Medical University, Lucknow.

Study duration: One year (2014-2015)

Study design: Prospectiveand retrospectivedescriptive study

DATA COLLECTION:

Newly diagnosed cases of acute myeloid leukemia were asked for personal identification details, disease symptoms, any presenting sign and symptoms of cytopenia like fatigue, weakness, dizziness, bleeding tendencies and infections and data about investigations already done.

Inclusion criteria for this study:

- Patients newly diagnosed with acute leukemia
- Patients who gave written informed consent for undergoing bone marrow aspiration.

Exclusion criteria for this study:

- Patients on chemotherapy
- Patients with coagulation disorder

PLAN OF STUDY

- Detailed clinical history for presenting symptoms was taken.
- We examined the patients for presence of features of anemia, any bleeding manifestations, gum hypertrophy, hepatosplenomegaly and lymphadenopathy.
- Following investigations were done in each patient
- Hemoglobin estimation, total and differential leukocyte counts and platelet counts
- General blood picture and blast identification and percentage
- Bone marrow aspirate examination for the presence and percentage of blasts.
- Cytochemical staining for Myeloperoxidase.

• Flowcytometric analysis of marrow aspirate for markers specific for myeloid as well as the aberrant markers.

• Cytogenetic study whenever permissible.

SAMPLE COLLECTION:

• 2 ml of peripheral blood sample was collected in EDTA vaccutainer for hemoglobin estimation and smears were prepared for total and differential leukocyte counts, platelet counts and general blood picture examination with blast counts.

• Bone marrow aspiration was done in all patients under local anesthesia using approximately 2ml of 2% lignocaine solution.

• Collected aspirate was used to prepare smears and the remaining sample was transferred to EDTA vaccutainer and refrigerated at 8˚C for flowcytometric analysis to be done within the next 24 hours.

EXPERIMENTAL DETAILS:

MORPHOLOGICAL EXAMINATION:

Leishman stain:

• **PRINCIPLE:** Leishman stain is a type of Romanowsky stain which distinguishes between the cellular components by subtle differences in the shades of staining. The dye has two components: Azure B and Eosin Y. Azure B reacts with the anionic molecules thereby staining the nucleic acid, proteins of cell nuclei, neutrophilic granules and basophilic granules. Eosin Y bind with the cationic molecules and hence stains the hemoglobin molecule and granules of eosinophils.

• **REAGENT USED AND STAIN PREPARATION:**

Prepared powdered Leishman stain (Thomas Baker) was used.

2.0 gm of powdered dye was dissolved in 1000ml methanol

This was warmed to 50˚C for 15 minutes with occasional shaking.

The solution was allowed to cool and kept for few days to allow maturation.

•**METHOD:** Air dried blood and bone marrow aspirate smears were placed on the staining rack and flooded with Leishman stain. After 2 minutes, the slides were flooded with double the

amount of water and left for 5-7 minutes. The slides were then washed with buffered water, air dried and examined.

• **INTERPRETATION:** The chromatin stains purple and nucleoli stain light blue. Cytoplasmic and granular staining is variable depending on the type of cells. Cytoplasm of granulocytic precursors stain pinkish. The granules of neutrophils and precursors stain red to purple. Eosinophils granules stain red to orange. Basophil granules stain purple black. Cytoplasm of lymphoid cells take bluish stain. Erythroid precursors show dark blue to pink cytoplasm in early to late normoblasts. RBCs stain pink in colour.

• The blasts were identified on the basis of their size, nuclear chromatin, nucleoli, cytoplasm and cytoplasmic granules,. Myeloblasts varied in size from slightly larger than mature lymphocytes to the size of monocytes or even larger, with moderate to abundant amount of cytoplasm staining dark blue to blue-grey. The nuclei were large, round to oval with finely granular chromatin and multiple nucleoli. Few azurophilic granules were occasionally present along with presence of Auer rods which stained purple.

CYTOCHEMICAL TEST:

Myeloperoxidase:

• PRINCIPLE: The enzyme myeloperoxidase (MPO) is present in the primary and secondary granules of neutrophils and its precursors, in granules of eosinophils and azurophilic granules of monocytes. It splits H2O2, which in the presence of a chromogen (3,3′diaminobenzidine as used here) gives an insoluble reaction product.

• **REAGENTS USED AND STAIN PREPARATON:**

Fixative: Buffered formal acetone (BFA)

We dissolved 20mg Na2HPO4 and 100mg KH2PO4 were in 30ml of distilled water. 45ml of acetone and 25ml of 40% formalin were added to the solution. Mixed well and stored at 4˚C. Used fresh fixative made every 4 weeks.

Buffer: Sorensen's phosphate buffer, pH 7.3

Stock solution was prepared by making 66mmol/l solution

of KH2PO4 (9.1gm/l) and Na2HPO4.2H2O (11.9gm/l). Then we mixed the above in a ratio of 19.2:80.8, thus giving the required buffer of pH 7.4

Hydrogen peroxide: H2O2, 30% w/v

Substrate: 3-3′ Diaminobenzidene (Sigma D-8001)(DAB)

Working substrate solution: Prepared by mixing 30mg of DAB in 60ml of phosphate buffer and added 120µl H2O2 and mixed well.

Counterstain: aqueous hematoxylin

- **METHOD:**

The air dried smears were fixed in cold BFA for 30 seconds. The slides were then rinsed thoroughly in gently running tap water and air dried. The smears were then placed in the working substrate solution and incubated for 10 minutes at room temperature. After the incubation period, the slides were washed in gently running tap water and counterstained in hematoxylin, rinsed, air dried and mounted for viewing.

- **INTERPRETATION:**

The presence of brown granular reaction product is considered as positive. Primitive myeloblasts are negative or show very faint positivity. Intensity of staining increases as they mature, with promyelocytes and myelocytes showing strong positivity with granules filling the cytoplasm. Auer rods were better visualized. Neutrophils show granular positivity and were considered as internal control. Eosinophils also stain strongly but their granules are larger in size as compared to neutrophils. Monocytes showed variable staining. Basophils that were rarely seen, were negative.

Early myeloblasts, lymphoblasts, lymphocytes, basophils and plasma cells are peroxidase negative. Red cells and erythroid precursors show diffuse brown cytoplasmic staining but were considered negative.

FLOWCYTOMETRY

Immunophenotyping was done by a multicolor flowcytometer. The instrument used was BD FACS Caliber (Becton Dickinson-Fluorescence Assisted Cell Sorter). We used appropriate

pairs of monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridinin-chlorophyl-protein (PerCP) and co-expression of different lymphoid and myeloid antigens was confirmed

- **PRINCIPLE:** The basic principle of flowcytometry relies on the passage of individual cells in a suspension through a tube at a constant velocity which is illuminated by a focused light source (laser beam) and study the light scattering pattern by detector positioned at specific angles to get information about the size and complexity of the cells. We label the cells with a specific antibody, tagged with a flourochrome, thus, we can use this principle to study various antigens present on the cells and thus define the lineage in leukemic blasts with better precision.

REAGENTS USED:

Fluorochrome labeled antibodies: Acute leukemia panel was used which included the following antibodies:

PerCP: CD45

FITC: CD2, CD7, CD10, CD20

PE: CD34, CD13, CD33, CD117, CD19, CD3, CD14

BD FACSLYSE solution: working solution was made by diluting with distilled water in the ratio of 1:10.

METHOD

Stain-Lyse-Wash method:

- The appropriate number of FACS tubes were labeled for the identification of the patient and the combination of fluorochrome conjugated monoclonal antibody.
- In each tube, 100µl of sample (bone marrow aspirate or whole blood) was pipetted.
- 20µl of antibody/antibody cocktail was added to each tube.
- The tubes were incubated in dark for 10-15 minutes.
- After the incubation time, 2ml of diluted FACSLYSE solution was added to each tube.
- The tubes were again incubated in the dark for 10-12minutes. This time period was crucial as incubation for a longer duration would degrade the cells of interest while a shorter time period

would lyse the RBCs incompletely thereby increasing the debris.

- The samples were then centrifuged at 200-300g for 3-5minutes. The supernatant was discarded, the pellet formed at the bottom of the tube was broken and the remaining cells were washed twice with sheath fluid (by adding about 2ml of sheath fluid, spinning at 200-300g for 3-5 minutes and then discarding the supernatant).

The cells were again resuspended in about 0.5ml of sheath fluid and run on a pre-caliberated flowcytometer.

DATA ANALYSIS

The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software. The values were represented in Number (%) and Mean±SD.

The following Statistical formulas were used:

1. Mean: To obtain the mean, the individual observations were first added together and then divided by the number of observations. The operation of adding together or summation is denoted by the sign Σ.

The individual observation is denoted by the sign X, number of observation denoted by n, and the mean by .

$$\overline{X} = \frac{\Sigma X}{\text{Number of observations } (n)}$$

2. Standard Deviation: It is denoted by the Greek letter Σ.

$$\sigma = \sqrt{\frac{\Sigma (X - \overline{X})^2}{n}}$$

3. Chi square test:

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Where O = Observed frequency

E = Expected frequency

4. Level of significance: "p" is level of significance

p > 0.05 Not significant

p <0.05 Significant

p <0.01 Highly significant

p <0.001 Very highly significant