Original Article

Correlation of morphologic and cytochemical diagnosis with flowcytometric analysis in acute leukemia

ABSTRACT

Introduction: The classification of acute leukemias has revolutionized over the years. Immunophenotyping of acute leukemia has gained popularity because of its influence on treatment and prognosis of the disease. The various antigens expressed by the leukemic cells can be assessed by flowcytometry (FCA) and can be used in rendering specific treatment and predicting the outcome of the different types of acute leukemia.

Aims: The main aim of this study was to compare the morphologic and cytochemical diagnoses with flowcytometric diagnoses in acute leukemia and to analyze the usefulness of FCA over morphology.

Results: In this study we analyzed 50 cases of acute leukemia and found concordance rate as high as 86% between morphologic/cytochemical diagnosis and flowcytometric diagnosis. Of these, complete concordance was seen in 58% of the cases and partial concordance was seen in 22% of the cases. Non-concordance was seen in only 4% of our cases. In remaining 16% of our cases FCA helped in sub classifying the acute leukemia where morphology and cytochemistry had failed to do so. CD19 and 20 were found to be consistent B-cell markers and CD3 was a very specific marker for T-cell leukemia. CD13 and 33 were important myeloid markers and were aided by other secondary panel of markers like CD14, CD117 and CD41.

Conclusion: FCA not only helps in confirming morphologic diagnosis in acute leukemia but also helps in assigning specific lineage to the blasts, particularly in acute lymphoid leukemia. Immunophenotyping is of utmost importance in classifying acute leukemia as it greatly influences the treatment and the prognosis.

KEY WORDS: Acute leukemia, CD markers, CD45, flowcytometry

INTRODUCTION

In view of the pitfalls in FAB (French American British) classification which was based on morphology, the WHO classification emphasized the importance of immunophenotyping and defined the myeloid and lymphoid malignancies by the antigenic features of the neoplastic cells. Flowcytometry (FCA) is a powerful technique by which such antigens can be identified. Identifying the lineage of the leukemic cells not only helps in assessing the course of the disease but also aids in rendering the most specific treatment to the patients. This is a 2 year retrospective study carried out in our lab in which we have compared the morphologic and flowcytometric diagnoses in 50 cases of acute leukemia and analyzed the correlation between the two.

MATERIALS AND METHODS

During the 2 year study period a total of 1,957 bone marrow specimens were received of which 142 patients were diagnosed as acute leukemia based on morphology and cytochemistry. Of these 142 patients, FCA was performed on 50 patient’s samples for further confirmation of the diagnosis.

The laboratory investigations analyzed included (1) examination of peripheral blood smears stained with Leishman’s stain, (2) examination of bone marrow aspirate smears (Leishman’s) and biopsy slides (H and E), (3) cytochemical stains-Myeloperoxidase, Sudan Black, Acid phosphatase and Periodic acid Schiff and (4) FCA using primary and a secondary panel of monoclonal antibodies.

The instrument used for FCA was FACS (fluorescence activated cell sorter) caliber-BD (Becton Dickinson and company). Specimens used were heparinized peripheral blood or bone marrow aspirate. Four milliliters of blood was collected in a 68 USB (universal serial bus) coated tube. The samples were received in the clinical lab and transported immediately to the FCA analysis center and processed within 24 h. EDTA (ethylenediaminetetraacetic Acid) sample was also collected simultaneously to analyze the...
blast percentage on peripheral smear. The technique used for sample acquisition was Lyse wash protocol. Fifty microlitre of heparinized marrow or peripheral blood (with a minimum count of $10^6$ wbc/mL) was collected in a 5 mL falcon tube and the respective antibody was added.

The primary panel of antibodies used were CD45 (for identifying blasts was added in all tubes), CD19, CD10, CD3, CD33, CD7, CD34, CD13. For some cases of AML (Acute myeloid leukemia) like AML M4/M5, CD14 and for AML M7, CD41 were also included in the primary panel. Secondary panel (CD117 and CD14) was used in few cases.

The sample was incubated for 30 min in dark and lysing solution was added and kept for 10-15 min and then centrifuged and the supernatant was removed. The pellet was washed with phosphate buffer solution, suspended in 1 mL Phosphate buffer solution and sample was acquired. The cells were stained with properly chosen fluorescent-labeled monoclonal antibodies and incubated under appropriate conditions.

The fluorescent probes used were Peridinin-chlorophyll protein used with CD45, Flouro iso thio cyanate, Phycoerythrin and, Allophycocyanin.

All the quality control measures were undertaken before starting the procedure such as checking the system pressure and vacuum gauges, checking the optical alignment, fluorescence standardization and color compensation of instrument and testing the antibody integrity by verifying quality controls.

Data interpretation and reporting was done by reporting hematopathologists. Blasts were identified based on forward scatter and their dim expression of dim CD45. They also showed a strong positivity for CD34. More than 20% expression of any antigen was considered as positive. Acute lymphoblastic leukemias (ALL) were sub classified based on their expression of CD10, CD19, CD3 and CD7 where CD19 is a B-cell marker and, CD3 and CD7 are T-cell markers. AMLs were identified by their expression of CD13 and CD33. In suspected monoblastic lineage leukemias, CD14 was used as an additional marker. A single case of AML M7 was included for which CD41 was also used in the primary panel.

**RESULTS**

Based on the morphology, of these 50 cases, 33 were classified as ALL and 12 were classified as AML. Five cases remained unclassified [Tables 1 and 2].

The following were the results of FCA in these 50 cases

a. ALL 34/50
   - B-ALL (B lineage acute lymphoblastic leukemia): 22 (64.7%) → CALLA (common acute lymphoblastic leukemia antigen) + 18 (81.8%), CALLA − 4 (18.2%)
   - T-ALL: 6 (17.6%) → CALLA + 5 (83.4%), CALLA − 1 (16.6%)
   - CALLA + ALL without expression of B or T antigens: 6 (17.6%)

b. AML 15/50
   - Only CD13 + 1 (6.67%)
   - Only CD33 + 3 (20%)
   - Both CD13 and 33 + 9 (60%)
   - CD13, 33 and 14 + 1 (6.67%)
   - CD41 + 1 (6.67%)

c. Mixed lineage leukemia 1/50

d. Acute leukemias with aberrant Ag expression
   - ALL with myeloid Ag → 8(16%), with CD7 expression → 1(2%)
   - AML with CD7 expression → 4(26.6%)

**Table 1: The results of the cytochemical staining in these 50 cases**

| Cytochemistry     | ALL                      | AML %        |
|-------------------|--------------------------|--------------|
|                   |                          | Positive in 91.6 (11/12) AMLs | Negative in 8.4 (1/12) AMLs |
| MPO and SBB       | Negative in all cases    |              |
| PAS               | Positive in 66.7% ALLs. | Negative in 21.2% ALLs. Inconclusive in 12.1% |
| Acid phosphatase  | Focal golgi positive in 38.5% T-ALLs | Did not show focal positivity in 62.5% T-ALLs |
| Oil red O         | Positive in 1 of 2 ALL L3 cases |              |
| MPO/SBB/PAS +     | 1/50(2%)                 |              |
| MPO/SBB/PAS negative | 3/50(6%)         |              |
| MPO/SBB/PAS could not be done | 1/50(2%) |              |

**Table 2: The morphologic types and subtypes of acute leukemia of the 50 cases**

| Type of leukemia (Morphologic diagnosis) | No of cases | Percentage |
|----------------------------------------|-------------|------------|
| ALL L1                                 | 13          | 26         |
| ALL L2                                 | 7           | 12         |
| ALL L3                                 | 2           | 4          |
| T-ALL                                  | 7           | 16         |
| ALL L2/T-ALL                           | 4           | 8          |
| AML M1                                 | 1           | 2          |
| AML M2                                 | 7           | 14         |
| AML M4                                 | 2           | 4          |
| AML M7                                 | 1           | 2          |
| AML not sub classified (M2/M4)          | 1           | 2          |
| Acute leukemia not sub classified       | 5           | 10         |

ALL= Acute lymphoblastic leukemia, AML= Acute myeloid leukemia, MPO=Myeloperoxidase, SBB=Sudan Black, PAS=Periodic acid Schiff
In two (4%) of our study cases there was complete discordance between the morphologic and flow cytometric diagnoses [Tables 3 and 4].

**Case 1. AML undifferentiated with aberrant CD7 expression**

Although morphology and cytochemistry was suggestive of ALL L2, however, on FCA, the B-cell markers were negative and there was strong CD33 expression with moderate CD7 expression and weak CD34 expression. Therefore, it was diagnosed as AML undifferentiated leukemia with aberrant CD7 expression.

The patient was given standard AML M0 therapy protocol with MRC 10, but succumbed on Day 7 of therapy following neutropenic sepsis.

**Case 2. Acute mixed lineage leukemia**

Cytochemical staining in this case showed occasional cell positivity on SBB (Sudan Black) and strong block positivity on PAS (Periodic acid Schiff). Hence morphologic diagnosis was given as AML M0/ALL L2. On FCA, more than 50% blasts expressed CD19, CD33 and CD13 therefore, it was diagnosed as Acute mixed lineage leukemia. The patient was treated with the COAP (cytophosphamide, vincristine, cytosine arabinoside, prednisone) (standard ALL) regimen. He responded and remained in remission during the 2-year follow-up.

**Cases with partial discordance**

In 11 (22%) cases of ALL, there was partial discordance between the morphologic and flow cytometric diagnoses with relation to B and T-cell lineage. Two cases of ALL L1/L2 turned out to be T-cell ALL on flow. Five cases of L1/L2 turned out to be CALLA + ALL without expression of any specific B-cell marker.

Three cases of T-ALL on morphology turned out to be CALLA + B-ALL on flow and one case of T-ALL was diagnosed as CALLA + ALL without expression of any specific T-cell marker [Tables 5 and 6].

In the five cases that were diagnosed as CALLA + ALL, ideally, a secondary panel of markers should have been used for B lineage consisting of cyCD22 and CD79a. However, this was not done due to non-availability of these markers. But the patients were treated as per the standard ALL protocol and remained in remission during the 2 year follow up.

Case no 2 (in Table No 6) was diagnosed as T-ALL on morphology but on FCA this case showed CD19 expression in only 20% of the blasts whereas CD7 expression was seen in 36.8% of the blasts but still it was categorized as B-cell ALL due to co-expression of CD10 and CD19, which indicates neoplastic proliferation of B-cells. CD7 was considered as an aberrant marker in this case.

Among four cases diagnosed as ALL L2/T-ALL based on morphology and cytochemistry, flow analysis identified three of them as B-cell ALL and one case as T-cell ALL. Therefore, in these four cases, FCA helped in identifying the correct cell lineage.

Two cases were diagnosed as ALL L3 based on morphology. On FCA, these cases expressed bright CD45 expression, very low to nil CD34 expression and, strong CD10 and CD19 expression. Hence, they were classified as mature B-cell ALL.

All the 12 cases diagnosed as AML on morphology were identified as AML on FCA also.
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MPO, SBB-Neg; PAS
FCA
MPO, SBB-
MPO, SBB-, PAS
FCA (%)
MPO, SBB-Neg;
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butyrate esterase), Periodic acid-Schiff, and acid phosphatase. Definite diagnoses were made for all 10 of their AML cases, whereas diagnoses were possible in only 79.4% patients with ALL when only morphology and cytochemical staining was used. In the rest of the cases, cytochemistry did not aid in diagnosis and hence they opted for FCA to render a definitive diagnosis. Hence, in their study, they stated that “Although cytochemical stains are essential to recognize the subtypes of AML, they are of limited use in differentiating the subtypes of ALL and that the FCA has become a standard tool for the assessment and management of patients with leukemia.”

The observations in our study also indicate that cytochemistry was of good help in diagnosing AMLs where as in ALL, FCA was required in addition to morphology and cytochemistry as 1/3rd of our ALL cases could not be diagnosed based on cytochemical stains.

In the current study the correlation between morphologic and FCA diagnosis showed complete concordance in 58% of the cases, partial concordance in 22% and non-concordance in 4% cases whereas in the remaining 16% FCA helped in establishing definite diagnosis where morphology had not helped. If we include the partially concordant cases even our study shows a concordance rate as high as 86% [Table 7].

A similar study was done by Kheiri et al.,[3] where they have compared cytochemical and flow cytometric diagnosis in 93 cases of acute leukemia. Their study has shown a lineage agreement of 95.8% of the cases. However, when non-diagnostic and bi-phenotypic diagnoses made by either methodology were included, complete agreement occurred only in 77.4% of the cases. In their study, 89.2% of the myeloid leukemias showed agreement between cytochemical staining and FCA, whereas 80% of the ALL showed agreement between the two modalities.

**Unclassified cases**

In five cases of morphologically unclassified acute leukemias, FCA helped in identifying the correct lineage of the blasts and aided in confirming the diagnosis of these cases, so that the treatment could be given accordingly.

**DISCUSSION**

The FAB classification for acute leukemia has been the major system of classification for more than 20 years. This system provided structured criteria for the diagnosis of a variety of morphologic and cytochemical subtypes of acute leukemia. However, studies[1] indicate that the majority of categories in the FAB system do not delineate significant disease groups based on morphology and cytochemistry in terms of patient survival.

In our study FCA analysis in 50 cases of acute leukemia were analyzed and compared with their morphologic diagnosis.

Cytochemical analysis of leukemia (PAS, MPO (Myeloperoxidase), SBB), when coupled with morphology rendered the diagnosis in >80% of our acute leukemia cases. In a setting of lack of facilities for immunophenotyping, morphology and cytochemical analysis best serve the purpose in diagnosis of acute leukemia. A total of 22/33 (66.7%) ALL cases and 11/12 (91.6%) AML cases could be assigned correct lineage based on morphology and cytochemical staining.

Cytochemical stains used in the study by Mhawee et al.[2] included Sudan-black, specific esterase (alpha-naphthyl ASD chlorooacetate esterase), non-specific esterase (alpha-naphthyl butyrate esterase), Periodic acid-Schiff, and acid phosphatase. Definite diagnoses were made for all 10 of their AML cases, whereas diagnoses were possible in only 79.4% patients with ALL when only morphology and cytochemical staining was used. In the rest of the cases, cytochemistry did not aid in diagnosis and hence they opted for FCA to render a definitive diagnosis. Hence, in their study, they stated that “Although cytochemical stains are essential to recognize the subtypes of AML, they are of limited use in differentiating the subtypes of ALL and that the FCA has become a standard tool for the assessment and management of patients with leukemia.”
In the study by Mhaweek et al.,[8] 80.32% concordance rate was seen between cytochemical staining and FCA. Their study consisted of 112 ALL cases, of which 89 (79.4%) cases were concordant, and nine AML cases, of which seven (77.7%) cases were concordant.

**Flowcytometry in acute leukemia**

Based on FCA, 50 cases of acute leukemia were reclassified into three groups, ALL, AML and mixed lineage leukemia. ALLs were further sub classified into B-cell, T-cell, CALLA + and CALLA − cases.

**Gating of blasts**

For the immunophenotypic analysis of acute leukemia, CD45 was used for gating of blasts. The availability of three-color flow cytometers and the discovery that leukemic blasts nearly always show less intense CD45 expression revolutionized the method of gating for leukemic analysis.

Studies have shown that a gating strategy using CD45 expression is the most superior marker for the identification of leukemic cell populations in peripheral blood or marrow specimens.[14–16]

Hence dim CD45 expression along with side scatter helps in the definitive identification of blast population in acute leukemias.

**Significance of CD34**

CD34 is normally expressed by immature hematopoietic cells as confirmed in a study by Basso et al.[9] Normal marrow contains less than 3% CD34 positive cells, making this an excellent marker for monitoring blast cell populations. Among leukemia, 45–65% AMLs, and 75% pre B-cell ALLs have been reported to be CD34 positive by Traweek et al.[10] Hence the major diagnostic application is due to its expression in leukemia of early myeloid lineage and lymphoblastic lymphoma.[11]

Neoplastic B-cell population in ALL L3 shows dim expression of CD34 as these cells are mature and loose the CD34 antigen expression during their maturation. Hence, lack of CD34 expression in the mature B-cells of ALL L3 is significant in its diagnosis however, the diagnosis also needs to be aided further with other findings like TdT (terminal deoxynucleotidyl transferase) negativity.[9]

**B-cell acute lymphoblastic leukemia**

57.6% of the cases were classified as B-ALL and were designated as such when >20% cells expressed CD19 Ag. CD19 has been used as the primary marker for identification of leukemia of B-cell lineage.[12] Diagnosis of B-ALL primarily relies on the reactivity of two Mo abs, CD19 and HLA-DR. Antibodies against the CD19 antigen have been especially helpful because CD19 is the earliest B-lineage-specific Ag presently known and it precedes the appearance of HLA-DR, CD10, CD20 and other B-specific antigens. Lack of reactivity with CD19, for all practical purposes, rules out a B-lineage of the leukemia. CD19 is present from the time of B-lineage commitment of the hematopoietic stem cell through the stages of pre-B and mature B-cell differentiation. It is finally down regulated during terminal differentiation of the B-lymphocyte into the plasma cell. Because CD19 expression is maintained during B-cell neoplastic transformation, CD19 expression is useful in the diagnosis of leukemia of B-cell lineage.[10]

Aguilera et al.[11] compared the sensitivity of B lineage markers cyCD79a, cyCD22, CD19, CD20 and CD22 in their study on 74 cases and showed that cyCD79a has 100% sensitivity and 80% specificity followed by cyCD22, which showed 97% sensitivity and 88% specificity. They concluded that these are highly sensitive markers for B-cell ALL cases. Hence, at least one of these markers is recommended in the primary panel of antibodies for identification of B-cell ALL. Children with precursor B-ALL show more intense expression of the CD10 and less intense expression of CD20, CD45 and CD34.

Mature B-ALL show a unique immunophenotype characterized by relatively bright and homogenous CD10 and dim CD34 expression. These correspond to ALL L3 of FAB classification.

In our study, two cases were diagnosed as ALL L3 on morphology, and both these cases showed dim CD34 expression on FCA.

**T-cell acute lymphoblastic leukemia**

In the current study 18.18% of the cases have been grouped as T-cell ALL based on FCA. The monoclonal antibodies used were CD3 and CD7. CD3 is normally expressed on T-cells and thymocytes and is diagnostic of hematopoietic neoplasms of T-cell lineage.[11]

Aguilera et al.[11] compared the sensitivity of T lineage markers cyCD3, CD2, CD5, CD7 and CD1 and CD3 in their study on T-ALL cases and showed that cyCD3 has 100% sensitivity and 98% specificity followed by CD7 with 100% sensitivity but lesser specificity. Hence they concluded that cyCD3 is the best marker for T-cell ALL cases. According to other studies,[12–14] CD7 appears to be the pan-T-cell Ag, and has been detected in T-ALL with frequencies >95% to 100%.

The presence of CD7 positive blasts is by no means synonymous with a diagnosis of T-ALL because a large number of acute leukemia of B-lymphocytic and myeloid lineage has been associated with the expression of CD7 on the cell surface.[15] Traweek et al.[16] have reported CD7 expression in AML cases. They reported the possibility of association of CD7 with blast immaturity due to its association with M0, M1 and M5a. Hence, a diagnosis of T-ALL should be made with great caution in

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**Table 7: The concordance rates in various other studies**

| Studies       | Concordance rates % (lineage assignment concordance rate %) |
|---------------|-------------------------------------------------------------|
| Kheiri et al.[3] | 77.4 (95.8)                                                  |
| Mhawech et al.[2] | 80.32                                                        |
| Current study  | 58 (86)                                                      |
the presence of reactivity with CD7 because a large number of acute leukemia of B-lymphocytic and myeloid lineage has been associated with the expression of CD7 on the cell surface. A diagnosis of T-ALL should generally be made when other T-cell markers (e.g., CD2, CD3, and CD5) are also present in addition to the CD7 antigen. Although classifications attempt to represent various levels of T-cell maturation, Roper et al. found no unique clinical features among the subgroups and no difference in remission duration or survival.

CALLA antigen

78.8% of the cases in the current study expressed CD10 antigen and hence were called CALLA + B-ALL or T-ALL. CD10 (CALLA) Ag, found commonly in childhood B-cell leukemia, is an especially useful marker for the diagnosis of precursor B-cell ALL and for detecting early relapse in precursor B-cell ALL, as it is present only on a very small fraction of normal cells. By multipara metric analysis, approximately one CALLA + cell among 100,000 normal lymphocytes can be detected, providing a sensitive technique for early detection. The level of CD10 expression is of prognostic significance, as it correlates with chromosomal abnormalities. High CD10 levels (>3 × 10^3/cell) are characteristic of hyperdiploidy, low CD10 levels (1.8-4 × 10^3/cell) correlate with translocations between chromosomes 1 and 19 ([t(1;19)], and undetectable CD10 levels (<1.2 × 10^3/cell) are common in ALL patients with a translocation between chromosomes 4 and 11 ([t(4;11)(q21;q23)] translocation). CD10 positivity has been associated with good prognosis, whereas its negativity is reported to be associated with poor prognosis. CD10 is seen commonly in association with B lineage but it is not uncommon to find in T-ALL.

CD10 has been reported in 27% of T-ALL cases in the study by Dowell et al. against 80% cases in our study. Dowell et al. compared the immunologic and clinico-pathologic features of CALLA-positive and CALLA-negative T-ALL in their study and showed that patients with CALLA-positive and CALLA-negative T-ALL had similar clinico-pathologic features at diagnosis. Patients with CALLA-positive T-ALL were more likely to achieve a complete remission (95% vs. 83%) and tended to have an increased duration of event-free survival than did patients with CALLA-negative T-ALL.

17.6% of our cases expressed only CD34 and CD10, and therefore could not be assigned a B or T-cell lineage and have been called CALLA-positive ALL. However, further evaluation of these cases with a secondary panel of specific markers like cyCD22 and CD79a for B-ALL and cyCD3 for T-ALL could not be done due to the non-availability of these markers. Okabe et al. have evaluated 15 such cases in their study and tried to classify these cases based on molecular studies that included immunoglobulin and T-cell receptor genes. Their study revealed that these cases showed gene rearrangements at Jκ genes and supported the view that most CALLA-positive cells are neoplastic B-cell precursors. In our study, we could not perform a genotypic study due lack of lab facilities.

A similar case was reported by Desai et al. In their study the CALLA-positive ALL developed in a pediatric patient with hypoplastic/aplastic anemia. The patient went into spontaneous remission for AA/HA when treated but developed ALL post chemotherapy. He responded to standard ALL therapy and was in remission for 4 weeks. However, he returned with a relapse after 7 months.

Acute lymphoblastic leukemia with aberrant myeloid antigens

In the current study 21.2% ALL cases expressed myeloid antigens CD13 and or CD33, CD13 being more common. 5.26% B-ALL cases had an aberrant expression of CD7. Studies have reported that the co-expression of myeloid cell surface antigens is found in approximately 30-45% of childhood and adult B-lineage ALL, and these neoplasms are termed myeloid surface antigen-positive (My+) ALL. CD34 is normally expressed in T-cell lineage antigens and hence was called CALLA-positive ALL. However, further evaluation of these cases with a secondary panel of specific markers like cyCD22 and CD79a for B-ALL and cyCD3 for T-ALL could not be done due to the non-availability of these markers. Okabe et al. have evaluated 15 such cases in their study and tried to classify these cases based on molecular studies that included immunoglobulin and T-cell receptor genes. Their study revealed that these cases showed gene rearrangements at Jκ genes and supported the view that most CALLA-positive cells are neoplastic B-cell precursors. In our study, we could not perform a genotypic study due lack of lab facilities.

Flowcytometry in AML

AMLs comprised 28% of the cases in the current study. CD45, immunologic marker for gating purpose for the identification of myeloid blast cells, has been used in the current study as per the present consensus. Along with it CD34, CD33 and CD13 have been used in our primary panel of markers. The study by Basso et al. indicated that in cases where CD45 is not suitable for gating purposes because of the marked heterogeneity of bone marrow cells or the limited number of blasts present in the sample, CD13, CD33 and CD34 may be of great help.

AML cases in our study expressed only CD13 in 1/15 (6.67%), only CD34 in 3/15(20%) and both in 10/15 (66.67%) cases. The remaining one (6.66%) case was an AML M7, and it did not show CD13 or CD33 expression but showed an 88.75% CD41 expression.

CD33 is normally expressed by myeloid progenitor cells and is diagnostic of leukemia of myeloid lineage. CD13 is normally seen on myelomonocytes and is diagnostic of myeloid lineage leukemias. Aguilera et al. compared the sensitivity and specificity of the myeloid markers cyMPO, MPO, CD13, CD14 and CD33, and observed that cyMPO showed sensitivity of 100% and 97.5% specificity and thus concluded that it is the most specific marker for AML. Studies by Kaleem et al. compared the immunologic and clinico-pathologic features of CALLA-positive and CALLA-negative T-ALL in their study and showed that in cases where CD45 is not suitable for gating purposes because of the marked heterogeneity of bone marrow cells or the limited number of blasts present in the sample, CD13, CD33 and CD34 may be of great help.

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showed that CD33 is a more sensitive but less-specific marker than CD13 for AMLs.

One case in our study was classified as AML M7 on morphology. FCA showed CD41 positivity along with an aberrant CD7 expression. CD41 is normally expressed by megakaryocytes and platelets, and is diagnostic of acute leukemia of megakaryocytic lineage (AML, FAB-M7).\(^1\)

The secondary panel of Abs for AML in our study included the monoclonal antibodies, CD117 and CD14. CD14 is normally expressed by monocytic cells and its diagnostic application is the identification of myelomonocytic leukemias, particularly of FAB M4 and M5 subclasses.\(^1\) However, the study by Dunphy et al.\(^{27}\) showed that although CD14 is a monocyte-specific marker, depending on the FCI technique and the epitopes analyzed, CD14 is often absent or frequently diminished in expression in AMLs with monocytic differentiation, and concluded that CD64 is a more sensitive and specific marker for distinguishing AMLs with a monocytic component (i.e., AMML and AMoL) from other AML subtypes.

CD117 is a marker of myeloid precursor cells and its major diagnostic application is in the diagnosis of acute myeloid leukemias.\(^1\) CD117 was used in five cases with unclear morphology. Of these five cases, four were ALL and one was acute undifferentiated AML. In all these five cases, CD117 expression was negative.

A study was done at the TATA Memorial Hospital (February 2006), India, by Gujral et al.\(^{28}\) and they concluded that FCA studies are important in those cases of acute myeloid leukemia where blasts do not show Auer rods and are negative for MPO and NSE stains. Thus the subtypes like AML-M0 and, AML-M7 (may be NSE\(^+\)), need FCA for a definitive diagnosis. Classical cases of AML do not require expensive FCA studies.

The secondary panel of markers recommended by the British committee\(^{29}\) for the myeloid lineage are CD33, CD41, CD42, CD61 and anti glycophrin A. The consensus group recommends CD15, CD16, CD41, CD42b, CD61, CD64, CD117, anti cyMPO and, anti glycophrinA. However, in general all the AMLs on FCA express CD13, CD33, and CD117, and are negative for HLA-DR.

**Aberrant antigen expression in AML**

CD7 was seen to be expressed by 28.58% of AML cases. Lo Coco et al.\(^{30}\) reported common association of CD7 in AML. Traweek et al.\(^{31}\) reported the possibility of association of CD7 with blast immaturity in their study due to its association with M0, M1 and M5a. Studies by Sissy et al.\(^{32}\) suggest that CD7 expression in AML should be interpreted with cytophenetic as it may be associated with unfavorable cytogenetic.

Studies by Drexler et al.\(^{33}\) on AMLs expressing lymphoid-associated Ags showed that there is no cytophenetic anomaly specific for Ly\(^+\) AML; expression of lymphoid-associated antigens (with the exception of CD7) on AML blasts lacked prognostic; significance; CD7\(^+\) AML appears to be a particular subset of malignant myeloid progenitors and suggested that, in general, Ly\(^+\) AML may not represent a biologically distinct form of leukemia as these cases have similar clinical features and respond to therapy in a comparable manner.

The reason for the striking differences between conflicting reports on aberrant expressions is not clear. None of our four cases relapsed in a period of 2-year follow-up.

**Mixed lineage leukemia**

In the current study, one case that was inconclusive on morphology and cytochemistry, was assigned acute mixed lineage leukemia on FCA. Mixed lineage leukemia is defined as simultaneous distinct populations of leukemia cells of more than one lineage.\(^{34}\)

The mixed lineage leukemia was classified as AML M0/ALL L2 based on morphology as cytochemical staining showed MPO, SBB occasional cell positivity and PAS was block positive. FCA showed >50% blasts expressing B-cell markers (CD19) and myeloid markers (CD33 and CD13). Hence, this case was diagnosed as mixed lineage leukemia. Without the aid of FCA this case would have remained unclassified or misdiagnosed.

Traweek et al.\(^{35}\) showed that the precursor B-ALL is more prone to mixed lineage Ag expression. The study by Rubnitz et al.\(^{33}\) showed that the overall survival rates for mixed lineage leukemia were no different from AML, although they were inferior to that of children with ALL. Our case remained in remission with standard ALL therapy protocol in 2-year follow-up.

**Our study implicates the following merits and demerits of flowcytometry in the diagnosis of acute leukemias**

- In 58% of our completely concordant cases, FCA helped in confirming the morphologic diagnosis.
- In 22% of our partially concordant cases and 4% of non-concordant cases, FCA aided in identifying the correct lineage of the blasts and thus prevented misdiagnosis on morphology in these cases. FCA is particularly of help in identifying B and T-cell ALLs and also in measuring the expression of CALLA Ag which has prognostic implications in ALL patients.
- In 16% of our cases, morphology and cytochemistry could not help in rendering definitive diagnosis. FCA proved to be of utmost help in classifying and sub classifying these cases so that the patients could receive the respective therapy protocols.
- FCA was not found to be of much help in diagnosis of ALL L3 unless facility for measuring TdT expression is available.
- Similarly FCA was also found to be of not much help in the diagnosis of AML M1, M2, M3 and M4. These categories of AMLs are better identified on morphology.
- However, FCA can be of help in diagnosis of AMLM6 and M7 if specific antibodies like glycophorin A and CD41,
respectively, are available. FCA can also be useful in the diagnosis of doubtful cases of AML M5a/b by using specific antibodies like CD14 and CD64.

- The most important application of FCA is in identifying mixed lineage and bi-phenotypic leukemias and leukemias with aberrant Ag expression as these cannot be possibly diagnosed only on morphology and cytochemistry.

CONCLUSION

Thus, the study implicates that FCA can be of great help in the diagnosis of acute leukemias particularly ALL provided we have the facility for broad panel of primary and secondary markers and proper trained manpower to carry out the procedure and to interpret the results.

On comparison of morphologic and flow diagnoses, it was found that there was complete concordance in 58% cases, partial concordance in 22% of the cases and non-concordance in 4% cases between both the modalities. In the remaining 16% of the cases, where morphology failed to give any diagnosis, flow cytometric analysis could aid in rendering a confirmed diagnosis.

CD19 and CD10 were found to be the most useful markers in identification of B-ALLs, however, the literature has mentioned CD79a and cyCD22 to be the more reliable markers for B-ALLs which could not be used in our setting due to the unavailability of these markers.

CD3 and CD7 were consistently expressed by T-ALL. However, cyCD3 has been reported to be the best marker for T-ALLs. CD13 and/CD33 helped in identification of myeloid leukemias. However, cyMPO has been reported to be the best marker for the identification AML blasts but it was not available in our setting. Flow analysis did not prove superior to morphologic analysis in identifying AML, except in AML M6 and M7 (CD41 expression was seen in AML M7). The most common aberrant antigens expressed were CD13 and 33 in ALL and CD7 in AML.

Hence, FCA helped in lineage assignment particularly in ALLs, and it also helped in classifying the unclassifiable cases based on morphology and cytochemistry, like the mixed lineage and undifferentiated leukemias, and also in detecting aberrant expression.

REFERENCES

1. Riley RS, Massey D, Jackson-Cook C, Idowu M, Romagnoli G. Immunophenotypic analysis of acute lymphocytic leukemia. Hematol Oncol Clin North Am 2002;16:245-99, v.
2. Mhawech P, Buffone GJ, Khan SP, Gresik MV. Cytochemical staining and flow cytometry methods applied to the diagnosis of acute leukemia in the pediatric population: An assessment of relative usefulness. J Pediatr Hematol Oncol 2001;23:89-92.
3. Traweek ST. Immunophenotypic analysis of acute leukemia. Am J Clin Pathol 1989;99:504-12.
4. Stelzer GT, Shults KE, Loken MR. CD45 gating for routine flow cytometric analysis of human bone marrow specimens. Ann N Y Acad Sci 1993;677:265-80.
5. Borowitz MJ, Guenther KL, Shults KE, Stelzer GT. Immunophenotyping of acute leukemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukemic blasts in three-color analysis. Am J Clin Pathol 1993;100:534-40.
6. Sun T, Sangaline R, Ryder J, Gibbens K, Rollo C, Stewart S, et al. Gating strategy for immunophenotyping of leukemia and lymphoma. Am J Clin Pathol 1997;108:152-7.
7. Basso G, Buldini B, De Zen L, Orfao A. New methodologic approaches for immunophenotyping acute leukemias. Haematologica 2001;86:675-92.
8. Traweek ST. Immunophenotypic analysis of acute leukemia. Am J Clin Pathol 1993;99:504-12.
9. Jabbour EJ, Fadrel S, Kantrajan HM. Adult acute lymphoblastic leukemia. Mayo Clin Proc 2005;80:1517-27.
10. Scheuermann RH, Racila E. CD19 antigen in leukemia and lymphoma diagnosis and immunotherapy. Leuk Lymphoma 1995;18:385-97.
11. Paredes-Aguilera R, Romero-Guzman L, Lopez-Santiago N, Burbano-Ceron L, Camacho-Del Monte O, Nieto-Martinez S. Flow cytometric analysis of cell-surface and intracellular antigens in the diagnosis of acute leukemia. Am J Hematol 2001;68:69-74.
12. Garand R, Vannier JP, Béné MC, Faure G, Favre M, Bernard A. Comparison of outcome, clinical, laboratory, and immunological features in 164 children and adults with T-ALL. Leukemia 1990;4:739-44.
13. Link M, Warnke R, Finlay J, Aymon M, Miller R, Dilley J, et al. A single monoclonal antibody identifies T-cell lineage of childhood lymphoid malignancies. Blood 1983;62:722-8.
14. Morishima Y, Kobayashi M, Yang SY, Collins NH, Hoffmann MK, Dupont B. Functionally different T lymphocyte subpopulations determined by their sensitivity to complement-dependent cell lysis with the monoclonal antibody 4A. J Immunol 1982;129:1091-8.
15. Zutter MM, Martin Pj, Hanke D, Kidd PG. CD7+ acute non-lymphocytic leukemia: Evidence for an early multipotential progenitor. Leuk Res 1990;14:23-6.
16. Roper M, Crist WM, Metzgar R, Ragab AH, Smith S, Starling K, et al. Monoclonal antibody characterization of surface antigens in childhood T-cell lymphoid malignancies. Blood 1983;61:830-7.
17. Childs CC, Hirsch-Ginsberg C, Walters RS, Andersson BS, Reuben J, Trujillo JM, et al. Myeloid surface antigen-positive acute lymphoblastic leukemia (My+ ALL): Immunophenotypic, ultrastructural, cytogenetic, and molecular characteristics. Leukemia 1989;3:777-83.
18. Lavabre-Bertrand T, Janossy G, Ivory K, Peters R, Seeker-Walker L, Porwit-MacDonald A. Leukemia-associated changes identified by quantitative flow cytometry. I. CD10 expression. Cytometry 1994;18:209-17.
19. Dowell BL, Borowitz MJ, Boyett T, Edward C, Russell, Falletta T, et al. Immunologic and clinopathologic features of common acute lymphoblastic leukemia antigen-positive childhood T-cell leukemia. Cancer 1987;59:2020-6.
20. Okabe M, Matsushima S, Fukuhara T, Tanaka M, Sakurada K, Kakinuma M, et al. Non-T, non-B acute lymphoblastic leukemias: Cellular origin based on molecular analyses of immunoglobulin and T-cell alpha- and beta-chain receptor gene rearrangements. Tohoku J Exp Med 1987;152:197-207.
21. Desai N, Vohra P, Pati H, Choudhry VP. Hypoplastic anemia: A preleukemic state in acute lymphocytic leukemia. Indian Pediatr 1991;28:1186-9.
22. Khalidi HS, Chang KL, Medeiros LJ, Brynes RK, Slovak ML, Murata-Collins JL, et al. Acute lymphoblastic leukemia. Survey of immunophenotype, French-American-British classification, frequency of myeloid antigen expression, and karyotypic abnormalities in 210 pediatric and adult cases. Am J Clin Pathol 1999;111:467-76.
23. Bradstock KE. The diagnostic and prognostic value of immunophenotyping in acute leukemia. Pathology. 1993;25:367-4.
24. Bradstock KE, Kirk J, Grimsley PG, Kabral A, Hughes WG. Unusual immunophenotypes in acute leukaemias: Incidence and clinical correlations. Br J Haematol 1989;72:512-8.
25. Unal S, Cetin M, Tuncer AM, Gümrük F, Yetgin S. The prognostic impact of myeloid antigen expression in pediatric acute lymphoblastic leukemia patients. Turk J Pediatr 2008;50:533-6.
26. Kaleem Z, Crawford E, Pathan MH, Jasper L, Covinsky MA, Johnson LR, et al. Flow cytometric analysis of acute leukemias. Diagnostic utility and critical analysis of data. Arch Pathol Lab Med 2003;127:42-8.
27. Dunphy CH, Tang W. The value of CD64 expression in distinguishing acute myeloid leukemia with monocytic differentiation from other subtypes of acute myeloid leukemia: A flow cytometric analysis of 64 cases. Arch Pathol Lab Med 2007;131:748-54.
28. Gujral S. Role of Flow Cytometry in Diagnosis of Hematolymphoid Malignancies. 4th conference on evidence based management of cancer in India, Available from: http://www.TATA Memorial Hospital_, Mumbai-India.mht. [Last accessed 2006 Feb 25].
29. Bain BJ. Diagnosis and classification of acute leukemia (chapter 29). In: Hoffbrand AV, Catovsky D, Edward GD, editors. Post graduate Haematology. 5th ed. Blackwell Publishing; 2005. p. 483.
30. Lo Coco F, De Rossi G, Pasqualetti D, Lopez M, Diverio D, Latagliata R, et al. CD7 positive acute myeloid leukaemia: A subtype associated with cell immaturity. Br J Haematol 1989;73:480-5.
31. El-Sissy AH, El-Mashari MA, Barsuni WY, El-Swaayed AF. Aberrant lymphoid antigen expression in acute myeloid leukemia in Saudi Arabia. J Egypt Natl Canc Inst 2006;18:244-9.
32. Drexler HG, Thiel E, Ludwig WD. Acute myeloid leukemias expressing lymphoid-associated antigens: Diagnostic incidence and prognostic significance. Leukemia 1993;4:489-98.
33. Rubnitz JE, Onciu M, Pounds S, Shurtleff S, Cao X, Raimondi SC, et al. Acute mixed lineage leukemia in children: The experience of St Jude Children’s Research Hospital. Blood 2009;113:5083-9.

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