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Immunophenotyping of Acute Lymphoblastic Leukemia in Pediatric Patients by Three-color Flow Cytometric Analysis

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SUMMARY **Immunophenotyping of acute lymphoblastic leukemia (ALL) in children using three-color flow cytometry was carried out at Chulalongkorn Hospital, Bangkok, Thailand. Of 38 patients with acute lymphoblastic leukemia, 65.8% were identified as common ALL, 15.8% were B-ALL, and 18.4% were T-ALL. Of these 38 cases, there were 4 cases of infantile leukemia. Relapsed cases of leukemia were found most in B-ALL up to 3 out of 6 cases and to a lesser extent in T-ALL (1 of 7 cases) and c-ALL (1 of 25 cases). Our data showed the CD markers expression for common ALL (c-ALL) were $CD19^+/10^+$(100%), $CD20^+$ (24%), $CD22^+$ (100%), HLA-DR^+ (70.1%), and $CD34^+$ (58.8%). CD markers expression for B-ALL were $CD19^+$ (100%), $CD20^+$ (33.3%), $CD22^+$ (80%), and HLA-DR^+ (80%). CD markers expression for T-ALL were $CD3^+$ (42.9%), $CD5^+$(100%), $CD7^+$ (85.7%). Myeloid aberrant expressions were found in c-ALL (25-37.5%), B-ALL (20%), and T-ALL (14.3%). The significance of the aberration is discussed. The immunophenotyping classification of ALL as c-ALL, B-ALL, and T-ALL is useful in prognosis and treatment.**

The French American British (FAB) classification,[1] based on morphology and cyto-chemical reactions has been used in the diagnosis of leukemias. It is, however, dependent largely on the intensive experience of the examiners. The advent of monoclonal antibodies together with immunocytochemistry staining have made the classification of immunophenotyping of leukemia possible.[2] The advantage of the latter classification is that acute lymphoblastic leukemia (ALL) can be further sub-classified into subtypes of B lineage or T lineage. The B lineage can be further subclassified into CALLA+ or common-ALL, CALLA- or B-ALL, and matured B-ALL (FAB:L3).[3] The subtypes based on CALLA (CD10) are useful in prognosis.[4,5,6] This is not possible in the conventional FAB-morphologic classification. However, cellular immunological staining has several drawbacks, *e.g.* subjective, experience required, impractical in multiple marker staining, etc. These drawbacks have been overcome by flow cytometry technology and therefore it has been widely used in the diagnosis of leukemia. Recently, Borowitz *et al.*[7] have improved the immunophenotyping of acute leukemia by using three-color immunofluorescence analysis. It eases multiple immunologic marker staining as well as the quantitation of positive cells. The above have implications on the findings of the lymphoid aberrant in AML[8,9] and fluorescent intensity in ALL[10] which are important in predicting therapeutic outcome. In this report we have used three-color flow cytometry in immunophenotyping the leukemia in childhood at Chulalongkorn Hospital.

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MATERIALS AND METHODS

Specimens

Patients in this study were admitted to the hematology section of the Department of Pediatrics at Chulalongkorn Hospital from March 1997 to December 1998. Bone marrow aspiration was collected in heparin-RPMI-1640 media and push through a 21-gauge needle to break the clumps. Bone marrow-mononuclear cells (BMMC) were separated by Ficoll Hypaque density gradient centrifugation.

Antibodies

The antibodies used in this study were panels of mouse monoclonal antibodies in Acute Leukemia Immunophenotyping kit (Becton Dickinson), except for anti-glycophorin A (GPA) which was obtained from Immunotech Corp. Anti-IgG2a and IgG1 are mouse isotype control antibody cells. Anti-CD10 is an antibody to CD10 antigen, expressed on early B progenitor cells, early T-lymphocytes, and also on germinal center cells. Anti-CD19 is an antibody to CD19 antigens, expressed at all stages of B lymphocytic maturation. Anti-CD20 is an antibody to CD20 antigen, expressed on B lymphocytes, synchronous with expression of cell-surface μ chain. Anti-CD5 is an antibody to CD5 antigen, expressed on all thymocytes and mature T-lymphocytes. Anti-CD3 is an antibody to T-cell receptor (TCR) complex, and is specific for T-lymphoid lineage in ALL. Anti-CD22 is an antibody to CD22 antigen which is expressed in most B cell leukemias. Anti-CD7 is an antibody to CD7 antigen, expressed throughout T-lymphocyte differentiation. Anti-CD33 is an antibody to CD33, expressed on myeloid precursors. HLA-DR is an antibody to HLA-DR antigen, expressed in acute B-lymphoid, T-lymphoid, myeloid, and undifferentiated leukemia. Anti-CD13 is an antibody to CD13 antigen, expressed on neutrophils, basophils, monocytes. The majority of patients with acute myeloid leukemia (AML), expressed the CD13 antigen, but less frequently than CD33 antigens. CD34 is an antibody to CD34 antigen, presentation on immature hematopoietic precursor cells. CD14 is an antibody to CD14 antigen, recognized a human monocyte/macrophage antigen. CD61 is an antibody to CD61 antigen, found on all normal resting and activated platelets. CD11b is an antibody to CD11b, recognized a human leukocyte antigen that is the C3bi complement receptor. CD71 is an antibody to CD71 antigen that is specific for the human transferin receptor. GPA is an antibody to glycophorin A, present on human red blood cells and erythroid precursors.

Immunological staining

A volume of 50 μl of 5 x 10^6/ml of viable BMMC was reacted with 5 μl of anti-CD45-Per CP as the third color in all tubes. In addition, volumes of 10 μl of each pairs of the fluorescein isothiocyanate (FITC) conjugated (1st color) and the phycoerythrin (PE) conjugated (2nd color) reagents, listed in Table 1 were added into each tube. After 30 minute incubation in room temperature, they were washed 2 times with 2 ml of phosphate buffer saline (PBS). Finally, they were fixed in 0.5 ml of 1% paraldehyde for 30 minutes.

Flow cytometric analysis

Samples were analyzed on FACS-Calibur (Becton Dickinson). The blast cells were gated by CD45-Per CP versus SSC plot. The gated cells were enumerated for CD markers by dual-parameters (FITC versus PE) plot in each tube. The percentages of cells expressing the markers were recorded from the quadrant statistic. Criteria for positivity were having greater than 20% positive cells for lymphoid markers (CD3, 5, 7, 10, 19, 20, 22, HLA-DR) and greater than 30% positive cells for myeloid markers (CD11b,

Table 1 Three-color parameters of antibodies to CD markers in acute leukemia profile

Tube no.	1st color (FITC)	2nd color (PE)	3rd color (Per CP)
1. Unstained/Auto-fluorescence	-	-	CD45
2. Controls	IgG2a	IgG1	CD45
3. CD10/19	CD10	CD19	CD45
4. CD20/5	CD20	CD5	CD45
5. CD3/22	CD3	CD22	CD45
6. CD7/33	CD7	CD33	CD45
7. HLA-DR/13	HLA-DR	CD13	CD45
8. CD34/14	CD34	CD14	CD45
9. CD61/11b	CD61	CD11b	CD45
10. CD71/GPA	CD71	GPA	CD45

13, 14, 33, 34, 61, 71, GPA). Assignment to the leukemia immunophenotypes was based on the expression of at least two core lineage markers of the corresponding immunophenotypes.

RESULTS

Common-ALL (c-ALL)

As shown in Table 3, c-ALL which is the major type of ALL was identified in 25 of 38 cases or 65.8% of the total ALL cases. In Table 4, the major CD markers are CD19+/ CD10+ and CD22+/CD3- at 100% expression of each, followed by CD13-/HLA-DR+ (70.1%), CD14-/CD34+ (58.8%), and CD5-/CD20+ (24%). Interestingly, there is aberrant expression of T-cell lineage such as CD5+/CD20- (4%), CD5+/CD20+ (4%). In addition, there are 25% of CD33+/CD7- and 37.5% of CD13+/ HLA-DR+ expressions of the myeloid lineages in the diagnosed c-ALL's.

B-ALL

We could identify B-ALL in 6 out of 38 cases, or 15.8% of the total ALL cases. The major CD markers are CD19+/CD10- (100%), CD22+/CD3- (80%), CD13-/HLA-DR+(80%), and CD5-/CD20+ (33.3%). We also found 1 case of B-ALL that expressed CD5+ as well as CD33+ and CD13+/HLA-DR+.

T-ALL

We identified 7 cases out of 38 ALL cases, accounting for 18.4% of the total ALL cases. The major CD markers are CD5+/ CD20- (100%), CD33-/CD7+ (85.7%), and CD22-/CD3+ (42.9%). We found myeloid aberrant expression in 1 case. There were expressions of CD34+ and CD71+; 2 cases in each. We also found 1 case expressing HLA-DR+ which was a full blown relapsed case of T-ALL.

DISCUSSION

In a total of 38 ALL's admitted to the Pediatric Department, we could identify 65.8% to be c-ALL's. However, of these 33 were newly diagnosed ALL. Of the newly diagnosed ALL, 72.7% were c-ALL. This is less than the reports in childhood B-ALL (90%)[11] and in adult B-ALL (88%).[9] This subtype all expressed CD19+/10+ and CD22+/CD3-. In c-ALL, there are 37.5% myeloid aberrant expression of CD13+/HLA-DR+ and 25% of CD33+/CD7-. The percentages of aberrant myeloid markers are similar to that reported by Loken *et al.*[11] and Sobol *et al.*[9] There were 2 cases of c-ALL which might have been biphenotypic leukemia[12,13,14] owing to their co-expression of CD13+ and CD33+ with high percentages of

Table 2. CD marker-profile for acute lymphoblastic leukemia

Immunophenotypes	CD markers
ALL: c-ALL	CD10, CD19, CD20, CD22, CD34, HLA-DR
: B-ALL	CD19, CD20, CD22, CD34, HLA-DR
: T-ALL	CD3, CD5, CD7

Table 3. Immunophenotypes of childhood acute lymphoblastic leukemia at Chulalongkorn Hospital (1997-98)

Status	Number	No. of patients (percentage)		
		Common ALL	B-ALL	T-ALL
Newly diagnosed	33	24 (72.7%)	3 (9%)	6 (18.2%)
Relapsed	5	1 (20%)	3 (60%)	1 (20%)
Total	38	25 (65.8%)	6 (15.8%)	7 (18.4%)

Table 4 Marker expression of acute lymphoblastic leukemia in pediatrics at Chulalongkorn Hospital (1997-1998)

CD markers	Number of patients giving positive results* (Percentages of each subtypes)		
	c-ALL	B-ALL	T-ALL
CD19+/CD10-	1/25 (4%)	6/6 (100%)	0/7 (0%)
CD19-/CD10+	0/25 (0%)	0/6 (0%)	0/7 (0%)
CD19+/CD10+	25/25 (100%)	0/6 (0%)	0/7 (0%)
CD5+/CD20-	1/25 (4%)	1/6 (16.7%)	7/7 (100%)
CD5-/CD20+	6/25 (24%)	2/6 (33.3%)	0/7 (0%)
CD5+/CD20+	1/25 (4%)	0/6 (0%)	0/7 (0%)
CD22+/CD3-	24/24 (100%)	4/5 (80%)	0/7 (0%)
CD22-/CD3+	0/24 (0%)	0/5 (0%)	3/7 (42.9%)
CD22+/CD3+	0/24 (0%)	0/5 (0%)	0/7 (0%)
CD33+/CD7-	6/24 (25%)	1/5 (20%)	0/7 (0%)
CD33-/CD7+	0/24 (0%)	0/5 (0%)	6/7 (85.7%)
CD33+/CD7+	1/24 (4.2%)	0/5 (0%)	0/7 (0%)
CD13+/HLA-DR-	1/24 (4.2%)	0/5 (0%)	1/7 (14.3%)
CD13-/HLA-DR+	17/24 (70.1%)	4/5 (80%)	1/7 (14.3%)
CD13+/HLA-DR+	9/24 (37.5%)	1/5 (20%)	0/7 (0%)
CD14+/CD34-	1/17 (5.9%)	0/4 (0%)	0/5 (0%)
CD14-/CD34+	10/17 (58.8%)	1/4 (25%)	2/5 (40%)
CD14+/CD34+	1/17 (5.9%)	0/4 (0%)	0/5 (0%)
CD11b+/CD61-	1/14 (7.1%)	1/4 (25%)	0/5 (0%)
CD11b-/CD61+	1/14 (7.1%)	0/4 (0%)	0/5 (0%)
CD11b+/CD61+	0/14 (0%)	0/4 (0%)	0/5 (0%)
GPA+/CD71-	0/15 (0%)	1/4 (25%)	0/5 (0%)
GPA-/CD71+	1/15 (6.7%)	1/4 (25%)	2/5 (40%)
GPA+/CD71+	0/15 (0%)	0/4 (0%)	0/5 (0%)
Total cases 38 (100%)	**25** (65.8%)	**6** (15.8%)	**7** (18.4%)

*Greater than 20% of positive cells for lymphoid antigens or 30% of positive cells for myeloid antigens

positive cells, 77.49-95.74% and 73.02-97.85%, respectively. Based on the percentage of the co-expressing cells, a possible argument that there might have been two populations of different cell lineage co-existing within the same blast-gate is less likely. On the other hand, the existence of lineage promiscuity or infidelity (termed by Greaves and co-workers)[13] is more likely. It was reported that the myeloid antigen expression in ALL was identified in a high risk group of patients with adult ALL.[9] Interestingly, there was one patient (4%) co-expressing T-B cell markers ($CD5^+/20^+$). The co-expression of T-cell markers in B-cell lineage ALL were reported to be 11% in childhood ALL[11] and 5.3% in adult ALL[9]

We were able to identify 6 out of 38 (15.8%) of ALL-patients as B-ALL. These B-ALL; 3 cases were relapsed cases and two of them were infantile leukemia. The major B-cell markers were CD19+ (100%), CD22+ (80%), CD20+ (33.3%), and HLA-DR+ (80%). Our findings may give rise to the fact that the expression of CD22 antigen is earlier than CD20 but later than CD19 expression in the sequence of B-lineage maturation in leukemia. We also noticed that the percentages of CD19/10 and CD22 positive cells were high (70-95%) as compared to that of CD20 positive cells (30-50%). Whether this is due to the reactivity of the particular clones of antibodies used or the results of the quantity of different antigens expressing on the leukemic cells is to be determined. Among these B-ALLs, T-cell marker aberration was found to be 16.7% of CD5+ It was reported to be 11% of CD5 marker in B-ALL.[7] We also found 20% of myeloid aberration in B-ALL patients.

There were 18.4% of ALL-patients identified as T-ALL. The

T-cell markers were 100% of CD5+, 85.7% of CD7+, 42.9% of CD3+, and 33.3% of CD34+. These are slightly different from the reports of Loken *et al.*[11]; 100% of CD5+, 80% of CD3+ and CD7+, 20% of myeloid and B-cell markers. We also found 14.3% of CD13+ and HLA-DR+ aberration in T-ALL.

An immunophenotyping classification of ALL, e.g., c-ALL, B-ALL and T-ALL is practical in prognostic purposes by correlating to the therapeutic outcome. In particular for the c-ALL-patients all response to the treatment. We could observe only 1 case of c-ALL that relapsed within 18 months following complete remission after administration of standard regimen in our follow-up studies by flow cytometry (manuscript in preparation). It is of interest that this case was 1 of the 4 cases of infantile leukemia.

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