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Original Research Article

Co-relation between Morphology and Special Stains with Flow Cytometry in the diagnosis of Acute Leukemia

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Abstract: Acute Leukemia accounts for approx 0.15-0.60% of total medical admissions in hospitals in India. It is still diagnosed mainly on the basis of morphology and cytochemistry in many centres, because of its ease and it remains the pillar of diagnosis where flow cytometry is not available. In our present study, the aim is to evaluate the positivity of special stains Periodic acid Schiff (PAS) and Sudan Black B (SBB) in peripheral blood smear and bone marrow aspirate, correlate the results with the final results obtained through flow cytometry, and to determine the sensitivity and specificity of these special stains in the diagnosis of acute Leukemia. We included 343 new cases of acute leukemias in which morphological diagnosis using special stains has been made and primary and secondary markers have been applied for immuno phenotyping and a definitive diagnosis was made. We analysed the sensitivity and specificity, and positive and negative predictive value of the special stains in establishing the diagnosis of acute leukemia. SUDAN BLACK (SBB) was positive in 72.7% cases of acute myeloid leukemia (AML) and negative in 27.3% cases. PAS was positive in only 28% of cases of Acute Lymphoblastic Leukemia (ALL) and it was negative in 72% cases in our study. Morphology plus cytochemical staining with PAS and SBB was able to correctly diagnose 93.38% cases of AML. PAS stain was positive in only 27.4% cases of ALL.
Keywords: Acute Leukemia, Cytochemical Staining, Flow Cytometry

INTRODUCTION

Acute Leukemia accounts for approx 0.15-0.60% of total medical admissions in hospitals in India[1]. Acute leukemias are a heterogenous group of malignancies with varying clinical, morphological, immunological and molecular features. AML accounts for approx 79% cases in adults as compared to children (21%) while ALL is commoner in children (72%) as compared to 28% in adults [2]. AML accounts for less than 15% cases in children less than 10 years, 25-30% between 10-15 years and 80-90% in adults. The average life time risk of developing ALL in a person is less than 1 in 750. Approx 0.5% of men and women will be diagnosed with AML at some point of their lifetime [2].

Despite advances in other areas, microscopic examination of Wright stained blood smear and bone marrow aspirate remains fundamental in haematological diagnosis, in addition to special stains. In acute leukemia, the morphologic identification of cells is sometimes difficult, due to marked similarity between earlier precursors of different cells series. In cases of poorly differentiated acute leukemias the morphologic features may be equivocal, requiring additional studies. In these cases the cytochemical stains are of great help in recognizing the type of precursor cells especially when there is asynchronism between nuclear and cytoplasmic maturation [3].

Cytochemistry is worth in the diagnosis and classification of acute leukemia. Concordance rate as high as 86% between morpho/cytochemical diagnosis and flow cytometry has been found [4]. Of these, complete concordance was seen in 58% of cases and partial concordance in 22% cases. A study showed that cytochemical staining should be available for those cases in which flow cytometry fail to yield a definite diagnosis [5].

In studies, PAS sensitivity ranging from 40.3% to 66.7% has been found in ALL cases [4, 6-9]. SBB is most commonly used and most valuable in distinguishing AML from ALL. Sensitivity of up to 100% and specificity of 86.67% have been found in a study [6]. Another study showed sensitivity of SBB to be around 78.2% in AML cases [7]. In this study, morphology and SBB positivity could correctly diagnose 93.9% cases of flow cytometry proven cases of AML whereas with PAS positivity, this combination was able to diagnose 60% cases of ALL correctly.

MATERIAL AND METHOD

The study was conducted in the department of pathology, Gujarat cancer research institute, Ahmedabad, India. Total 343 new flow cytometry proven cases of acute leukemia were included in the study in which cytochemical staining was applied and a morphological diagnosis has been done. Cases of all age groups were included in the study.

Bone marrow aspiration was done from all the patients with prior consent and Wright stained smear was prepared for microscopic examination, Cytochemical staining and immuno phenotyping. Peripheral blood was also collected in EDTA vacutainer for the same and French-American-British classification was used as the criteria for classification of acute leukemia. Cytochemical staining was done with both PAS and SBB in the entire bone marrow smear.

Principle for PAS reactivity:

Periodic acid specifically oxidizes glycols groups to produce stable di-aldehydes. These dialdehydes give a red reaction product when exposed to Schiff's reagent. The PAS stain reacts primarily with glycogen, generating a fuchsia coloured precipitate. Lymphoblasts in ALL often have prominent course granular or block PAS staining.

Principle for SBB reactivity

SBB stains a variety of lipids, including neutral fat, phospholipids and steroids. It is a lipophilic dye that binds irreversibly to an undefined granule component in granulocytes, eosinophils and some monocytes. It can't be extracted from the stained granules by organic dye solvent and gives comparable information to that of MPO staining.

Two observers assessed the PAS and SBB reaction in all the cases by counting two hundred blast cells at least on each slide. Results were recorded as a percentage of such cells showing block positivity for PAS stain. 5% was taken as a cut-off above which the reaction was considered positive [6]. For SBB, 3% blasts or above with positive Sudan positivity were taken as a cut off [8]. Based on the morphological examination of the marrow and/or blood smear and cytochemical staining results, a morphological diagnosis was made and cases were classified according to FAB classification. After that, flow cytometric analysis of all the cases was done.

All the bone marrow or peripheral blood samples were analysed by FACS Canto II flow cytometer (6-color, 2-Laser, BD Biosciences USA) and analysed using FACS Diva software. At least 30,000 total cells were acquired, and the side scatter versus CD45 PerCP dot plot was used for blasts gating. The percentage of positive blast cells more than 20% was considered positive for the surface or intracellular markers used. The monoclonal antibodies used in the primary panel were:

CD45 (Per CP), CD22 (FITC), CD34 (PE), CD5 (PE Cy7), CD10 (APC), CD19 (APC-H7), CD7 (FITC), CD13 (PE), CD33 (PE Cy7), CD117 (APC), HLA-DR (APC-H7), MPO (FITC), cCD79a (PE), cCD3 (PE Cy7) and TdT (APC) and in the secondary panel were CD11b (PE Cy7), CD11c (PE), CD14 (APC-H7), CD15 (FITC), CD2 (FITC), CD4 (PE Cy7), CD8 (APC-H7), CD1a (PE), CD41a (PE), CD41b (FITC), and CD61 (FITC). The CD45 was used for blast gating for both surface and cytoplasmic markers. The antibodies were procured from BD Biosciences, USA.

For surface markers, respective antibody (20 µl) mentioned above was added in six-color combination to the bone marrow or peripheral blood (100 µl, 1×10^6) and incubated for 15 min. After incubation, 2 ml of erythrocyte lysing solution (1:10 dilution with double distilled water; BD Biosciences, USA) was added and incubated for 15 min at room temperature. Then, cells were centrifuged at 400 *g* for 5 min and supernatant was discarded. Remaining pellet was washed twice with phosphate-buffered solutions (PBS) and then resuspended in 500 µl of PBS. For cytoplasmic markers, 2 ml lysing solution was added to 100 µl of bone marrow or peripheral blood to lyse red blood cells and incubated for 15 min. After centrifugation, to the pellet, 1 ml perm/wash buffer was added to permeabilize the cells for intracellular staining and incubated for 20 min. After centrifugation, to the pellet, respective antibody (20 µl) was added to the pellet and incubated for 15 min. Then, 2 ml PBS was added and the samples were centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the pellet was

resuspended in 500 µl of PBS. For surface and cytoplasmic markers, negative control tubes were run simultaneously with the addition of sample and CD 45 antibody.

Acquisition and data analysis

The cytometer setup and tracking beads were (BD Biosciences, USA) used for daily calibration of the instrument. The samples were then acquired in FACS Canto II flow cytometer (6-color, 2-Laser, BD Biosciences, USA) and analyzed using FACS Diva software (BD Biosciences, USA). At least 30,000 total cells were acquired, and the side scatter versus CD45 PerCP dot plot was used for blasts gating. The percentage of positive cells more than 20% was considered positive for that surface or intracellular markers.

RESULTS

Out of total 136 flow cytometry proven cases of AML, PAS was negative in 132 cases (97%) and positive in 04 cases (2.9%).Table 1 depicts that SBB was positive in 99 cases (72.8%) and negative in 37 cases (27.2%). Out of these 37 cases, 3/3 (100%) Cases of AML M_0 were SBB negative. Further, out of 8 cases of AML M_4, 2 cases (25%), and out of 10 cases of AML M_5, 4 cases (40%) were SBB negative. Further, out of these 136 cases of AML, morphology plus cytochemistry was able to diagnose correctly 127 cases of AML. Rest 9 cases, morphologically diagnosed as AML, turned out as ALL on flow cytometry. Thus 93.38% cases of AML could be correctly diagnosed on morphology and cytochemical staining with PAS and SBB.

Out of total 207 cases of ALL, PAS was positive in 58 cases (28%) and negative in 149 cases (72%). SBB was positive in 02 cases (0.96%) and negative in 205 cases (99.9%) as depicted in table 1. Further, out of total 161 cases of Flow proven cases of B ALL, 44 cases (27.32%) were PAS positive and 117 cases (72.68%) were PAS negative. 2 cases (1.24%) were SBB positive and rest 159 cases (98.76%) were SBB negative. Out of 47 cases of T ALL, 14 cases (29.78%) were PAS positive and 33 cases (69.2%) were PAS negative. All 47 cases of T ALL (100%) were SBB negative. Morphology alone was able to diagnose correctly 155/161 cases of B-ALL (96.27%) and 47/47 cases of T-ALL (100%).

Table 1: Diagnostic performance of PAS in ALL and SBB in AML

Special Stains		Diagnosis		Sensitivity	Specificity	Ppv	Npv
		ALL (207 cases)	AML (136 cases)				
PAS	NEGATIVE	149	132	27.4%	96%	92%	46%
	POSITIVE	58	04				
SBB	NEGATIVE	205	37	72.8%	99%	98%	84%
	POSITIVE	02	99				

Ppv=Positive predictive value, Npv=Negative predictive value

Table 2: PAS Positivity in ALL and SBB in AML [4, 6-8].

Studies	PAS in ALL	SBB in AML
Sushma *et al.;*	66.7%	-
AAM Deghady	40.3%	100%
Liqaa M *et al.;*	60%	78.2%
A.gupta *et al.;*	62%	66%
Current study	27.4%	72.8%

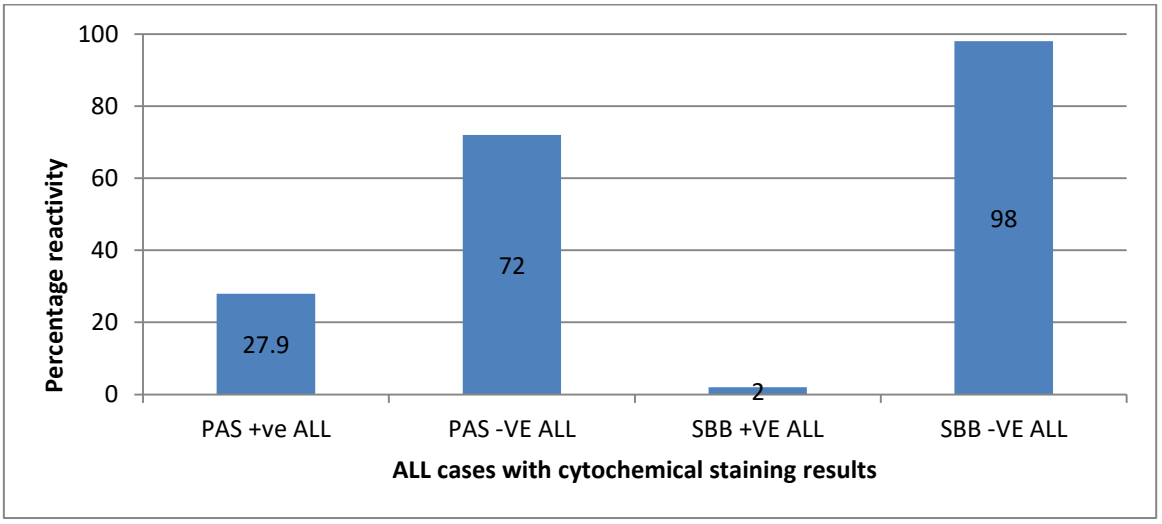

Fig-1: Reactivity of PAS and SBB in ALL

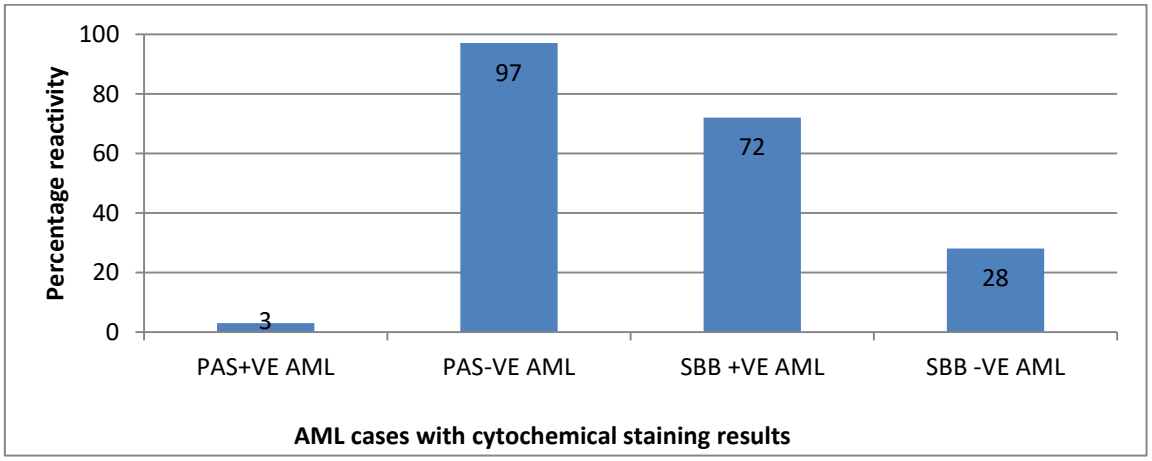

Fig-2: Reactivity of PAS and SBB in AML

Cases of Acute Leukemia negative for both PAS and SBB

Out of 343 cases of Acute Leukemia, 182 cases were negative for both PAS and SUDAN B, But proven to be either AML or ALL with flow cytometric analysis. Thirty three AML cases out of 136 (24%) were negative to both of these stains whereas 116 B-ALL cases out of 161 (72%) and 33 T-ALL cases out of 47 (70%) were negative to both PAS and SBB.

DISCUSSION

In our study, upto 93.3% cases of AML can be correctly diagnosed with the help of morphological examination and special stains. The sensitivity of SBB in AML cases was 72.7%, which is at par with various studies [4, 6-8]. Where its sensitivity was found to be in the range of 60% to 100%. Ashish gupta *et al.;* showed SBB positivity to be 66% in their study which is slightly lower than our current study. Liquaa M *et al.;* showed SBB positivity to be 78.2% in their 79 cases of acute leukemia which is at par with our study [7]. AAM Deghady showed that all 15 cases of acute leukemia were SBB positive , thus sensitivity of SBB was 100% in their study of 15 cases. In Our current study, there were 136 cases of AML, which showed the positivity of SBB to be 72.8%, highlighting the importance of SBB staining in AML. Only 4/136 (2.9%) cases of AML under our study were PAS positive. Snower DP *et al.;* showed 4 cases out of their 21 AML cases (19.04%) to be PAS positive in their study [9]. Many SBB negative cases of AML in our current study were either AML M0, AML M5a or M5b.This also emphasises the role of SBB in the sub-classification of AML cases. Only in 2 cases out of 207 ALL cases, SBB turned out as false

positive (0.96%). Several studies show that SBB may be positive in cases of ALL, though very rarely. SBB positivity has been reported in 1.6% cases of ALL in study by Stass A, Pui CH, Melvin S *et al.;* [10].

As far as role of PAS staining in ALL is concerned, our study showed limited usefulness and little added benefit to morphological examination and PAS positivity was observed in only 27.4% cases of ALL, but one useful finding was that its false positive rate is quite low (2.94%) and it was positive in only 4 cases out of 136 cases of AML. Studies show that PAS reactivity is specific but less sensitive for ALL diagnosis [11]. Ashish gupta *et al.;* showed PAS positivity to be 62% while Liquaa M *et al.;* showed PAS positivity to be 60%.

CONCLUSION

Being cheap, simple and requiring no use of special instruments, cytochemical stains are very much important in developing countries for the diagnosis of acute leukemia, especially in cases of AML where SBB correlate well with the immuno pheno typing markers as evident by its high sensitivity and specificity. Our study with SBB positivity in AML also correlates well with that of the results obtained by flow cytometry. Sensitivity of SBB improves further in AML M_1 upto AML M_4 and its results are very much comparable to that of flow cytometry results. On the other hand, our study with PAS revealed very little value in the diagnosis of ALL cases and many other studies also shows that it is a less sensitive test in the diagnosis of ALL. One possibility of low sensitivity of PAS in ALL might be geographical variation too.

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