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Medical College, Pakistan

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Shahid Pervez
Aga Khan University, shahid.pervez@aku.edu

M. Khurshid
Aga Khan University

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Classification and Immunophenotyping of Acute Leukemias: A Prospective Study

Pages with reference to book, From 103 To 106

S. Pervez, M. Khurshid (Department of Pathology, The Aga Khan University Hospital, Stadium Road, Karachi.)

Abstract

Over a period of 3-1/2 years, 86 cases of acute leukemia were analyzed by immunohistochemical (IHC) means on ficoll separated cytospin preparations of peripheral blood and/or bone marrow samples. Antibodies included in the panel were specific against Tdt, HLA-DR, CD19/CD2O/CD22, CALLA (CD1O), CD2, CD11C as well as against Ig heavy chains. Of 86 cases analyzed, 48 cases were of ALL, (25 of common pre-B ALL, 15 of pre-B/NULL and 8 of T ALL phenotype), twenty-four (24) out of 86 cases were of nonlymphoblastic (AML/AMML) type. In six cases, there was suggestion of a mixed lineage, while in 8 cases there was inconclusive diagnosis. Mean age was lower in common ALL sub-set of ALL as compared to pre-B/NuIl gmup (i.e., 8 vs 12 years), while in non-lymphoblastic group it was 36 years. T cell phenotype was invariably seen in young adults, who usually presented with a mediastinal mass (JPMA 47:103,1997.

Introduction

Uptil recent past, the hallmark for the diagnosis of acute leukemias has been the morphology and cytochemistxy. However, due to major developments inthefieldofinirnunology, like recognition of cell surface markers[1] and development of monoclonal antibodies (MABs)[2], the classification of acute leukemias is now widely based on combined morphological, cytochemical and immunophenotypic approach[3]. Immunohistochernical (IHC) analysis usually allows accurate information concerning the lymphoid or myeloid nature of leukemia, whetherit is of mature orimniature differentiation and of B or T cell lineage[4]. This analysis is mandatoiy when the leukemic blasts are morphologically undifferentiated and negative forthecytochemical reactions characteristic of myeloid cells. In these cases, immunophenotypic analysis may classify whetherthe case corresponds to one of the sub~types of ALL orto poorly differentiated acute myeloid leukemias[5]. In addition to this, particularly in ALL, immunologic classification appears to be capable of defining prognostically significant sub-gmups[4]. Acute leukemias can be analyzed by immunofluorescence on viable unfixed cells in suspension or by immuno-enzymatic techniques like immunoperoxidase and immuno-alkaline phosphatase (APAAP) on fixed cells on slide and by flow cytometry using cell suspension[6]. We have been using inimunoperoxidase technique for analyzing acute leukemias. When a blood or bone marrow sample is sent to us for analysis, usually two questions are asked, i.e., lymphoid ormyeloid nature of acute leukemia and identifying prognostically significant sub-sets inparticularwithin ALL group.

Materials and Methods

In a prospective study, 86 cases of acute leukemias were analyzed over a period of 3-1/2 years (1992-1995). Immunohistochemical analysis was carried out on peripheral blood and bone marrow samples. Most analysis (83%) were carried out on peripheral blood samples which were actually preferred if it contained atleast 50% blast population. This was essential, as if the proportion of normal cells increased, the phenotypic analysis of the leukemia became difficult and was complicated by the

staining pattern of normal cells. A panel of antibodies was always used for each specimen to increase the sensitivity and specificity of the tests. It was concluded that no single reagent can define adequately the phenotype of leukemic cells and reporting physician must be aware of the cross reactivity problem7. 2-5 cc of peripheral blood/bone marrow aspiration samples were received in heparimzed tubes. All specimens were invariably processed 2-4 hours after venepuncture and refrigerated samples were never accepted to avoid the chances of inclusion of degenerated cells. After obtaining complete and differential counts, mononuclear cells were separated by ficoll density centrifugation and washed thrice in RPM1 medium. This procedure usually avoids any RBCs and neutrophilic contamination, as both of these populations are rich in endogenous peroxidases. After adjustments in cell count, cytospin preparations were made. Atleast 10 such preparations were made on each occasion and immediately fixed in acetone for 10 minutes. These cytospin preparations were then either used immediately or stored at -70°C foil wrapped until stained.

Antibodies

The panel of antibodies used for leukemia analysis included Tdt (terminal deoxynucleotidyl transfemse), CD20, CD22, CD 19, CD10 (CALLA), CD2, CDI 1C (P150,95) and immunoglobulin heavy chain (IgG and 1gM), all from Dakopotts, Denmark.

Immunoperoxidase procedure

Cytospin preparations were briefly dipped in 0:3% H202 - methanol for 10 minutes to block endogenous peroxidase. After brief wash in tns buffer (pH 7.2), possible background reactions (due to non-specific protein binding sites and FC receptors) was blocked by incubating sections with 10% normal swine serum (NSS) for 15 minutes. No washings were perfonned afterthis step. Afterdraining excess serum sections were then incubated with primaiy antibodies appropriately diluted in phosphate buffered saline (PBS) with 1% NSS. Dilutions for various antibodies were Tdt (1:5), CD20, CD22 and CD19 (1:25), CD10 (1:20), CD2 and CD11C (1:10), IgG and 1gM (1:500). Primary antibody incubations were kept overnight in a humid chamber at 4°C (refrigerated), Next morning, after washing intris buffer (3x5 min each), sections were incubated with 2nd layerfor 1/2 hour at room temperature. After another 3 washings (5 mineach) in counterstained lightly withHarris hematoxylin, differentiated in acid alcohol, dehydrated, cleaned and mounted in synthetic mountant.

Results

Blood samples were used in 83% of the total acute leukemias analyzed. This was preferred because of easier approach to obtain blood sample, consistency in volume obtained and less cross reactivity. The reagents used to characterize and distinguish acute leukaemias are outlined in Table I.

Table I. Guide to phenotypic profiles in acute leukemia.

	Tdt	HLA-DR	CD19/CD20/CD22	CALLA (CD10)	Cμ (IgM)	SIg	T11 (CD2)	CD11C
Common ALL	+	+	+	+	-	-	-	-
Pre-B/Null ALL	+	+	+	-/+	-/+	-	-	-
T ALL	+	-/+	-	-	-	-	+	-/+
AML	-	+/-	-	-	-	-	-	+/-

+/-= Majority of cases positive (80%).
-/+= Rare cases positive (0-20%).
Tdt= Terminal deoxynucleotidyl transferase.
Cm= Cytoplasmic mμ (IgM) chain.
SIg= Surface immunoglobulin.
>10%= CALLA (CD10)+cells places DR+ALL in the common sub-type.

Anti-Tdt was the main marker which was included in the panel to distinguish ALL fromAML. This antibody detects the nuclear enzyme terminal deoxynucleotidyl transferase (Tdt) withbrown nuclear staining (Figure 1).

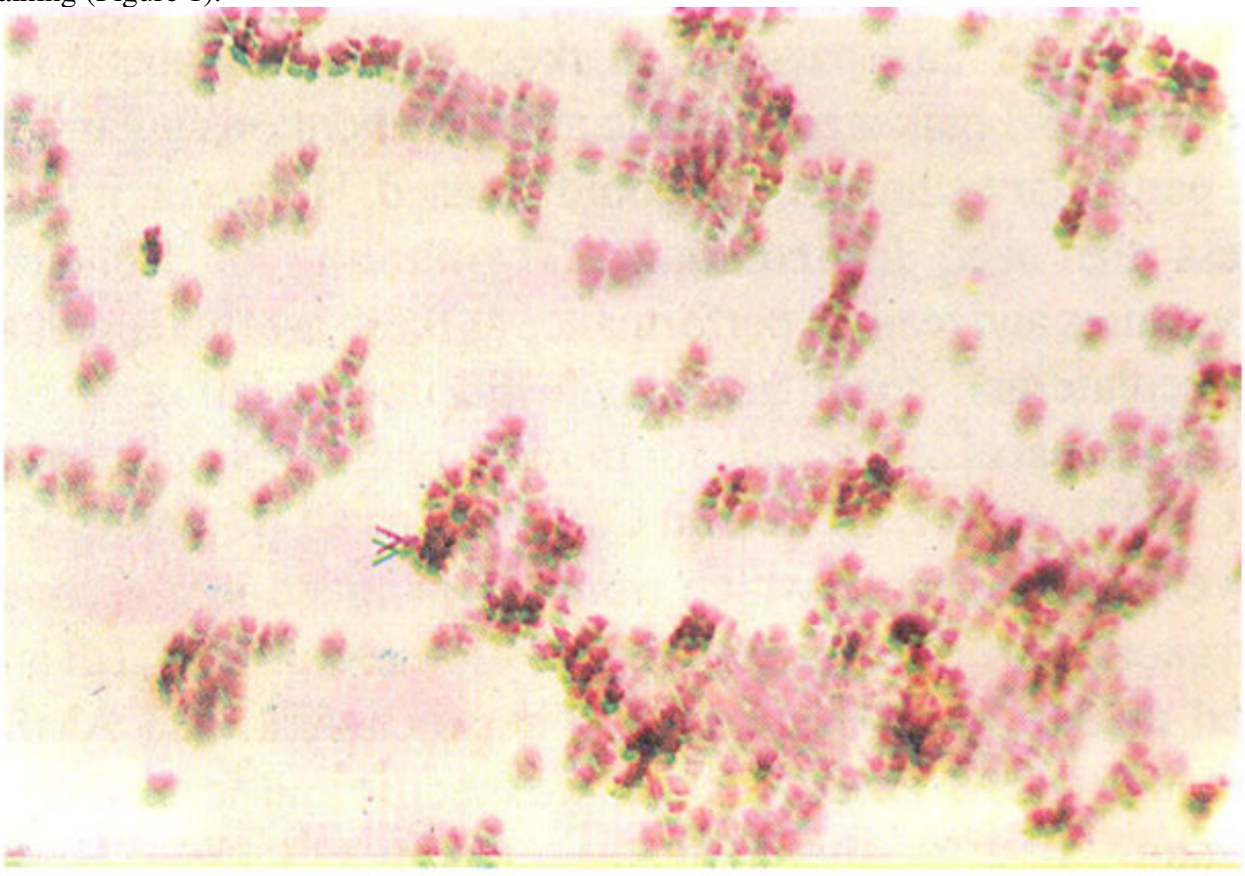

Figure 1. Peripheral blood cytospin preparation of a child stained with a monoclonal antibody against Tdt. Note strong nuclear staining (arrow) consistent with ALL.Immunoperoxidase staining, 10X.

Tdt showed 80% sensitivity to detect ALL while the remaining 20% cases which were finally labelled as pre-B ALL were reactive to B markers and non reactive to T (CD2) and myeloid (CD11c) markers. Four (4) B-associated (lymphoid) markers, CD 19, CD20, CD22 and CD10 (CALLA) were included in the panel. By utilizing these antibodies, we were able to categorize most non-T (pre-B) ALL. CD 19 showed the highest sensitivity and labelled 90% of pre-B ALL. This was followed by CD20 and CD22. CD10 (CALLA) labelled 56%ofpre-B ALL and these cases were classified as common ALL sub-set of pre-B ALL (a good prognostic indicator, Figure 2).

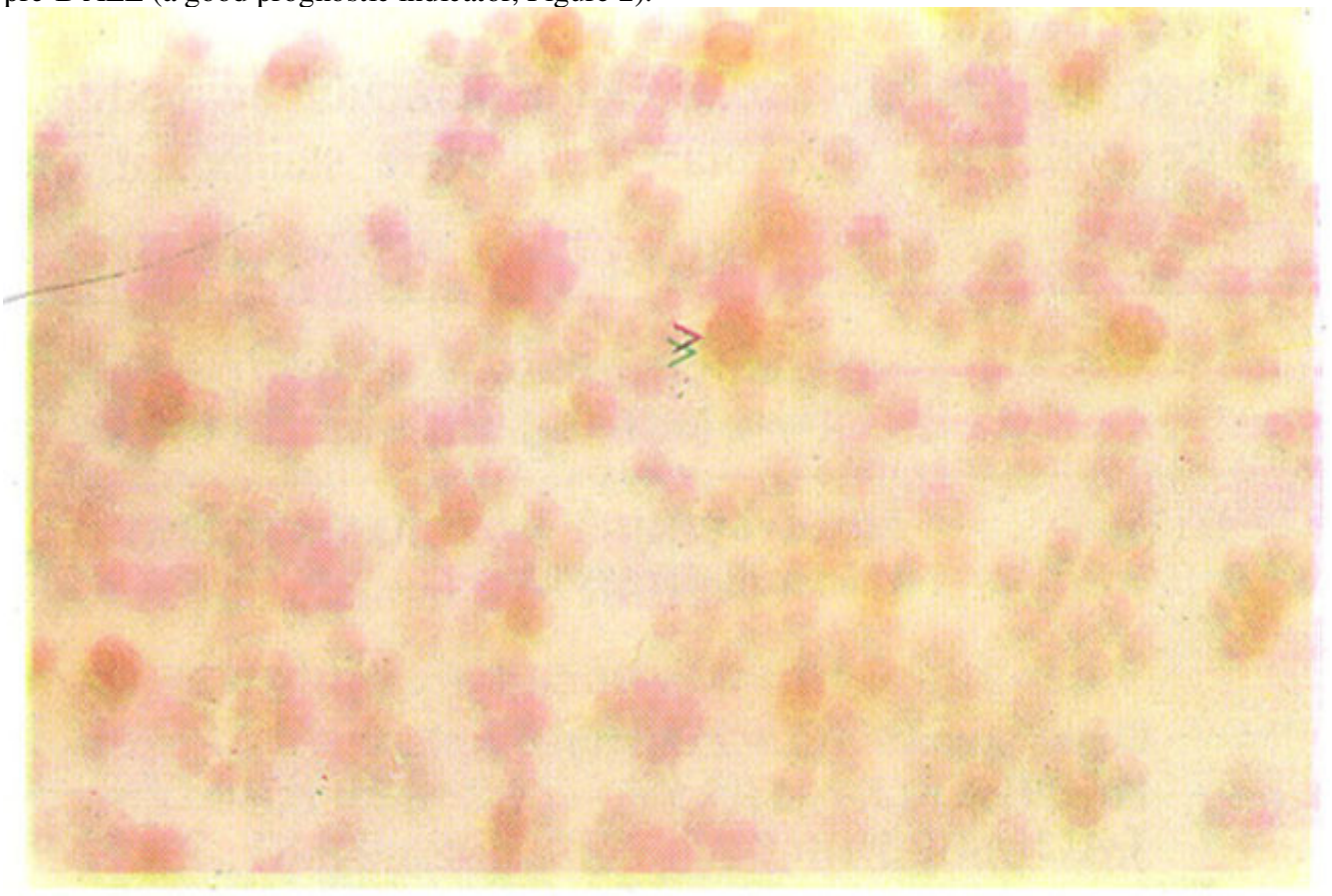

Figure 2. Peripheral blood cytospin preparation of a child stained with a monoclonal antibody against CALLA (CD10).Note strong membrane staining of some blast cells (arrow) consistent with common pre-B ALL Immunoperoxidase staining, 25X.

A rather non-specific marker, HLA-DR which was always included in the panel showed 100% reactivity with all non-TALL. This markerwas also used as positive control and the staining intensity of cells with this antibody helped us to assess the staining quality. Whenever, weak intensity of staining was obtained with this antibody, staining was repeated. T-ALL usually showed none or only weak focal reactivity to HLA-DR, while they invariably reacted with a single T cell marker (CD2) included in the panel. CD11c which was used as a myeloid marker was always interpreted withcaution, because of its cross-reactivity with some T-ALL or mature B cell (hairy cell) leukemias. Its reactivity with blast cells only in presence of negative Tdt and other B markers was taken as evidence of myeloid lineage leukemia. Antibody to 1gM was sometimes used to detect cytoplasmic ui chain (cyt 1gM) in CALLA negative ALL to confinn its pe-B phenotype. With this immunohistochemical technique, most acute leukemias (86%) were classified as lymphoid or myeloid. However, a small percentage (7%) of leukemias were suggested to be of bi-phenotypic or mixed lineage, in which the blasts expressed a constellation of myeloid and lymphoid antigens. Nine (9%) percent cases were unclassifiable. In these

cases, all the markers used in the screening were negative except focal positivity for DR only (Table II).

Table II.

Total cases=86 (acute leukemias)		
ALL=48 (56%)	Mixed =6 (7%) Unclassifiable 8 (9%)	AML= 24 (28%)
——— Common pre-B ALL = 25 (29%)		
——— Pre-B/Null ALL = 15 (17.5%)		
——— T-ALL = 8 (9.5%)		
	Mean age	
Common pre-B ALL	..	8 years
Pre-B/Null ALL	..	12 years
T-ALL	..	33 years
Non-lymphoblastic (AML/AMML)	..	36 years

For all the markers used in the panel, them is no consensus onthe cut off pointwhichwill indicate thatasample is positive for the marker or not. However, a cut off point of 10% was recommended when assessment was made using immunocytochemistiy6, so if with a particular antibody for instance anti-Tdt >10% blast cells were stained, it was interpreted as positive. Mean age was lower in common ALL sub-set of pre-B ALL as compared to pm-B/Null ALL group(i.e., 8 vs 12 years), while mean age in non- lymphoblastic group was 36 years. T cell phenotype was invariably seen in young adults (mean age 33 years) who usually presented with a mediastinal mass (Table II).

Discussion

Recent advances in immunology have led to important insight into leucocyte differentiation and the cellular origin of leukemias. Leukemias arise from dysregulated clonal expansion of immature lymphoid or myeloid progenitor cells that are blocked at a particular stage of differentiation. Most leukemic cells share features with normal lymphoid or myeloid progenitors. By establishing the pattern of developmentally relevant antigen expression, one can classify a leukemic cell according to normally recognized maturation sequences. Since the myeloid leukemias are more easily studied with morphological and cytochemical criteria than are lymphoidleukemiasandas they are much more heterogenous, our panel mainly included lymphoid markers alongwith anti-Tdt by which a distinction can be made between most ALL and Lymphoid markers also helped in determining pmgnostically

significant subsets of ALL.
With the passage of time, the classification of acute leukemias is now widely based on combined morphological, cytochemical and immunophenotypic approach. Difficulties are however, frequently encountered inreachinganacceptable degree of diagnostic concordance between different laboratories because of variations in the techniques used in terms of methodology, reagents and equipment as well as interpretation. This issue was recently addressed in depth by International Council for Standardization in Hematology (ICSH) [3]. In proposing the 'core panel' of markers for immunophenotyping, the ICSH makes no specific recommendations regarding the particular technique that could be used. However, it does stress the need for continuous monitoring of technical procedures and reagent performance as these are fundamental to accurate and consistent diagnostic interpretation. ICSH minimum panel of reagents for immunophenotyping includes Tdt, CD22, CD 19, CD10 and CD2 besides CD13 and CD33 (myeloid associated), which were not included in our panel and another myeloid marker CD! lCwas used instead. Tdtis a good marker to make distinction between ALL and AML. Although it is not absolutely lineage specific and according to literature, 10-20% AML may show reactivity to Tdt[9]. However, in ourseries no suchcross reactivity was observed. Inthis study, sensitivity of this markerto stainbothB and TALL was approximately 80%, whic his consistent with other studies[8]. In various studies, 50-75% of the ALL expressed CALLA (CD 10) antigen[10]. In our study, 56% of pre-B phenotypic leukemias expressed this antigen, which is well established as a favourable prognostic indicator[9]. The T-ALL are important to recognize and they are considered to be the high risk group". CD2 proved to be a sensitive and specific marker for this group while this was mostly associated with HLA-DR negativity. In this study, 9% of the cases were of T cell phenotype which appear to be close to western figures (10-20%). CDIIC appeared to be a rather less specific marker for myeloid leukemia and it was difficult to interpret this marker in isolation. However, the use of a panel of antibodies helped to reach to a conclusion in most cases. In a small sub-group of cases, leukemic blast cells expressed both lymphoid and myeloid associated antigens. In literature, this is variously explained as the result of aberrant regulation of gene expression, malignant transformation of pluripotent stemcells or rare progenitor cells that normally co-express features of more than one lineage[12,13]. These cases were classified as either myeloid antigen positive ALL or lymphoid antigen positive AML (mixed lineage). One of our cases which was immunophenotypically typical ALL showed Philadelphia chromosome positivity. Some investigators believe that myeloid antigen positive ALL is associated with a poor prognosis[14,15] , however, others have found that this type of leukemia responds well to intensive therapy[16]. It was also shown that lymphoid antigen positive AML may respond to therapy directed to lymphoid cells if the initial treatment is unsuccessful[16,17].
In summary, this study demonstrates the increasing importance of immuno-histochemical characterization of undifferentiated leukernias and/or to identify prognostically significant sub-sets, in particular within ALL group.

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