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RESEARCH ARTICLE

HEMATOLOGICAL AND CLINICAL EVALUATION OF LEUKEMIAS, USING CYTOCHEMICAL STAINS AND IMMUNOPHENOTYPING

Abu Arshad A.[1], Narmatha M.[2] and Ulaganathan S.[3]

1. Assistant Professor, Department of Pathology,Government Mohan Kumaramangalam Medical College, Salem.TamilNadu.The TamilNadu Dr.MGR.Medical University,Chennai.India.
2. Assistant Professor, Department of Pathology,Government KAPV Medical College, Trichirappalli.TamilNadu. TheTamilNadu Dr.MGR.Medical University,Chennai.India
3. Assistant Professor, Department of Pathology,Government Mohan Kumaramangalam Medical College, Salem.TamilNadu. TheTamilNadu Dr.MGR.Medical University,Chennai.India.

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Abstract

Background: Leukemias are abnormal proliferation of hematopoietic cells, causing progressive infiltration of the marrow. It is the eleventh most common cancer in the world, and increasingly found now in developing countries. Two widely used classifications are used now, the FAB, and the WHO classification, which has got supplanted now, with increasing knowledge on cytomorphology and cytogenetics. This study, attempts to evaluate the role of cytochemistry as a cost-effective tool, in the various types of leukemias, and the role of immunophenotyping in a select few cases.

Aim:- The main aim of the study, was to assess the type, and subtype of leukemia, using cytochemistry, and to find their concordance with immunophenotyping in a select few cases, as a cost effective tool in diagnosing it.

Methods: 56 cases of leukemia, were identified by morphology and cytochemistry, using Sudan black B, and PAS stains. Immunophenotyping, was done in 6 cases selectively, and their concordance rate was determined.

Results:- Out of 56 cases of leukemia, 36 were acute, rest 20, were chronic cases. AML, accounted for 43% of the cases, CML at 33%, and ALL at 22%. Anemia was seen, more in acute leukemias, especially ALL, followed by AML. Total count values were seen high in CML, followed by AML. Platelet counts, were less in acute leukemias, especially ALL,followed by lowest in AML. Splenomegaly, was the commonest feature seen in 21 cases. Immunophenotyping, was done in 6 cases, 4 cases were concordant, showing a 67% rate.

Conclusion :- In a setting where there is a lack of facilities for flow cytometry, as in the developing countries, morphology combined with cytochemistry, still serves as the best means in diagnosing leukemia cases.

Corresponding Author:- Abu Arshad A.
Address:- Assistant Professor, Department of Pathology,Government Mohan Kumaramangalam Medical College, Salem.TamilNadu. TamilNadu Dr.MGR.Medical university,Chennai.India.

Introduction:-

Leukemias are neoplastic proliferation of hematopoietic cells, which form a major proportion of hematopoietic neoplasms, which are diagnosed worldwide. Leukemias, are classified into two broad groups, myeloid and lymphoid, based on the origin of the leukemic stem cell clone. They cause progressive infiltration of the bone marrow, and in certain forms the lymphatic tissues are particularly affected. Leukemia is the eleventh most commonest cancer in the world. In India, the number of new cases were 13 per 100,000 men and women per year. Two widely used classifications are used, one by the French, American, and British group called the FAB Classification, and other by the World Health Organisation (WHO Classification), based on the morphologic findings, genetic abnormalities, clinical and Immunophenotyping characteristics.[1,3,4,5,19,23] Overall annual incidence in the general population is 4 per 100,000 , with approximately 70% being Acute myeloid leukemia(AML). AML accounts for about 90% of the acute leukemias in adult population. Acute lymphoblastic leukemia(ALL) is primarily a childhood disease, and commonly seen at a peak age of 2 or 3 years. Chronic myeloid leukemia (CML) occurs typically between ages 40 and 60, and the Chronic lymphoid leukemia (CLL) occur mostly after 65 years of age. This study attempts to evaluate the role of cytochemistry in classifying the various types of Leukemia, and its subtypes, by a combination of morphology on the peripheral blood smear, as well as to correlate with the clinical and hematological findings of the cases, with available means in our laboratory. Cytomorphological assessment, based on FAB Classification was made, using Sudan Black B(SBB) stain and Per-iodic acid Schiff(PAS) stain.[2,21] Immunophenotyping, was done for a select few cases, where there was doubt, or ambiguity, and its concordance with the cytochemical staining patterns, and findings were taken into account. The study also aims to tell that the simple and cost effective method of cytochemical stains, are a valuable tool, in aiding the diagnostic methods in leukemia.

Aims And Objectives:-

1. To classify leukemias and subtype acute leukemias with the help of cytochemistry according to French American British(FAB) classification.
2. To study the clinical and haematological manifestations of the various types of leukemias.
3. The study also aims to tell that the simple and cost effective method of cytochemical stains, are a valuable tool, in aiding the diagnostic methods in leukemia.

Methods:-

Study design:
Prospective study.

Study population:
Peripheral smears of 56 cases of Leukemia.

Inclusion criteria:
Peripheral blood smear from all cases, of all age groups, who presented with clinical features, and abnormal hematological values suggestive of leukemia.

Exclusion criteria:
Other Hematological neoplasms, like Lymphomas and Multiple myelomas were excluded in the study.

Hematological examination:
Peripheral smear study was done first, by standard Romanowsky stains, mainly by Leishman's stain, and a presumptive diagnosis was made. Following which peripheral blood smears were subjected to special cytochemical stains, using Sudan Black B(SBB) and Periodic acid Schiff's stain(PAS).[2,3,4,7,10] The percentage of blast cells were enumerated, and the cytomorphology was studied based on their positive staining effects on the respective blood smears.. Thereby the types, and subtypes of leukemias were classified and reported as per FAB Classification. Relevant clinical history was obtained from each case, with paramaters relating to Splenomegaly, Hepatomegaly, and lymphadenopathy. Hematological investigations were done on a three part automated cell counter, and values pertaining to Hemoglobin, Total count, and Platelet count were also noted. In six cases, where in, doubt and ambiguous nature was thought of, immunophenotyping using flow cytometry, and standard panel markers, was undertaken.

Statistical analysis:
With the data collected, statistical analysis was made. Clinical and Hematological correlation was done, and immunophenotyping concordance patterns with cytochemical stained smears were noted.[11,14]

Human participant protection:
The study was conducted after institutional ethical committee clearance, and with patients informed consent.

Results:-

The distributive nature of the different types and subtypes of leukemia, are seen in Table-1. Of the total 56 cases, 24 cases(43%) were AML, 17 cases(30%) of CML, 12 cases(22%) of ALL, and 3 cases(5%) of CLL, were seen. In AML, 13 cases were of M2(55%), 6 cases(25%) were M1. In ALL, 6 cases were seen, each in L1 and L2, 50% equally distributed. CML, had 9 cases(52%) in the chronic phase, with 3 each in accelerated phase(18%) and blast crisis(18%). One case of Juvenile CML, and another case of CNL, was also seen.

Table 1:- Distribution of patients according to types and sub types of leukemia.

TYPES AND SUBTYPES OF LEUKEMIA	**NO. OF CASES AND PERCENTAGE (%)**	
AML (n=24)	**NO. OF CASES**	**PERCENTAGE (%)**
M0	2	8
M1	6	25
M2	13	55
M3	1	4
M4	2	8
M5 M6 M7	0	0
ALL (n= 12)		
L1	6	50
L2	6	50
L3	0	0
CML (n=17)		
Chronic phase	9	52
Accelerated phase	3	18
Blast crisis	3	18
Chronic Neutrophilic leukemia(CNL)	1	6
Juvenile CML	1	6
CLL (n=3)	3	100

Table -2, provides the different statistics for the hematological parameters according to the types of leukemia. The maximum mean hemoglobin level was observed in CLL, followed by CML, AML ,and ALL, the least. The mean Total count, was maximum in CML, followed by ALL, AML and CLL. The mean platelet count ,was high in CML, followed by CLL, ALL, and least in AML. Using One way Anova , by SPSS, the analysis of the differences in the group means, resulted in a 'p' value of < 0.0001, and it was highly significant across the leukemia types.

Table 2:- Different Hematological parameters according to the types of leukemia.

Parameters	**AML (n = 24)**	**ALL (n = 12)**	**CML (n = 17)**	**CLL (n = 3)**
Mean SD : Hb (gm/dl)	5.533 ± 2.77	4.975 ± 1.24	6.724 ± 1.68	8.601 ± 2.150
Mean SD : TLC (per µl)	56166.667 ± 52432.25	72343.166 ± 70265.325	106258.824 ± 82563.265	31900 ± 28564.325
Mean SD : Platelet Count (/cmm)	58268.635 ± 55625.012	68916 .011 ± 34865.854	226647.059 ± 123562	162666. 66 ± 143652.21

With regards to hemoglobin, the highest mean levels were seen with cases having lymphadenopathy, and the lowest in cases with splenomegaly. The difference in mean Hb levels by one way Anova, resulted in a 'p' value of 0.52. In the case of total count, the highest value was observed in patients with splenomegaly, and the least was seen in cases with hepatomegaly, the ' p' value obtained, was 0.54. And with platelet count, the highest mean values obtained were in patients with lymphadenopathy, followed by hepatomegaly and the least in splenomegaly. The 'p' value obtained here was 0.51.There was no statistically significant differences, between the group means , and the clinical features, as determined by one way ANOVA.This is seen in Table-3.

Table 3:- Comparison of clinical features with reference to haematological values.

Signs	**Hb (gm/dl)**	**TLC (/ul)**	**Platelet Count (Lacs /cu.mm)**
Lymphadenopathy (n = 7)	6.36 ± 0.43	69246.799 ± 33672.368	130200.565 ± 8349.952
Hepatomegaly (n = 16)	6.03 ± 1.23	63226.735 ± 30276.269	108775.755 ± 51687.363
Splenomegaly (n = 21)	4.88 ± 2.14	47874.394 ± 2163.351	103181.282 ± 32269.779
p -Value	**0.52**	**0.54**	**0.51**

Immunophenotyping Results

6 cases underwent flow cytometry,using peripheral blood samples, with 2 cases
using regular AML panel and 3 cases with ALL panel, and with one CML panel, for suspected and doubtful cases. Though they showed cytochemical stain positive, the results were obtained , with the plots on the side scatter graph. 4 cases, were concordant with the cytochemical stained smear diagnosis. But 2 cases of ALL, were partially concordant, and were requiring bone marrow aspirate samples for flow cytometry. Flow concordance percentage was 67%, and the remaining 33% was not in concordance, for peripheral blood sample.

1) AML M2 showed CD 13, CD,33,CD,117 positivity
2) AML M1 showed CD13, CD33 positivity
3) ALL L1 showed CALLA positivity with CD10 gated blasts.
4) ALL L2 showed mild blasts, but required a bone marrow aspirate analysis.
5) CML showed CD117, CD13,CD33,CD34 positivity in the gated blasts.
6) ALL L1 showed lymphocytosis, required a bone marrow aspirate analysis.

Discussion:-

Leukemias, as such are now common, and affects all ages and genders. It requires a multi parameter approach for its diagnosis[18], which includes cytomorphology study, with phenotypic and genotypic studies.[17] In the present study, 56 cases, were evaluated, by studying their morphology, using cytochemical stains, as well as clinical features, and with 6 cases for concordant Immunophenotyping, using lineage specific panels of markers. Of the 56 cases, 36 cases were acute leukemias(64%), of which 24 were AML(43%), and 12 were ALL(22%). The rest 20 cases were chronic cases(36%), of which 17 were CML(30%),which include one case of Chronic neutrophilic leukemia, and 3 were CLL cases(5%). This clearly shows Acute leukemias, being more common than chronic ones. In the acute group, AML was more common than ALL, and in the chronic group CML was commoner than CLL. After subtyping of acute leukemias with cytochemistry, the study showed in AML, 2 cases of M0(8%), 6 cases of M1(25%), 13 cases of M2(55%), 1 case of M3(4%), 2 cases of M4(8%). No cases of M5,M6 and M7 were detected. This showed that AML M2 was the commonest, in the FAB classification, and AML showed higher predominance than ALL or CML. However, CML cases were found to be second commonest, this could be due to population bias, and its limitations in the study. Hemoglobin values, were less than 6gm% in 83% of ALL cases, and 71% in AML cases. 35% of CML cases, and 34% of CLL cases, had less than 6gm%, this clearly indicates, severe anemia as a co existing disorder, in cases of acute leukemia.[1,7] Total count values, were seen in high ranges in CML, with more than 100,000/µl, in 58% of cases, and as much as 29% of them had more than 200,000/µl, followed by AML, which had 46% of cases upto 50,000/µl. ALL had 33% upto 50,000/µl. CLL had 67% of cases upto 11,000/µl, and 33% less than 4000/µl. This signifies that elevated counts will have less mature cells, and more of the immature variety.[1,2] Platelet counts, in general are lower in Acute leukemias, and normal to higher or rarely lower in Chronic leukemias.[1] This was interpreted in the study, and AML had 75% cases with lesser values below 50,000/cu.mm, and ALL had

71% cases, below that level. CML had normal to higher values of more than 150,000/cu.mm in 76% of their cases.CLL had more than 66% of their cases with more than 100,000/cu.mm. This signifies that acute leukemias, end up with less production of platelets, with decreased survival, causing thrombocytopenia. CML, usually has normal ranges in the chronic phase, but in the blast phase, they fall below the normal range just like in the acute cases. A thrombocytopenic picture in CML, always signals that an accelerated phase has started its process, leading to blast crisis.[1]

Anova analysis showed the ' p' value to be statistically significant across the group of means of the laboratory values, in the hematological parameters. A clear picture of the hematological values in assessing the Total count, Platelet count and Hemoglobin is essential in the diagnostic aid of leukemias. Various clinical presentations are seen in leukemias, assessment of splenomegaly, hepatomegaly and lymphadenopathy, were taken into account in this study. Splenomegaly was the commonest feature seen in 21 cases, followed by hepatomegaly in 16 cases, and 7 cases had lymphadenopathy. One particular feature seen here was a lesser platelet count , a lower hemoglobin value, and a total count less than 50.000/µl, in cases which had splenomegaly. There was no statistical significant differences, between the hematological value group means and the clinical features, as obtained by one way Anova. The varying nature of the clinical features, were seen. CML invariably had splenomegaly in all their cases, and hepatomegaly was mixedly seen in AML , as well as CML and ALL. CLL cases, all had lymphadenopathy. One case of AML M3, had disseminated intravascular coagulopathy, and one other case of CML in chronic phase, had visual disturbances. It is important to note that, in this study, the resources were limited, and cost effective measures had to be undertaken for the diagnosis. In a country like ours, where sophisticated laboratories having flow cytometric analysis for immunophenotyping, are limited in number, are a costly affair.[7] Hence, here a select few doubtful cases, were taken to undergo Flow cytometric analysis. Six cases were taken into account. The main purpose was to see their concordance, with cytochemistry. 4 cases were concordant with the flow cytometric analysis, one ALL, one CML, and two AML cases were found to be concordant, with cytochemical findings, the other two ALL cases required a bone marrow aspirate, and secondary markers for the lineage identity,which as per our study was out of our limitations. 67% were concordant, and effective in diagnosing the cases in our study. Flow analysis, uses a viable single cell suspension for the method, this consideration is important to note that hemodiluting effects can alter the analytical process, and can result in differences in results.[1,18] Quality control and maintenance of equipment is highly essential, which should be scrupulously followed.[1,14,16] By and large, a practical method, in our setting would be, to do a basic peripheral blood smear with routine stains, and with cytochemistry and morphology, a diagnosis can be made. In case of ambiguity or lineage specific identification, for the follow up of therapy and prognosis, flow cytometric analysis and cytogenetics would be of immense help.[15,18]

Conclusion:-

With resource limitations and considering the fact, that at present, Flow cytometry is either not available, or beyond the capacity of poor patients in our country, this study was carried out, with the aim that cytochemistry would be a cost effective method, in diagnosing leukemias. .A leukemia diagnosis protocol, is a need of the hour, considering the economics involved. So a simple and effective means is to be in place for routine morphology, cytochemistry , so as to give a correct diagnosis, and to make it easily performable, in centres devoid of flow cytometric analysis. Current therapeutics and prognostic factors, rely more on flow cytometry and cytogenetics, and the WHO has classified leukemias on its basis. This has prompted a new beginning in identification of the types and morphology. Hence a simpler adaptive format, with cost effective means can be devised in countries, where there are least, or no facilities, and access to them is made available.

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