FLOWCYTOMETRIC ANALYSIS

OF ACUTE LEUKEMIA

by

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CERTIFICATE

This is to certify that the dissertation entitled

"FLOWCYTOMETRIC ANALYSIS OF ACUTE LEUKEMIA"

is the bonafide record of research work done by

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ABSTRAK

Satu kajian telah dibuat keatas 84 pesakit kanak-kanak dan dewasa yang mengalami penyakit leukemia akut antara Januari 2003 sehingga Januari 2005. Kes-kes tersebut telah diklasifikasikan secara morfologi dan immunofenotip. 93 pesakit telah didiagnosakan mengalami leukemia akut. Walaubagaimanapun hanya 84 rekod yang lengkap dan dapat dianalisa secara keseluruhan.

Berdasarkan klasifikasi FAB, 43 pesakit diklasifikasikan sebagai Myeloid Leukemia Akut (AML) dan 41 pesakit adalah Limfoblastik Leukemia Akut (ALL). Jenis-jenis leukemia yang diklasifikasikan secara immunofenotip adalah AML sebanyak 51%, B-ALL (31%), T-ALL (14%) dan Acute Biphenotypic Leukemia (4%).

Kajian ini diharapkan dapat memberi kesan informasi kepada bidang kesihatan terutama dalam pengdiagnosan penyakit akut leukemia dan seterusnya memberi manfaat kepada semua yang terlibat dalam memberi rawatan terbaik kepada pesakit akut leukemia khususnya.

ABSTRACT

A review has been made of the 84 paediatric and adult cases of acute leukemia between January 2003 until January 2005. These cases were classified morphologically (using FAB classification) and immunophenotypically. A total of 93 paediatric and adult cases were diagnosed, however only 84 records of patients were retrievable from Haematology Lab record books.

By FAB classification, 43 cases were classified as Acute Myeloid Leukemia (AML) and 41 cases as Acute Lymphoblastic Leukemia. Acute Leukemia was immunophenotypically classified as AML with 51%, B – ALL (31%), T – ALL (14%), Acute Biphenotypic Leukemia (4%).

Contribution of Flow Cytometry analysis to patient is dynamic and evolving with clinical relevance of biologic parameters and also for the prognostic information. This study was hope to give some benefit in the information which could be used in health area especially in the diagnosed and treatment of acute leukemia

1.0 INTRODUCTION

1.1 Acute Leukemia

Acute leukemia is usually aggressive diseases in which the malignant transformation causes accumulation of early bone marrow haemopoietic progenitors, called blast cells. It is a heterogeneous group of malignancy with varying clinical, morphologic, immunologic and molecular characteristics. Many distinct types are known to carry predictable prognoses and warrant specific therapy. Distinction between lymphoid and myeloid leukemia, most often made by flow cytometry, is crucially important. Several advances in flow cytometry, including availability of new monoclonal antibodies, improved gating strategies and multiparameter analytic techniques have all dramatically improved the utility of flow cytometry in diagnosis and classification of acute leukemia.

Acute leukemia is defined as the presence of over 30% of blast cells in the bone marrow at clinical presentation. It is further subdivided into acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) on the basis whether the blasts are shown to be myeloblasts or lymphoblast. In most cases, the clinical features and morphology on routine staining distinguish ALL from AML. Specialized tests are needed to confirm the diagnosis of ALL or AML and to subdivide the cases into their different subtypes. These include cytochemical stains, immunophenotyping by flow cytometry, chromosomal studies, and molecular studies.

The first accurate description of a case of leukemia was given in 1827 by Velpeau. His patient, a florist and lemonade seller age 63 years had abandoned himself and fell ill in 1825 with a pronounced swelling of the abdomen, fever and weakness. He died soon after

admission to hospital and at autopsy, was found to have a massive enlargement of the liver and spleen. The blood was thick and described as resembling the colour of the yeast of red wine.

1.2 Epidemiology of acute leukemia

1.2.1 Age and sex

Acute leukemia occurs at all ages, but its frequency rises steeply with increasing age beyond 50 years. Acute myeloblastic leukemia (AML) accounts for the great majority of adult cases while acute lymphoblastic leukemia (ALL) predominates among children. Generally, acute leukemia is more common in males than females.

1.2.2 Ethnic and social class differences

Acute leukemia in all ages is more common in whites than in blacks. These differences are thought to reflect differences in environmental exposure. Acute leukemia is also observed more commonly in higher than lower socio-economic groups.

1.2.3 Exposure to radiation

The capacity of ionizing radiation to cause leukemia has long been recognized. Following the 1945 atom bomb explosion in Hiroshima and Nagasaki, Japan, leukemia incidence among exposed survivors is increased markedly. It might have begun as early as one and a half years after the bombing. The increased incidence was demonstrated only amongst those with an estimated exposure of 100 centiGray or greater.

1.2.4 Genetic

Certain groups which are associated with genetic abnormality have greater than normal risk for developing acute leukemia. The risk for patients with Down Syndrome is about 1%. Siblings of children with leukemia have a four-fold higher risk. In identical twins if one of the child is diagnosed as having leukemia the chances of developing leukemia in the other twin is approximately 20% within six months of the diagnosis.

1.2.5 Geographic differences

Although the incidence of leukemia differs significantly between countries but the differences is not much and rarely exceed two fold. Of the different types of cancer, leukemia probably shows the least of geographic variation.

1.3 Flow Cytometer

The flow cytometer (FCM) has been hailed as a new product of technical revolution, but the concept of FCM has existed for over 50 years, and cell counters have been used extensively for more than 20 years. Nevertheless, the emergence of the fluorescence detector in the new generation of cytometers greatly enhanced their versatility. The availability of a great variety of monoclonal antibodies finally pushed FCM to the forefront.

FCM has the advantage of being more efficient, sensitive, accurate and reproducible than manual techniques. With FCM, multiple specimens can be simultaneously processed with a panel of 10 or more, and tests can be completed within several hours. When there is sufficient specimen, FCM counts 3,000 to 5,000 cells for the study of each antigen compared with 100 to 200 cells counted in manual techniques. The major merit of FCM is its capability

of measuring multiple parameters forward light scatter, side scatter and fluorescence) simultaneously, and the data thus obtained can be stored for further analysis. In addition, the electronic gating gadget enables the study of separate cell groups without evoking tedious isolation techniques.

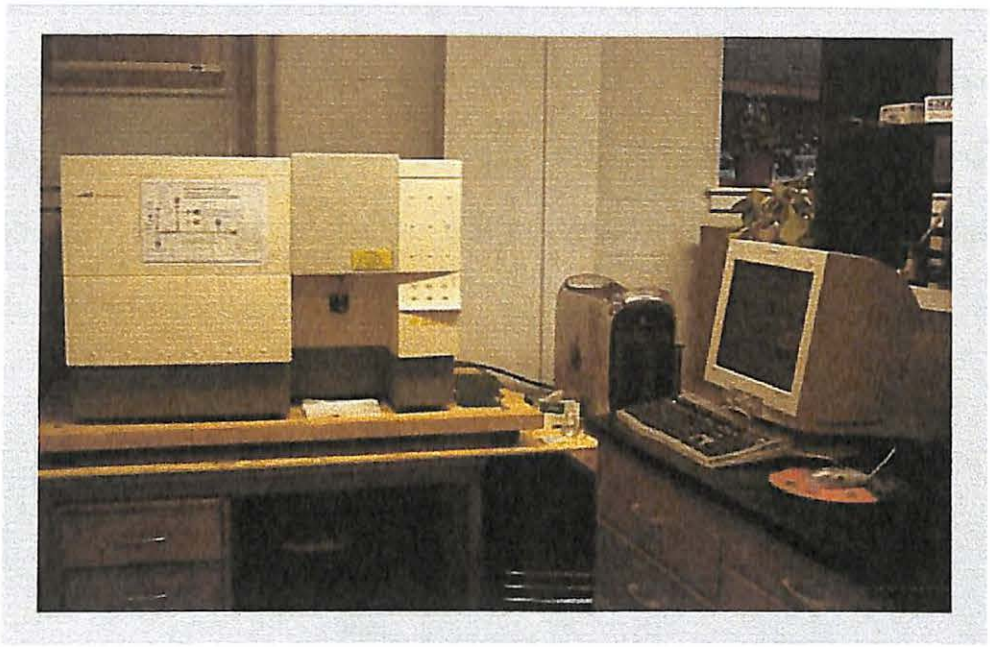

Figure 1.1 : Flowcytometer

1.3.1 Flow Cytometric immunophenotyping In Acute Leukaemias

Flow cytometry is a method to differentiate and count cells and microparticles. It is a process in which the measurements are made while the cells or particles pass, preferably in single flow through the measuring apparatus in a fluid stream. According to Howard Shapiro: "Cytometry refers to the measurement of physical and/or chemical characteristics of cells or, by extension, of other biological properties".

Flow cytometry uses fluorescent lights which the flourochrome absorbs energy from laser then releases the absorbed energy by vibration and heat dissipitation. It will than showed the relatives sizes of cells, the relative granularity and fluorescence intensity.

Components of a flow cytometry are fluidics, optics and electronics. Fluidics to introduce and restrict cells for interrogation, optics to generate and collection of lights signals and electronics to convert optical signals to an electronic form and also to digitize signals for computer analysis.

Although morphology itself is useful in the classification of acute leukaemias, immunophenotyping is better in diagnosing the types of acute leukaemia more accurately. Immunophenotyping uses the antigens on the cell surface to react with matching monoclonal antibodies (mAbs). Several hundred monoclonal antibodies have been assigned to Clusters of Differentiation (CD) groupings by the International Workshops on Leukocyte Differentiation Antigens studied by Kishimoto et al.

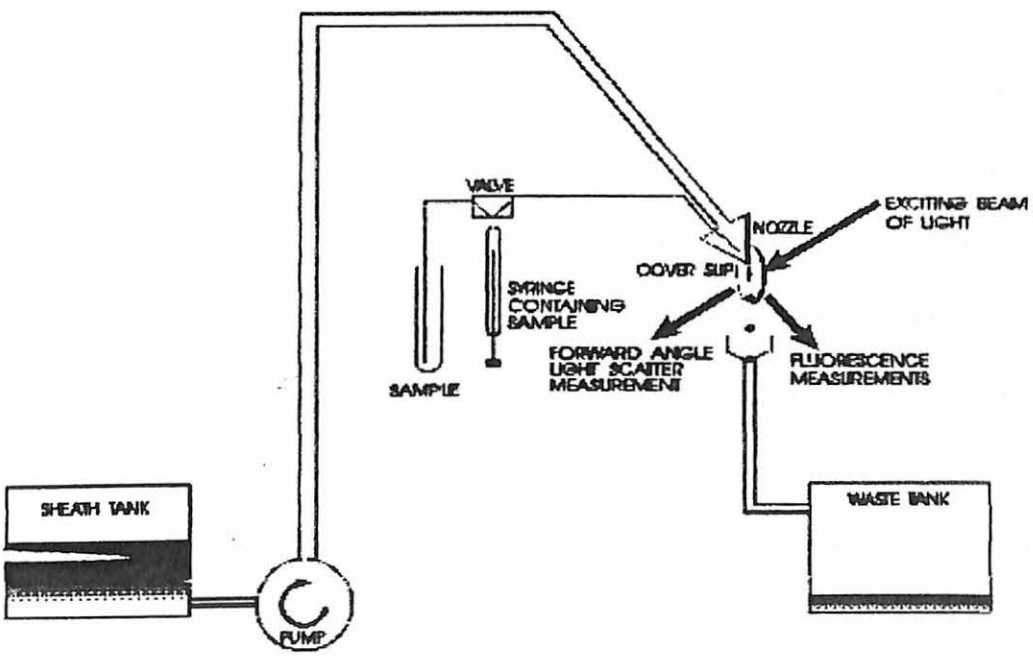

Figure 1.2 : Basic of flowcytometer

Clinical flow cytometers are designed to measure light scattered by a cell at two different and distinct angles, while at the same time measuring one or more fluorescence emission signals. All measurements are performed while the cells are injected, single file, through a flow cell. In order to arrange cells in such a configuration, the cells are pushed through a coaxial liquid stream. The cells are in the core stream, which is surrounded by a second stream called the sheath stream. The sheath stream is actually a tube or cylinder of cell-free fluid surrounding the core stream which contains the cells aligned in single file. This phenomenon is called hydrodynamic focusing. The velocities of the two streams are adjusted to obtain laminar flow. The sheath flow is adjusted to position the cells in the proper observation region in the center of the core stream in a stable flow environment. As the stationary laser light intercepts cells one at a time, the positioning of each cell must be precise. The inside diameter of capillary in the flow chamber was 75-200μm. The size of the cells that must pass through the flow system predetermines the diameter of the orifice of the flow cell to be used. There is a trade-off between a narrow orifice, which provides an ideal single-file cell stream in the flow cell, and a wide orifice, which virtually never clogs. The observation region which is also called the interrogation zone is the spot where the moving cell intercepts the stationary laser light. The instrument is equipped with beam focusing lenses to provide a focused light bundle on cells as they pass the observation point

The optical system of the flow cytometer is designed to make measurements of illuminated cells. Scattered light, an intrinsic parameter, occurs when light is deflected off cells, and is detected simultaneously in two different directions. Light is collected at 90° (orthogonal light) from the axis of the light beam, and in a forward direction blocker, or

obscuration bar, is essential to assure that only the forward scattered light is collected and that the beam light at 0° is stopped.

Fluorescence signal detection on clinical flow cytometers is an extrinsic cellular parameter. The distinction between intrinsic and extrinsic cellular parameters will be covered in more detail later in this review. In most instruments, the collection of the fluorescence signal is shared with the orthogonal light collection system. Various optical mirrors and filters are used to reflect and absorb light, respectively. The filtered light signal is deflected into a photomultiplier tube (PMT). This device converts light energy into electric current. Most clinical instruments operate with a single laser and are capable of detecting two or three different flourochromes simultaneously.

Fluorescent dyes that have been successfully direct-coupled to monoclonal antibodies fall into three categories. These are low-molecular-weight organic dyes, biological pigments, and the tandem dyes systems (TDS), with energy transfer between two chemically bound dyes. There are commercially available dyes from all three of these categories.

Signal processing and signal acquisition professes to be the least user-friendly attributes of clinical flow cytometry. With current instruments, most of the acquisition decisions are reduced to algorithms that are preset protocols selected by the operator. The onboard software is designed to handle this sequence of operations during data acquisition. To detect various cell clusters, a wide dynamic range of signal detection is required. Heterogeneous populations of cells can be visualized in a single dot plot, and morphologically or immunologically different clusters can be resolved.

Each detection system on a flow cytometer represents a parameter. In order to acquire dual light scatter, and one set of fluorescence signals, the minimum of a three-parameter

acquisition is required. Five-parameter acquisition is preferred so that both linear scatter data is available to the investigor from list mode. In this mode of flow cytometer storage mode, data from all the acquired parameters of the cells analyzed are stored on an electronic media. On most instruments, fluorescence signals are collected only in logarithmic-scale format. Conventional clinical immunophenotyping protocols traditionally trigger on two light scatter parameters simultaneously. Initially, cell sorters were designed to trigger on a single-parameter fluorescence signal.

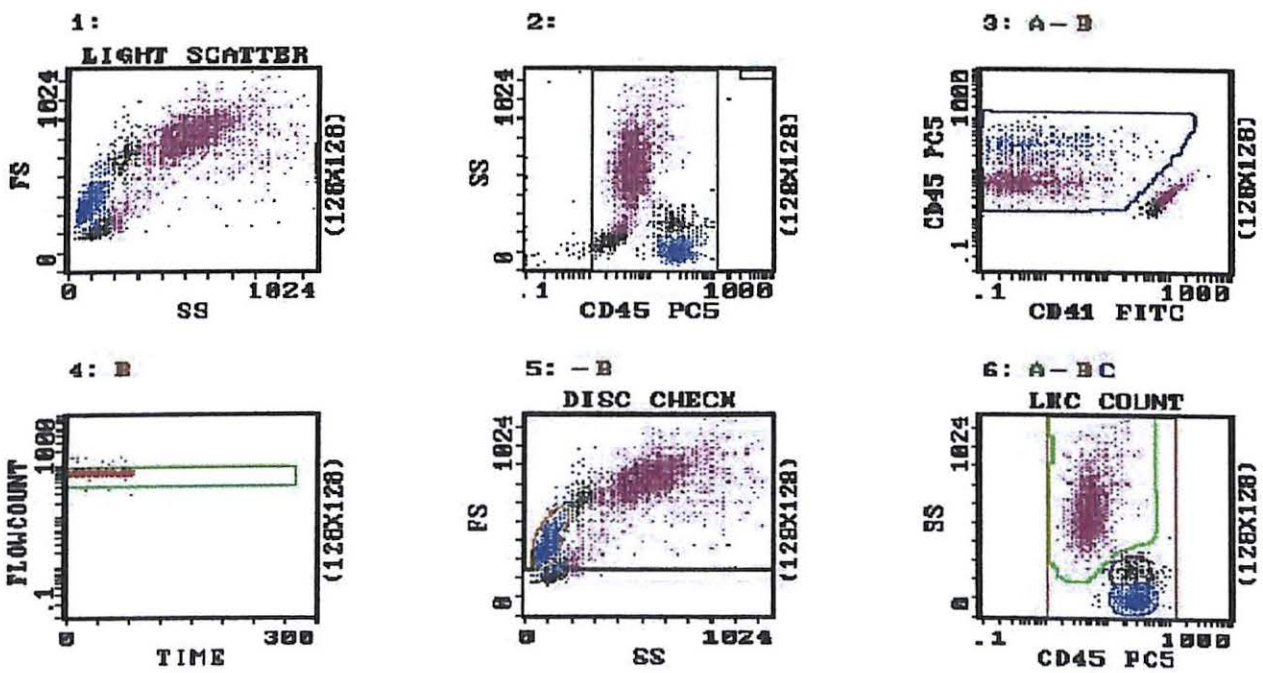

Figure 1.3 : Examples results of flowcytometry analysis

2.0 LITERATURE REVIEW

2.1 General

Many studies critically evaluate the diagnostic usefulness of flow cytometric immunophenotyping of acute leukemia and provide guidelines for the judicious use of immunomarkers and proper interpretation of results (Bruce H. Davis et al, 2000, Gregory T. Stelzer et, al, 1997 and Edward G.Weir et al, 2001).

One of the most relevant and recent studies, is a study done in Kuala Lumpur Hospital from the year 1997-2002 for immunophenotyping of acute leukemia. This study used 990 acute leukemia cases which were collected within a period of 5 years. It showed that ALL constituted 50% of all cases, AML 48.9% and biphenotypic leukemia is 1.1% (K mariammah et al, 2003).

Another study was done in Kelantan State using 45 cases of childhood acute leukemia (Menon BS et al, 1998). This study showed that ALL constituted 80% of all childhood cases of acute leukemia, while AML only 20%.

Aside from identification of blasts, flow cytometry was found to be especially useful in correct identification of AML M_0, APL (AML-M_3) from AML-M_1/M_2, deoxynucleotidal transferase terminal (TdT)-negative ALL unusual variant, such as transitional B-cell ALL and biphenotypic acute leukemia (Zahid Kaleem et al,2003).

Flow cytometry was also found to be useful in the detection of the atypical blasts phenotypes existing in 31% of newly diagnosed patients with acute leukaemia with co-expression of CD19 and CD2 markers in AML, co-expression of CD33 marker in ALL , co-

expression of CD19 marker in T cell ALL, and biphenotypic (mixed-lineage) leukaemia (Piatoska-Jakubas B et, al, 1996).

2.2 Classification of Acute Leukemias

The French-American-British (FAB) classification has been used as the basis for the classification of acute leukemia for many years. However, the proposed World Health Organization (WHO) classification has made many changes on the FAB classification (Daniel Catovsky et al, 2002). The FAB classification divides ALL into L_1, L_2 and L_3 but the WHO classification considers that the division of L_1 and L_2 does not serve any clinical purposes and merges them into precursor B-cell and precursor T-cell ALLs. L_3 is retained as Burkitt cell leukemia. In AML, the original FAB categories, M_0, M_1, M_2, M_3, M_4, M_5, M_6, and M_7, are now under the tittle of AML not otherwise categorized, which also includes acute basophilic leukemia and acute panmyelosis with myelofibrosis (J.A Whittaker et al). In addition, there are AML's with recurrent cytogenetic translocations; AML with multilineage dysplasia; and AML with myelodysplastic syndrome, therapy related; and biphenotypic acute leukemia. The addition of these new entities is due to their clinical relevance.

2.3 Acute Myeloblastic Leukemia (AML)

2.3.1 Acute myeloid leukemia without morphologic or cytochemical evidence of differentiation (AML M_0).

Leukemia that are devoid of detectable MPO should be classified as AML M_0 only in the absence of lineage-restricted lymphoid and megakaryocytic antigens. Although the cells

of most M_0 express CD13 or CD33, some cases may lack these antigens. The expression of CD117 by leukemic cells is strongly suggestive of AML (Tsieh Sun, 2002).

2.3.2 Acute Myelocytic Leukemia with little differentiation (AML M_1)

AML M1 cases commonly express MPO, CD 13, CD 33, CD 34, CD 65, CD117 and HLA-DR but in variable combinations. Expressions of CD4, CD15 and CD66 are less frequent. By using cytochemical, stains of Myeloperoxidase (MPO) and chloroacetate esterase (CAE) were positive, whereas α-naphtyl butyrate esterase (NBE) and terminal deoxynucleotidyl transferase (TdT) were negative in both the bone marrow and peripheral blood specimens (Daniel Catovsky et al, 2002).

The basic requirement for the diagnosis of AML is that more than 30% of all nucleated marrow cells are blasts and less than 50% are erythroid precursors except for erythroleukemia (Knox-Macaulay HHM et al, 2000). Cytochemical staining can be totally negative in AML, and sometimes the staining is difficult to interpret or inconclusive. Therefore, immunophenotyping is helpful in substantiating the diagnosis, reliable predictors for prognosis (Tsieh Sun, 2002).

2.3.2.1 Features for Laboratory Diagnosis of AML-M1 (Tsieh Sun, 2002).

- ✓ Greater than 90% myeloblasts in the bone marrow or peripheral blood
- ✓ Greater than 3% MPO-positive blasts in the bone marrow
- ✓ Blasts positive for CAE but negative for NBE
- ✓ Blasts positive for CD33/CD13, HLA-DR
- ✓ Blasts negative for CD14, CD15, CD41/CD61, Glycophorin A

2.3.3 Acute Myeloblastic Leukemia with Maturation (M2)

Leukemic blast cells commonly express MPO, CD34, CD65 and HLA-DR but CD13 and CD33 expression is characteristically very weak. Myeloperoxidase (MPO) and chloroacetate esterase (CAE) were positive, and α-naphtyl butyrate esterase (NBE) was negative for blasts in the bone marrow.

The major diagnostic criterion for AML-M_2 is based on the blast count in the bone marrow. Peripheral blood showed many blasts containing multiple Auer rods. This subtype is usually associated with favorable prognosis (Edward G.Weir et al, 2001).

2.3.3.1 Features for Laboratory diagnosis of Acute Myelogenous Leukemia-M2 :

- ✓ 20-90% of myeloblasts present in bone marrow
- ✓ Less than 20% monocytic precursors in the bone marrow
- ✓ Less than 5 x 10^9/L monocytic components in the peripheral blood
- ✓ Cytochemical stain for blasts
  - Positive for myeloperoxidase and choloroacetate esterase
  - Negative for α-naphthyl butyrate
- ✓ Monoclonal antibody panel :
  - Positive for CD13, CD15, CD33,HLA-DR
  - Negative for CD14

2.3.4 Acute Promyelocytic Leukemia (M_3)

Strongly express MPO, CD13, CD33 and CD65 but usually do not express CD34 or HLA-DR. CD 4 and CD 56 are seldom detected (Daniel Catovsky et al, 2002). Leukemic

cells from both the peripheral blood and bone marrow were positive for myeloperoxidase (MPO) and choloroacetate esterase (CAE) stains but negative for α-naphtyl butyrate esterase (NBE) stain. CAE stain also demonstrated single or multiple Auer rods in leukemic cells. AML-M_3 was originally designated hypergranular promyelocytic leukemia by the FAB group (Edward G.Weir et al, 2001).

2.3.4.1 Features for Laboratory Diagnosis of AML-M_3 (Tsieh Sun, 2002).

- ✓ Presence of more than 20% hypergranular (or microgranular) promyelocytes in the bone marrow
- ✓ Presence of multiple Auer rods in the cytoplasm of leukemic cells
- ✓ Cytochemical staining: Strongly positive for myeloperoxidase and choloroacetate esterase, but negative for α-naphtyl butyrate esterase.
- ✓ Immunophenotyping:

Positive: for myelomonocytic antigens (CD13, CD15, CD33) but negative for monocytic antigen (CD14) and HLA-DR.

2.3.5 Acute Myelomonocytic Leukemia (M_4)

Blast cells of most myelomonocytic leukemia express MPO, CD4, CD13, CD14, CD33, CD 34, CD45, CD65 and HLA-DR. In adults, CD19 also frequently expressed *(Daniel Catovsky et al, 2002).* In the bone marrow and peripheral blood specimens, the myeloperoxidase (MPO), chloroacetate esterase (CAE), and α-naphtyl butyrate esterase (NBE) stains were all positive for leukemic cells.

AML-M_4 accounts for 20% of AML cases. The diagnostic criterion as defined by the FAB group is the presence of 30% of blasts in the bone marrow, including type 1 and type II myeloblasts, monoblasts and promonocytes (J.A Whittaker et al, 1987).

2.3.5.1 Features for Laboratory Diagnosis of AML-M_4 (Tsieh Sun, 2002).

- ✓ Presence of at least 20% myeloblasts-monoblasts-promonocytes in bone marrow
- ✓ Both myeloblasts and monoblasts/promoncytes should exceed 20%
- ✓ If monocytic component is less than 20% in bone marrow:
  - Monocyte count in peripheral blood should be greater than 5 x 10^9/L
  - Serum lysozyme level should exceed three times the normal value
- ✓ Myeloperoxidase positive cells must be more than 3%
- ✓ Chloroacetate esterase and α-naphtyl butyrate esterase-positive cells were roughly more than 20% each
- ✓ Immunophenotype
  - Positive: CD13, CD14, CD33 and HLA-DR
  - Negative: CD41/CD42/CD61 and Glycophorin A

2.3.6 Acute Monoblastic Leukemia (M_5)

Expression of CD14 is largely restricted to cells of the monocytic lineage but is often absent in pediatric M_5 cases. In both bone marrow and peripheral blood specimens, more than 3% of blasts showed myeloperoxidase (MPO) staining, and more than 80% of blasts in the bone marrow were monoblasts that were positive for α-naphtyl butyrate esterase. None of

the immature cells were stained with chloroacetate esterase (CAE). The terminal deoxynucleotidyl transferase was negative in both specimens (J.A Whittaker et al, 1987).

The FAB criteria for the diagnosis of AML-M_5 require 80% or more of the nonerythroid cells in the bone marrow to be monoblasts, promonocytes, or monocytes. If the predominant component (>80%) is monoblasts, the condition is designated M_5a, whereas the predominant components should be promonocytes and monocytes in M_5b (Daniel Catovsky et al, 2002).

The immunophenotype is slightly different between M_5a and M_5b. CD14 expression is more consistent in M_5b cases, whereas in M_5a about one-third of cases show absence or low percentage of CD14 positivity. Another study has showed that CD117 is frequently positive in M_5a and negative in M_5b cases, whereas CD4 is often positive in M_5b but negative in M_5a (Edward G.Weir et al, 2001).

2.3.6.1 Features for Laboratory Diagnosis of M_5 (Tsieh Sun, 2002).

- ✓ Presence of more than 80% monocytic component among the nonerythroid cells in the bone marrow
  - M_5a: 80% or more of moncytic components are monoblasts
  - M_5b: Predominantly monocytes and promonocytes
- ✓ Elevation of serum and urine lysozyme levels
- ✓ Cytochemistry
  - Myeloperoxidase: may or may not be positive
  - Nonspecific esterase: Strongly positive
  - Specific esterase and periodic acid-Schiff: Usually negative

- ✓ Immunophetype:
  - Positive for CD13, CD14, CD33 and HLA-DR
  - Negative for CD41, CD61 and glycophorin A.

2.3.7 Acute Erythroleukemia (M_6)

Leukemic erythroblasts usually express CD36, CD71 and Glycophorin A. Cells of myeloid component express CD13, CD33, and MPO. The leukemic cells (mainly the myeloblasts) showed positive staining for myeloperoxidase and chloroacetate esterase but negative staining for α-naphtyl butyrate esterase. The erythroblasts showed the typical block pattern in the PAS stained preparation. An immunoflourescence stain for terminal deoxynucleotidyl transferase was negative (Daniel Catovsky et al, 2002).

Erythroleukemia M_6 is a rare disease, accounting for 4% to 5% of AML. The FAB criteria for M_6 is the presence of at least 50% normoblasts (erythroblasts) among the total number of nucleated cells and 30% type I and type II blasts among the nonerythroid population in the bone marrow. Kowal-Vern et al. suggested that cases with pronormoblasts more than 30% should be considered erythroleukemia rather than myelodysplastic syndrome, because their clinical behavior is more consistent with leukemia than dysplasia.

M_6 cases are pleomorphic and sometimes unclassifiable morphologically. Immunologic identification of erythroblasts becomes necessary. Several antibodies can help identify the erythdoid series. Gupta and Dhond et al. used a panel of monoclonal antibodies specific for different developmental stages (erythrocyte burst-forming units, erythrocyte colony-forming units, normoblasts, and erythrocytes) and components (glycophorin A and H antigens) of erythroid cells and found that in most cases of M6, the phenotype of the

pronormoblasts was that of the intermediate stage of maturation. Transferin receptor antibody (CD71) has also been used to detect mature and immature nucleated erythrocytes.

2.3.7.1Features for Laboratory Diagnosis of M_6 (Tsieh Sun, 2002).

- ✓ M_6a: Presence of more than 50% of normoblasts among all nucleated cells and 20% of myeloblasts among nonerythroid cells in the bone marrow.
- ✓ M_6b: Presence of more than 80% of pronormoblasts and basophilic normoblasts but less than 20% of myeloblasts in the bone marrow.
- ✓ PAS positive in mature and immature nucleated erythroid cells.
- ✓ Glycophorin-A or other erythroid antibodies: The only reliable antibodies for immunophenotyping.
- ✓ React variably to myelomonocytic (CD13, CD33) and platelet (CD41) antibodies.

2.3.8 Acute Megakaryoblastic Leukemia (M_7)

The leukemic cells of most AML M_7 cases express CD41a and CD61. Most cases are positive for CD4 and CD 33. CD13, CD34, CD36, CD45, and HLA-DR are infrequently detected (Tsieh Sun, 2002).

The leukemic cells in the bone marrow were negative for myeloperoxidase and α-naphtyl butyrate esterase but positive for choloroacetate esterase and PAS stains. The PAS stain showed a typical peripheral pattern with strong staining in the cytoplasmic blebs. An immunoflourescence stain for the terminal deoxynucleotidyl transferase was negative (Edward G.Weir et al, 2001).

The FAB criterion for the diagnosis of M_7 is the presence of 30% or more megakaryoblasts in the bone marrow. It requires the identification of megakaryocytic cells not only by morphology but also by either the platelet peroxidase reaction on electron microscopy or tests with monoclonal or polyclonal platelet specific antibodies (J.A Whittaker et al, 1987).

The monoclonal antibodies most commonly used are CD41, CD61 and CD42. For myelomonocytic antigens, CD33 is frequently positive, but CD13 and CD14 are usually negative. CD 34, a hemopoeitic progenitor cell antigen and CD56, a neural cell adhesion molecule and natural killer marker, are frequently expressed on megakaryoblasts. The expression of HLA-DR is variable. However, recent study showed that CD2 and CD7 were relatively common in M_7 (Tiensiwakul P et al, 1999). The PAS stain demonstrated the characteristic peripheral pattern, which may represent the maturing platelets on the surface of the megakaryocytes or megakaryoblasts.

2.3.8.1 Features for Laboratory Diagnosis of M_7 (Tsieh Sun, 2002).

- ✓ Presence of 20% or more megakaryoblasts in the bone marrow.
- ✓ Excess of blasts with increased numbers of maturing megakaryocytes in the bone marrow biopsy, plus identification of megakaryoblasts in the peripheral blood and bone marrow aspirate by immunologic techniques
- ✓ Electron microscopic identification of platelet peroxidase in leukemic cells.
- ✓ Monoclonal antibodies: CD41, CD42, and CD61 are specific for megakaryoblasts
- ✓ Myelomonocytic markers: Positive for CD33 but negative for CD13 and CD14.

- ✓ PAS staining pattern in megakaryocyte-megakaryoblasts with periphery of cytoplasm with accentuation on cytoplasmic blebs.

2.4 Acute Lymphoblastic Leukemia (ALL)

2.4.1 Acute Lymphoblastic Leukemia of B-Cell Lineage

Terminal deoxynucleotidyl transferase (TdT) was positive in 100% of blasts in both the bone marrow and the peripheral blood specimens. Myeloperoxidase was negative. ALL is leukemia with proliferation of lymphoblasts involving both the bone marrow and the peripheral blood. Lymphoblasts may not be demonstrated in the peripheral blood in occasional ALL cases (a leukemic leukemia). About one third of patients have a total white cell count of less than 5×10^9 / L (Tsieh Sun, 2002).

There are some subgroups that consists of B-precursor ALL, pre-B ALL and B-ALL. These stages can be distinguished by using CD19, Cμ and surface immunoglobulin. B-precursor ALL shows only CD19, pre-B ALL expresses CD19 and Cμ, whereas B-ALL bears CD19 and surface immunoglobulin. The malignant nature of the ALL cells is determined by TdT, CD10 (common ALL antigen), andCD34 (hematopoeitic progenitor antigen) (Gerald E. Marti et al, 2001). TdT is present in most cases of ALL except for B-ALL. CD10 is seen in most cases of B-cell ALL. CD34 is present in B-precursor ALL but not in pre-B ALL and some cases of B-ALL. Additional antibodies that can be helpful in classifying ALL include HLA-DR, CD20, CD22, and CD24. Cytoplasmic CD22 appears earlier in the B-cell developmental stage than surface CD22 and consistently positive in B-ALL

2.4.1.1 Features for Laboratory diagnosis of B-cell ALL (Tsieh Sun, 2002).

- ✓ TdT positive for precursor-B and pre-B ALL
- ✓ CD10 positive, except for some B-ALL cases
- ✓ HLA-DR positive
- ✓ CD19 frequently present without CD20
- ✓ Cμ positive in pre-B ALL only
- ✓ Monoclonal surface immunoglobulin in B-ALL only

2.4.2 Acute Lymphoblastic Leukemia of T-Cell Lineage

T-cell ALL may show either L_1 or L_2 morphology, whereas L_3 is always of B-cell lineage. The cytochemical reactions in T-cell ALL are the same as B-cell ALL. When a high percentage of tumor cells stains for TdT, the diagnosis of ALL is certain. Although a small percentage of acute myeloblastic leukemia (AML) cases may show positive TdT, the percentage of positive leukemic cells is usually much lower. In other words, only a small subset of leukemic cells may express TdT in AML cases. PAS positivity helps establish the diagnosis, but a negative PAS reaction does not rule out ALL (Tsieh Sun, 2002).

The most comprehensive immunologic classification divides T-cell ALL into four immunophenotypes which are the pre-T, early cortical, late cortical and medullary. The pre-Tcell phenotype expresses only CD7, cytoplasmic CD3, and TdT without other T-cell antigens. The early cortical phenotype shows CD2, CD5, CD7 and strong TdT. The late cortical phenotype reveals CD1, CD2, CD5, CD7 and dual CD4/CD8 with minimal surface CD3 (Gerald E. Marti et al, 2001). The medullary phenotype shows CD2, CD3, CD5, CD7

and segregated CD4 or CD8. TdT is not commonly expressed in this phenotype. Cytoplasmic CD3 expressed in all stages.

2.4.2.1 Features For Laboratory Diagnosis of T-cell ALL (Tsieh Sun, 2002).

- ✓ Most cases are positive for TdT
- ✓ Pediatric cases are frequently negative for CD10 and HLA-DR
- ✓ CD7 is frequently the only positive T-Cell marker, but all T-cell markers can be present.
- ✓ Dual CD4/CD8 positivity is most common than other CD4/CD8 combinations.

Table 2.1 : Morphologic (FAB) classification of ALL (Daniel Catovsky et al, 2002)

	L_1	L_2	L_3
Size of blasts	Small,uniform	Large, variable	Medium to large, uniform
Amount of cytoplasm	Scanty	variable	Moderate
Cytoplasmic basophilia	Moderate	variable	Intense
Cytoplasmic vacuoles	Variable	variable	Prominent
Nucleus	Regular, occasional clefting, homogenous chromatin	Irregular, clefting common, heterogeneous chromatin	Regular, nonclefted, homogeneous, finely stippled chromatin
Nucleolus	0-1, inconspicuous	1 or more, prominent	2-5, prominent
Nuclear/cytoplasmic ratio	High	Low	Low

2.5 Supplementary Tests

With the proliferation of new monoclonal antibodies and the improvement of instruments, FCM has become increasingly useful. However, flow cytometer is still not an

entirely independent tool for the diagnosis of hematologic neoplasms. These are some multiparametric approach that had been used so that specific treatment can be tailored (Tsieh Sun, 2002).

- Cytochemistry
- Immunohistochemistry
- Immunogenotyping
- Southern Blotting
- Quantitative Polymerase Chain Reaction
- Cytogenetics
- Flourescence in situ Hybridization (FISH)
- Oncogenes

Cytochemistry is an integral part in the diagnosis of AML required by the French-American-British Cooperative Group. Myeloperoxidase (MPO) stain is the first screening test to distinguish AML from ALL. This enzyme is present in neutrophilic, eosinophilic, and monocytic lineage but not in lymphocytes. The MPO in eosinophils is resistant to cyanide, so that eosinophil and its immature forms can be identified this specific reaction. The peroxidase of megakaryocytes and platelets cannot be visualized by light microscopy, but it can be demonstrated by electron microscopy (Tsieh Sun, 2002).

Acid phophatases (APs) are a group of enzymes capable of hydrolyzing monophosphates esters in an acid environment. By electrophoresis, APs can be separated as seven nonerythrocytic isoenzymes. Isoenzymes 2 and 4 are present in neutrophils and