Evaluation of Efficacy of DAB for Phi Bodies in Acute Leukemia

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Original Research Article

ABSTRACT

Diagnosing the type and sub-type of Leukemia is very important as the therapy, prognosis and survival rate changes with each type and sub-type. In the present study French American British (FAB) morphological classification is used and 60 % cases of Leukemia were evaluated by studying the detailed clinical features, morphology on peripheral blood smear and bone marrow aspiration. Out of the 60 cases we found that 40 cases were of acute Leukemia and 20 cases of chronic Leukemia. Out of which 24 were of acute Myeloid Leukemia, 16 of acute Lymphoid Leukemia. There were 15 cases of CML and 5 cases of CLL. Phi bodies appear to be characteristics of immature Myeloid cells in Leukemia and are seen with a higher frequency than Auer rods in acute Myeloid Leukemia.

KEY WORDS: Phi bodies, Leukemia, DAB

INTRODUCTION:

Leukemia is a neoplastic proliferation of hematopoietic cells and forms a major proportion of hematopoietic neoplasm that are diagnosed worldwide. Leukemia is classified into two broad groups, Myeloid and Lymphoid, based on the origin of the leukemic stem cell clone[1].Diagnosing the type and sub-type of Leukemia is very important as the therapy, prognosis and survival rate changes with each type and sub-type.

Although the French American British (FAB) morphologic classification of Myeloid and Lymphoid neoplasm has been used since many years, the discovery of number of genetic lesions that predict the clinical behavior and outcome better than morphology alone.

It necessitates the incorporation of specific genetic data in the classification scheme[1]. The diagnosis of acute Leukemia entails a stepwise approach. First in the sequence and of importance is the distinction of acute Leukemia from other neoplastic diseases and reactive disorders. Second is differentiating acute Myeloid (AML) from acute Lymphoblastic (ALL) Leukemia. The third facet is the classification of AML and ALL into categories that define treatment and prognostic groups. In most cases, the first two facets of diagnosis of acute Leukemia can be achieved by careful morphological assessment of blood and bone marrow smears and marrow trephine biopsy sections[2]. By assessing the morphologic features together, a majority of cases of AML and ALL can be accurately diagnosed. In some cases of poorly differentiated acute Leukemia, however, the morphologic features may be equivocal, requiring additional studies [3].

The present study was conducted to assess the cytochemical reaction for Myeloperoxidase as a valuable tool in the classification of acute Leukemia, chiefly in the distinction between acute Myeloid and acute lymphoblastic leukaemia[4]. Auer rods[5] are usually visible with Romanovsky stains in blast cells from various acute Myeloid types, particularly those with myeloblastic-promyelocytic differentiation (Ml to M3 of the FAB classification).'They have been considered to result from aberrations of the primary (azurophil) granules of early granulocytic cells[6] and are often seen more easily in peroxidase preparations. Hanker et al[7], described a new type of rod, the Phi body, not visualized with Romanovsky dyes but only by means of a peroxidase reaction using 3, 3'diaminobenzidine (DAB), and recommended that they were also characteristic of acute Myeloid Leukemia. We have investigated the presence of Auer rods and Phi bodies in patients with acute Leukemia in order to clarify the relation of Auer rods to the so-called Phi bodies[8].

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AIMS & OBJECTIVES:

1. To classify Leukemia and subtype acute Leukemia with the help of Cytochemistry according to French American British (FAB) Classification.
2. To study the Routine MPO with Benzedine Hydrochloride and DAB in Acute Leukemia and its sub types [3,8].
3. To Demonstrate the Phi bodies in AML blast only when DAB is used[9].

MATERIALS & METHODS:

The hematological laboratory based study spanned over a period of 2 years. The peripheral blood smears & bone marrow aspirates of all morphologically diagnosed Acute Leukemia cases received in the Department of Pathology NKP SIMS LMH Nagpur were studied.

INCLUSION CRITERIA:

Patients of all age groups who presented with clinical features and abnormal hematological findings suggestive of Leukemia (Peripheral Smear and Bone marrow aspiration smear).

EXCLUSION CRITERIA:

The relevant clinical history was obtained in each case, routine blood counts performed and peripheral smear and bone marrow aspirates studied in detail. Smears were stained with standard Romanovsky stains (Lieshman stain) and studied for morphology of cells. Hematological investigations were done on a 5 part cell counter. Cytochemical stains such as Myeloperoxidase, Non specific esterase; Sudan Black B and Periodic acid Schiff were done wherever needed for typing acute Leukemia cases. Kits manufactured by FAR SRL from Verona Italy were used for Cytochemistry[3]. Leukemia was classified according to the FAB criteria[1] using May-Grunewald-Giemsa stain : Sudan Black B,naphthol-AS acetate esterase ± NaF, a-naphthyl acetate esterase ± NaF, acid phosphatase, and lysozyme.The Myeloperoxidase reaction was observed on peripheral blood smear from all acute Leukemia (24 Myeloid and 16 lymphoblastic) cases with the following reagents for comparative study (Figure 1 to Figure 12):

(1) Benzedine dihydrochloride (Figure 13) and (2) 3, 3'diaminobenzidine tetra hydrochloride (DAB), introduced by Graham and Karnovsky in 1966.[10] Substrates (1) used according to previous techniques.[11] (2) DAB was tested according to the simplified technique of Hanker et al[12] with minor modifications, as follows:

FIXATION:

Smears were fixed for One minute in a mixture of 1.25% glutaraldehyde and 1% formaldehyde in 0.1 M phosphate buffer (pH 7.3); rinsing in 0.9% NaCI.

INCUBATION:

Smears were immersed One minute in a medium (Freshly prepared before use) containing 5 mg DAB, 10 ml Tris HCI buffer (0.05 M, pH 7.6), and 0.1 ml 1% H_2O_2. Slides are then rinsed briefly in Tris-HCI buffer and immersed for 1 minute in 0.5% CuSO4 in Tris-HCl buffer and rinsed again in 0.9% NaCl.

COUNTERSTAIN:

Ten minutes in a 10% solution of Giemsa, than dry & mounted in DPX [8] (Figure 14).

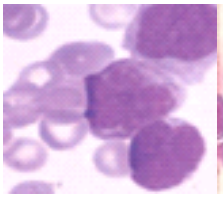

Figure 1: AML-M1, Myeloblast on PS (Leishman Stain)

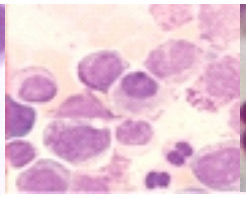

Figure 2: *AMLM2, Myeloblast on PS (Leishman Stain)*

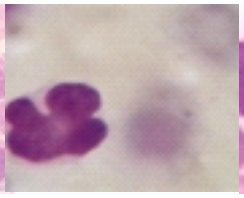

Figure 3: *AML- M3, Promyelocyte on PS, (Leishman Stain)*

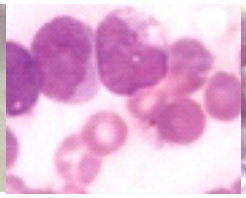

Figure 4: *AML-M4, Blast of Myeloid & Monocytic lineage (Leishman)*

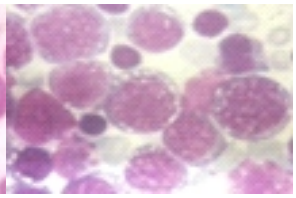

Figure 5: AML-M6, Blast on BM, (Leishman Stain)

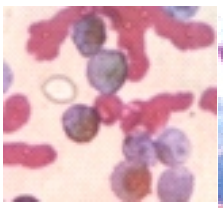

Figure 6: Blast with MPO Positive

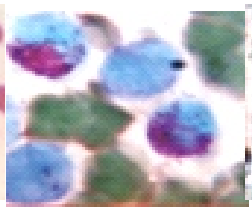

Figure 7: Blast with NSE Positive

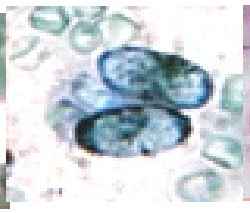

Figure 8: Blast with SBB Positive

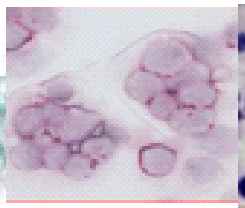

Figure 9: Blast with PAS Positive

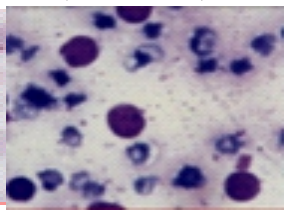

Figure 10: *A*LL-L1, Blast on PS (Leishman Stain)

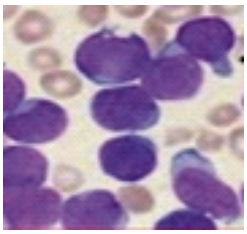

Figure 11: ALL-Hand mirror Blast on PS (Leishman Stain)

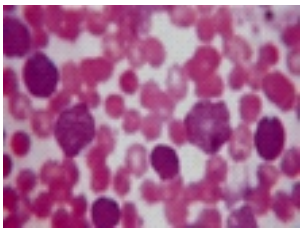

Figure 12: *ALL-L2, Blast on PS (Leishman Stain)*

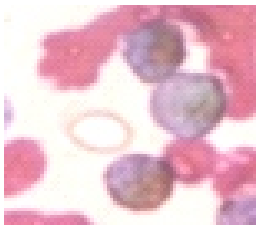

Figure 13: *Benzidin Method*

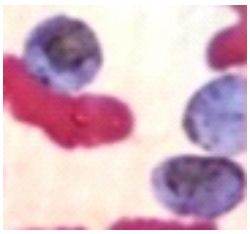

Figure 14: *DAB Method*

Table1: Distribution of patients according to type and Subtype of Leukemia (n=60).

Type and Subtype of Leukemia	Number (%)
AML (n=24)	
M1	3
M2	8
M3	7
M4	4
M5	2
ALL (n=16)	
L1	7
L2	9
CML (n=15)	
CP	10
AP	3
BC	2
CLL (n=5)	
CLL	4
PLL	1

Table 2: Distribution of Acute Leukemia as per Cytochemistry (n=40).

CYTOCHEMISTRY	AML	ALL
MPO+/SBB+/PAS-	21	00
MPO+/SBB+/PAS+	02	00
MPO+/SBB+/PAS-	01	00
MPO-/SBB-/PAS-	00	12
MPO-/SBB+/PAS+	00	03
MPO-/SBB-/PAS-	00	01
Total	24	16

RESULTS:

In the present study, 60 cases of Leukemia were evaluated by studying the detailed clinical features, morphology on peripheral blood smear and bone marrow aspiration. Cytochemistry was done to arrive at a final diagnosis.Table:1.Out of the 60 cases we found that 40 cases were of acute Leukemia and 20 cases of chronic Leukemia. Out of which 24 were of acute Myeloid Leukemia, 16 of acute Lymphoid Leukemia. There were 15 cases of CML and 5 cases of CLL. Acute Leukemia was more common than chronic Leukemia. After sub typing acute Leukemia cases with Cytochemistry, we got AML-M2 as the commonest type with 8 cases (00.2%), followed by M3 with 07cases (00.64%), M1- 3 cases (00.45%), M4- 4 cases (00.82%). We got no case of AML-M6, no cases of AML-M0 and M7. Kulshreshtha et al[7] in their study also got AML-M2 as the commonest subtype.

All the 24 AML cases were positive for Myeloperoxidase stain/MPO.

- 21cases stained positive for Sudan black/SBB along with MPO.
- 01case of AML-M4 stained positive for non-specific esterase/NSE stain and MPO.
- 02cases of AML Stained positive for Periodic Acid Schiff stain/PAS along with MPO and SBB of which 2 were diagnosed AML-M3 & 1 was diagnosed as AML-M6 (Morphologically).
- 16 cases of ALL were subjected to Cytochemistry out of which all cases were negative for MPO.
- 12 cases were PAS positive.
- 01 case was MPO, PAS and SBB negative but on the basis of morphology of blasts it was categorized as ALL-L2.This case was subjected to flow cytometry and they were T-ALL and B-ALL.[3]

All cases of acute Leukemia were considered for the analysis of peroxidase activity. As the results with substrates (.i.e. Benzedine hydrochloride with DAB).[13] only the comparison of both will be described here, as Phi bodies were visualized better with DAB and were single or multiple under observation, fusiform thin rods easily distinguishable from the relatively thicker Auer rods. The distinction between the two types of rods was not always possible, and for some of the comparisons we have scored the incidence of both rods together. Table 2 shows the results with both substrates in all the cases studied. The most striking difference was observed in the incidence of rods. Auer rods were detected three times more often with DAB (p< 0-02, Fischer's exact test), and Phi bodies were seen in over two-thirds of cases (p<0-001, Fischer's exact test), particularly in which no Auer rods were detected by either substrate, and they were not visible in the Romanovsky-stained films. Phi bodies were frequent in Ml- M2 and M4-M5 cases; Auer rods were slightly more common in M1-M2 (Figure 15 and Figure 16). The proportion of blast cells with Phi bodies ranged from < 1% to 15% in the positive cases; Auer rods ranged from 1 to 8%. None of the cases of acute Lymphocytic Leukemia tested, showed positivity in blasts or the presence of rods with either substrate[8] (Table 3).

Table 3: Comparison of Results with Benzedine Hydrochloride and Diaminobenzidine (DAB): n=40 (AML, ALL).

AML, ALL	No. of Cases	Benzedine hydrochloride	Diaminobenzidine (DAB)	
		No. of cases with Auer rods	No. of cases with :	
			Auer rods	Phi bodies
M1	8	2	3	3
M2	3	1	2	3
M3	7	-	1	2
M4	4	-	2	3
M5	2	-	-	-
Total	24	3(%)	8(%)	11 (%)
ALL	16	-	-	-

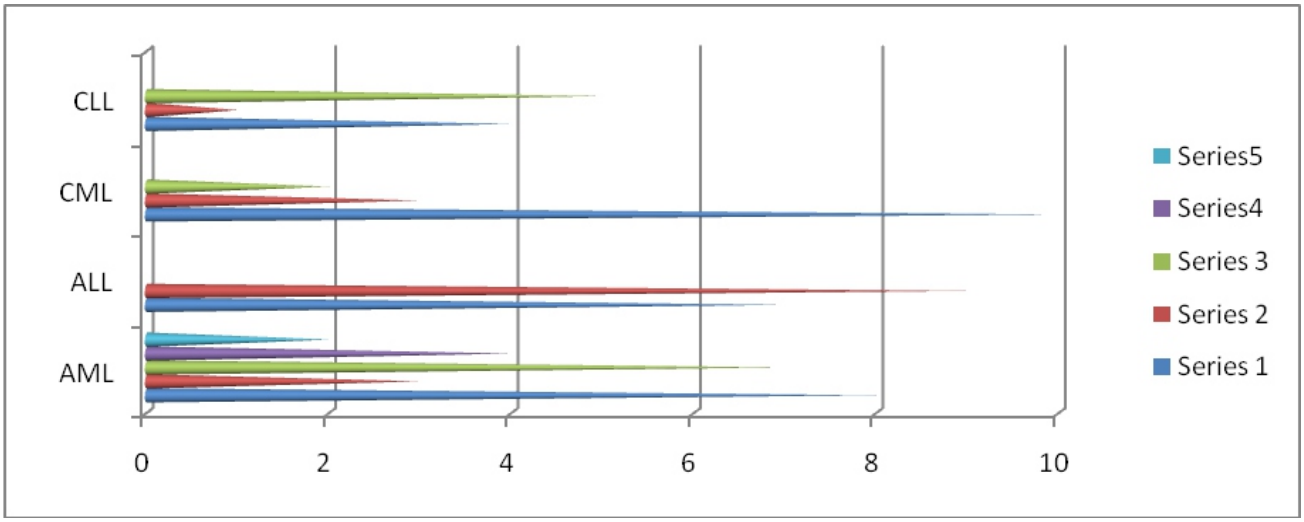

Figure 15: Distribution of patients according to type and Subtype of Leukemia.

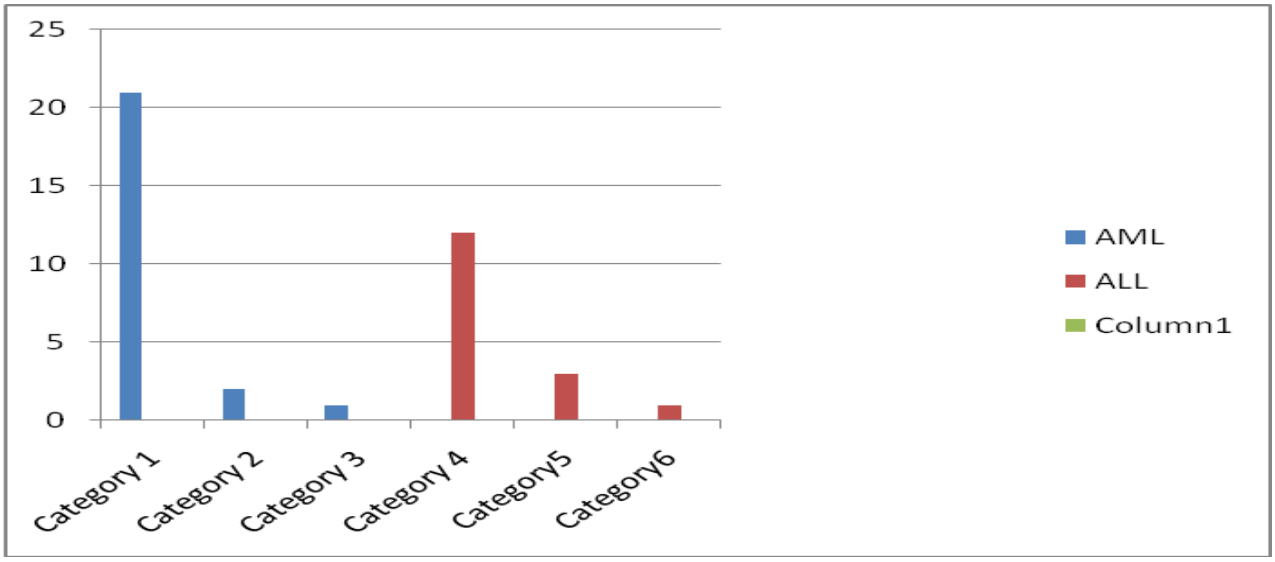

Figure 16: Distribution of Acute Leukemia as per Cytochemistry (n=40)

DISCUSSION:

We have confirmed the observation of Hanker et al.[7,14] that in a high proportion of AML cases spindle or fusiform shaped rods (Phi bodies) occur with a greater frequency than Auer rods when DAB is used as substrate for the peroxidase reaction,[15] Hanker designated the cytochemical reaction he described as hydroxyperoxidase[7]. The use of DAB reveals Phi bodies and is also a very sensitive method for demonstrating peroxidase activity in leukemic blast cells (Table 3). We therefore now use DAB routinely in preference to Benzedine.DAB is also used for [the demonstration of Myeloperoxidase activity at electron microscopy level][16, 17]. One of our M5 cases showed peroxidase activity only with DAB while the reaction was negative with Benzedine hydrochloride. Maximum interest is the relation of Phi bodies to Auer rods, partly discussed by Hanker et al[14]. Using the technique with DAB, it is sometimes difficult to distinguish them because both are strongly positive with this reagent. Phi bodies are not seen in lymph oblasts [8].

CONCLUSION:

We conclude that DAB with Benzedine enhances the catalase activity which indirectly reveals the presence of phi bodies in the Peripheral smear of Acute Leukemia, as against only DAB which reveals peroxidase granules and Aur rods. Phi bodies in the peripheral smear can give an indirect clue for ABL BCR positivity. Hence we follow the procedure of Hanker et al[7,14] i.e. the use of hydroperoxidase cytochemical reaction, which permits the

demonstration of Phi bodies in most AML cases and new evidence supporting the origin of those rods from catalase-containing granules.

More work is necessary, particularly at electron microscopic level with DAB well as parallel ABL BCR study to justify as well as to clarify the morphology of the Phi bodies and their relation with the traditional Auer rods[8].

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