LEUKEMIA IMMUNOPHENOTYPING

(Received 21 July, 1992)

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Immunophenotyping is, by definition, to classify and subclassify the cells by using monoclonal antibodies (Mabs) raised against cell surface antigens. These antigens may be helpful to discriminate morphologically indistinguishable cells. Immunophenotyping has been used in clinical practice for many years and it became an indispensable diagnostic aid for certain diseases.

The routine clinical applications of immunophenotyping are:

1 - Determining the origin and stage of differentiation of Leukemias and Lymphomas
2 - Detecting early recurrence of hematologic malignancies
3 - Monitoring chemotherapy response
4 - Diagnosing and monitoring congenital and acquired immunodeficiencies
5 - Diagnosing and monitoring autoimmune disorders
6 - Pre and posttransplantation monitoring and evaluation.

The focus of this review will be Leukemia immunophenotyping.

Diagnosis and Classification of Leukemia :

The conventional method of leukemia diagnosis and classification is based on morphologic and cytochemical studies. Although FAB system is widely accepted, recent data indicates that further criteria should be implemented in order to subclassify and determine the origin of leukemic blasts.

Thus, immunophenotyping and gene rearrangement studies have been introduced into the diagnosis and classification of hematologic malignancies.

The idea of immunophenotyping stems from the facts that hematologic neoplasms:

1 - represent normal hematopoietic precursors that are frozen at one stage of differentiation (maturation arrest)
2 - express only one lineage specific antigens i.e lymphoid cells carry only lymphoid antigens but not myeloid antigens and vice versa (lineage fidelity).

Although this concept is changed considerably with recent data it remains the main feature of leukemogenesis.

Thus it has become pertinent to use Mabs against mature and immature surface antigens to classify and diagnose the leukemias (1).

Around a hundred of Mabs are defined and each named with a cluster designation (CD) number.

The functional properties of the majority of surface antigens recognized by these Mabs are poorly established. However, some of them may directly play role in the leukemogenesis and/or provide information about more specific sites where developmental arrest occurs (2).

Before discussing leukemia immunophenotyping, normal ontogeny of hematopoietic cells in the bone marrow should be reviewed.

Ontogeny of Hematopoietic Cells:

The proliferation and differentiation steps of hematopoietic cells in the bone marrow are under the control of hematopoietic growth factors (Figure 1).

Among these, interleukin-3 (IL-3) interacts first and then granulocyte-macrophage colony stimulating factor (GM-CSF) interacts with committed progenitors, which become dependent upon late-acting factors such as granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF) and erythropoietin (EP) (3).

During proliferation and differentiation stages cells acquire and lose some surface antigens.

For instance B lymphoid maturation has four steps in the bone marrow and appearance and disappearance of antigens can be seen in all steps (4).

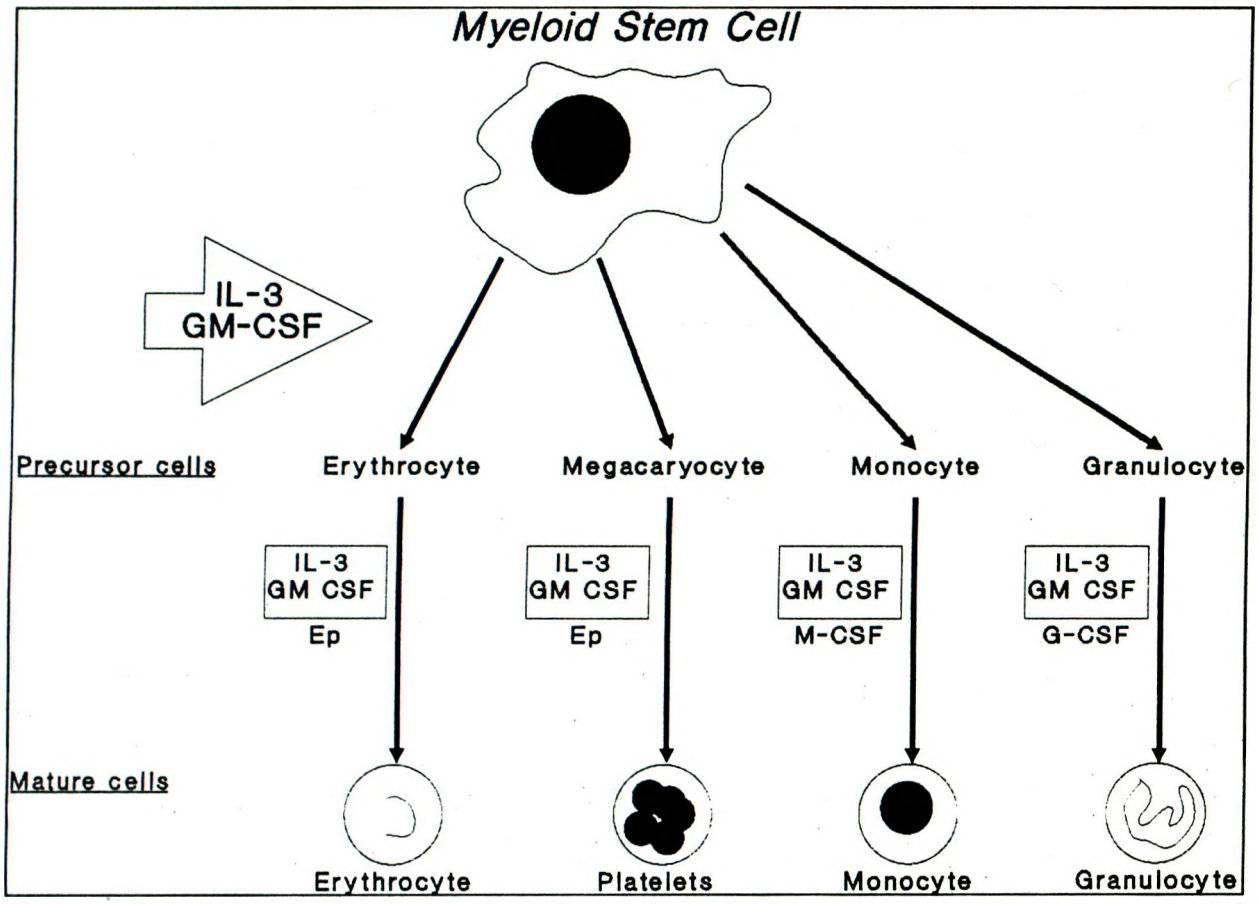

Fig 1: The effect of hematopoietic growth factors on the development of myeloid cells in the bone marrow : IL - 3: Interleukin -3, GM-CSF: Granulocyte - Monocyte colony stimulating factor, Ep: Erythropoietin, G-CSF: Granulocyte colony stimulating factor, M-CSF: Monocyte colony stimulating factor.

Figures 2 and 3 show the hypothetical scheme of lympoid and myeloid differentiation respectively (5). Since leukemia is basically a maturation arrest for unknown reasons with proliferation at an immature stage without going into terminal differentiation (6), the known composite phenotypes of precursor cells will provide to determine the cellular origin and maturational stage of leukemic blasts.

Leukemia can be classified by using the combinations of nonoverlapping lineage associated and stage specific Mabs.

Flow Cytometry

Mabs produced against surface antigens are labelled with fluorochrome dyes or with fluorescinated secondary antibodies; cells possessing the relevant antigen would be fluorescinated. Different dyes (fluorescein isothiocyanate: FITC, phycoerythritin: PE, Texas red) give different colors under fluorescein light. These cells can either be counted under conventional fluorescent microscope or with flow cytometry. The latter technology is less laborious, and less time consuming, more objective and repeatable. Flow cytometry is the combination of laser technology, fluorochrome cytochemistry and a broad array of Mabs along with computer processing.

The criteria of a flow cytometric analysis are:
1 - Size of the cells (Forward scatter: FSC)
2 - Granularity of the cells (Side scatter: SSC)
3 - Fluorescent emission (FL1, FL2, FL3)
4 - Fluorescent intensity.

As the first step of leukemia immunophenotyping the interested population of cells is gated according to the size and granularity. Leukemic blasts are usually larger than other mononuclear cells and are not granulated. Surface antigens of gated cells are then evaluated by using various Mabs and positive and negative cells are analyzed by using computer software.

The bitmap printouts maybe so striking in some cases showing a monoclonal proliferation of all gated cells (Figure 4).

There is abundant proliferation of CD19 (+) B cells whereas CD3 (+)T cells comprise only a minority of lymphocytes of this chronic lymphocytic leukemia (CLL) case.

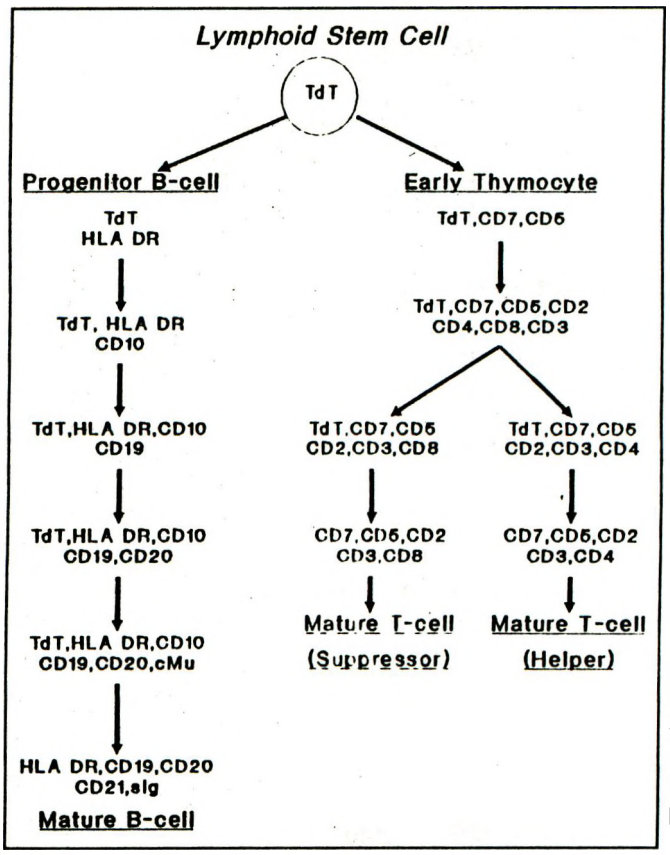

Fig 2: Hypothetical scheme of lymphoid cell maturation TdT: Terminal deoxynucleotdyl Transferase, sIg: surface Immunoglobulin, cMu: cytoplasmic Mu chain

It is also possible to determine the fluorescent intensity which reflects the density of the cell surface antigen. The relative intensity of CD45 which reacts with a panleucocyte antigen in different cell types has been described (7). Early acute lymphoblastic leukemia (ALL) cells are stained very dimly by CD45. The intensity is increased in late phase of the disease. CLL cells show more intense staining still being more pale than normal peripheral blood lymphocytes. Hairy cell leukemic blasts are stained most intensively with CD45.

Acute Lymphoblastic Leukemia (ALL) Phenotypes:

Flow cytometry improved the leukemia classification and ALL's gained a new classification terminology. The majority of childhood ALL is non-T ALL comprising 70-80% of cases which has been divided into 6 subgroups by Nadler et al according to surface phenotypes of blastic cells (8). This classification is based on the expression of HLA-DR, CD19, CD10, CD20 surface antigens and presence of cytoplasmic and surface immunoglobulins (Figure 5). CD10 (cALLa) is present on normal immature lymphoid and leukemic cells. Although this antigen is not confined to lymphoid cells and is shown on fibroblasts, endothelial cells and rarely on myeloid blasts; it is accepted as a milestone in the classification and as a prognostic criterium of ALL.

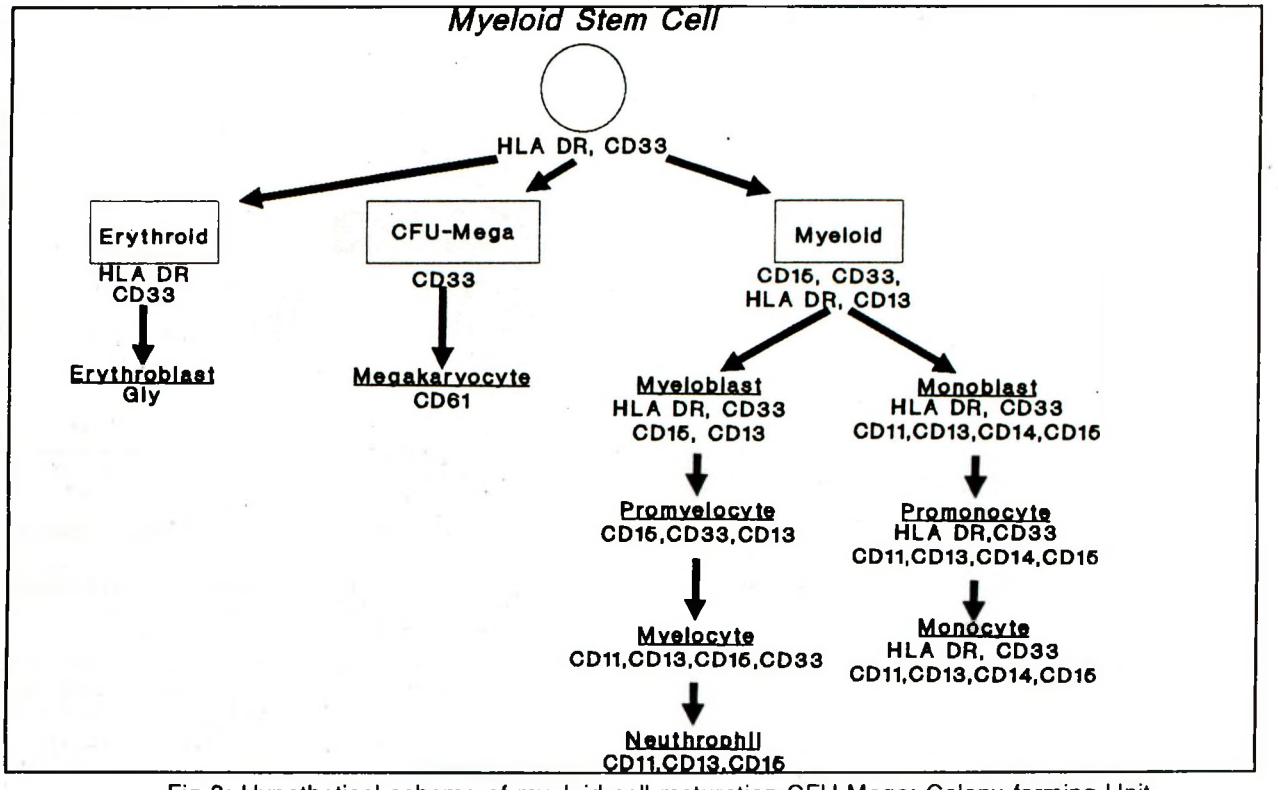

Fig 3: Hypothetical scheme of myeloid cell maturation CFU-Mega: Colony forming Unit-megakaryocyte

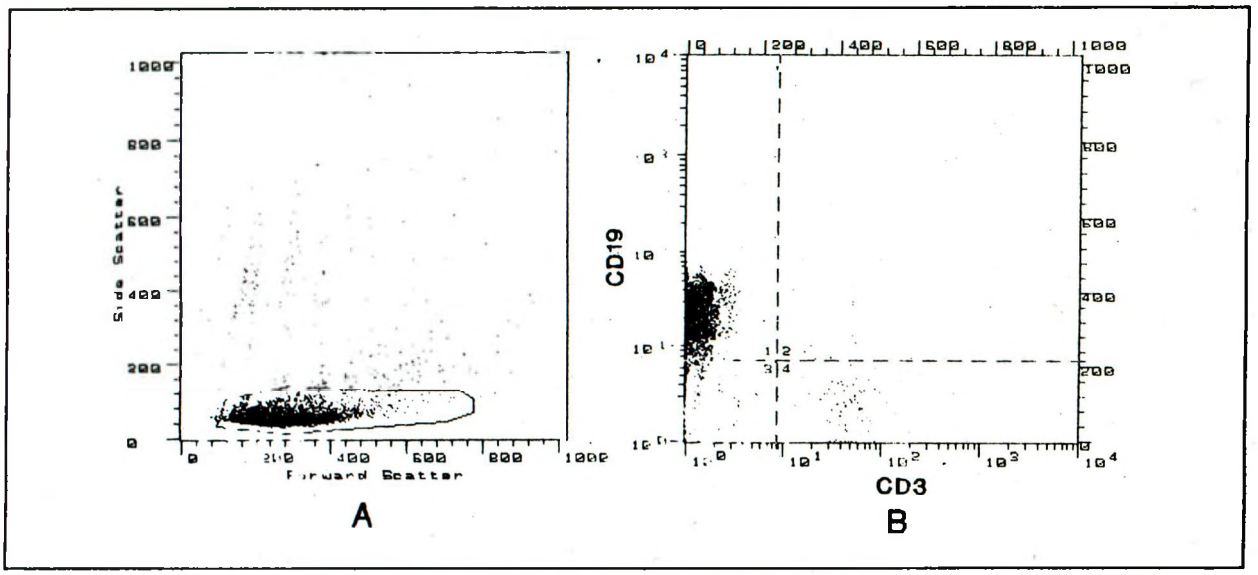

Fig 4: Flow cytometric analysis of a chronic lymphocytic leukemia case: A: Forward scatter and side scatter analysis shows that majority of cells are nongranulated with a relatively uniform size. B: of the gated cells the majority are CD19 (B cell). CD3 cells are very few.

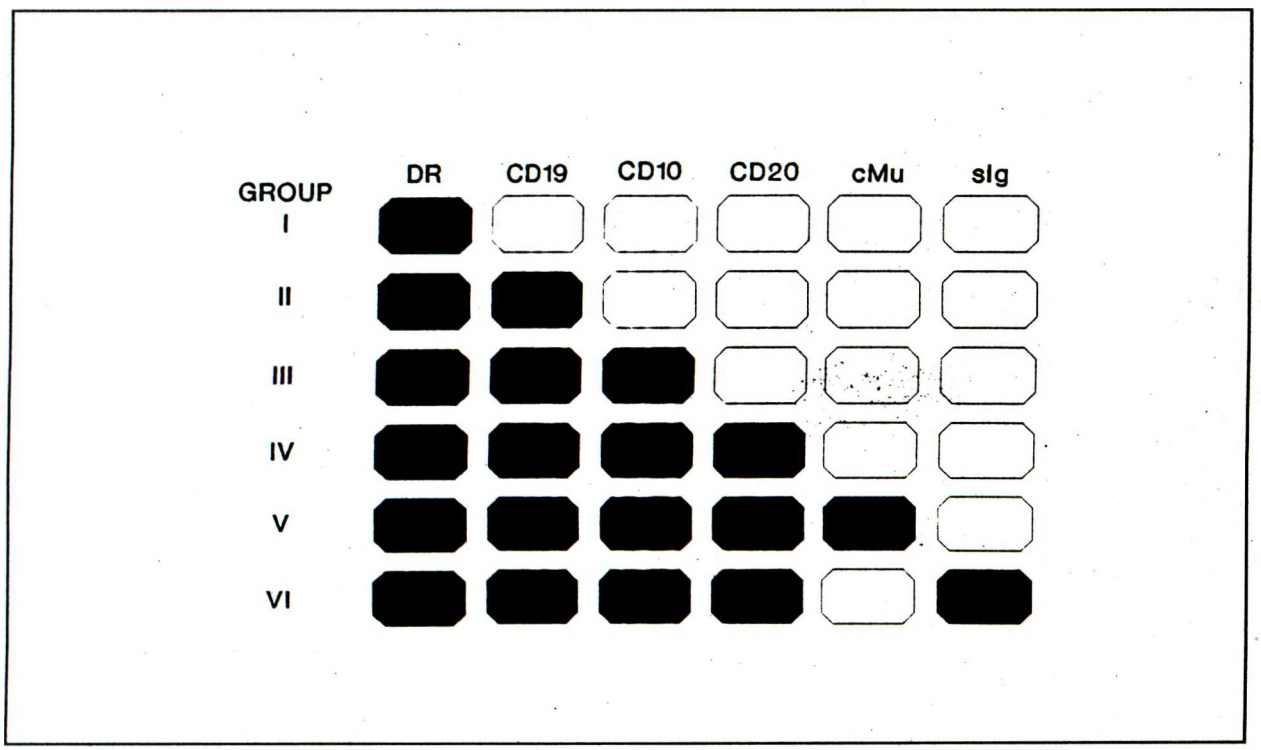

Fig 5: Nadler's classification of non-T ALL.

In contrast to Nadler's classification, cALLa disappears when surface immunoglobulin is expressed on the blast cells in other reports (9,10). The presence of surface immunoglobulin corresponds with FAB L3 morphology which is consistent with its poor prognosis.

A flow cytometric analysis of a cALLa (+) ALL case is shown in figure 6. cALLa is present only one in 100.000 cells in normal peripheral blood so the presence of this antigen is helpful for early detection of most childhood ALL and minimal residual disease.

T-ALL classification is suggested by Foon et al according to surface phenotypes (Figure 7).

T-ALL has more aggressive clinical pattern, with larger leukemic burden i.e high blast counts, and mediastinal masses. A case of group I T-ALL with CD7 (+), CD5 (+), CD4(–), CD8 (–) phenotype is shown in figure 8.

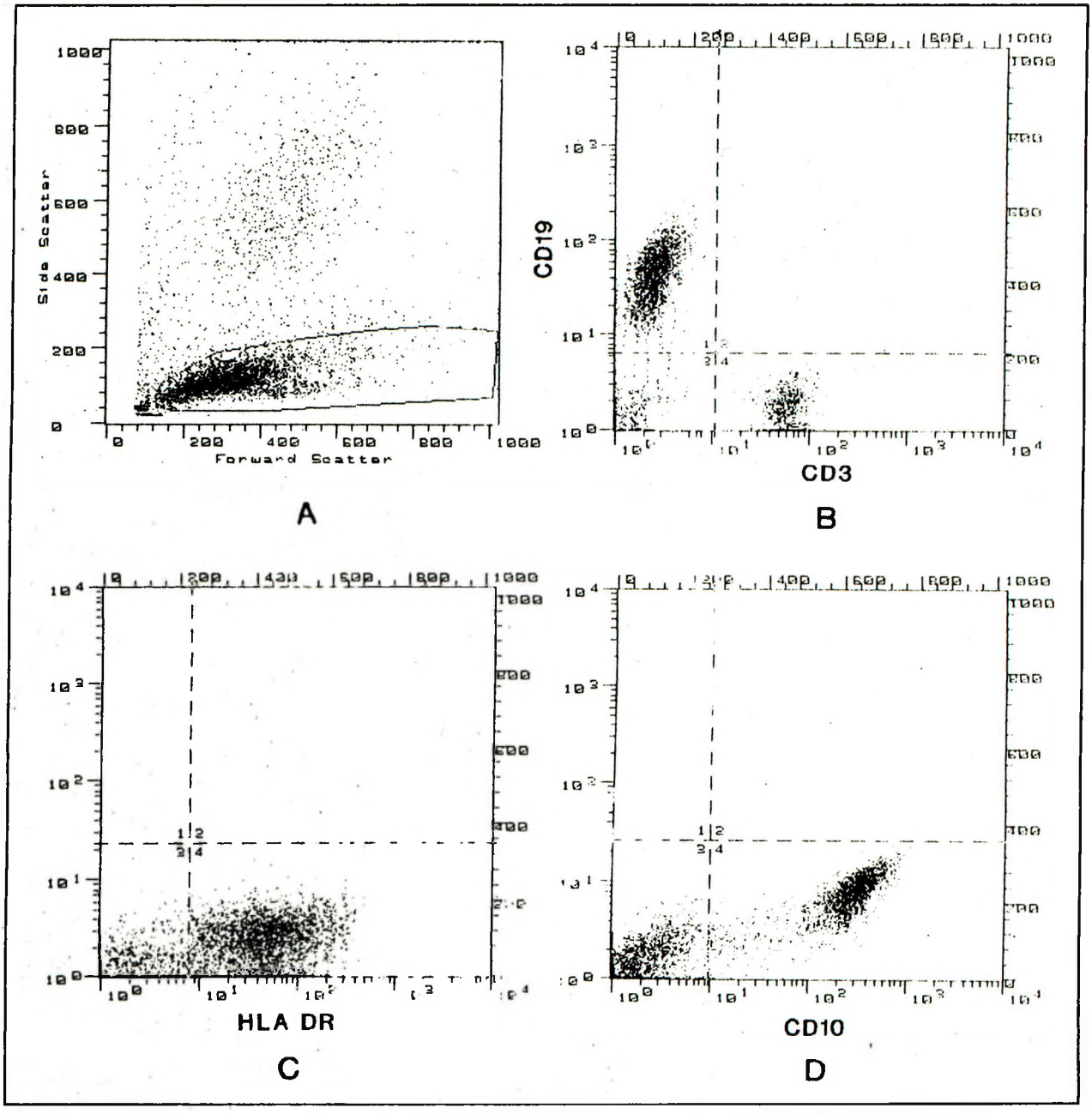

Fig 6: cALLa (+) ALL: A: gated cells B: Majority of cells are CD19 (+) C:HLADR (+) D:CD10 (+)

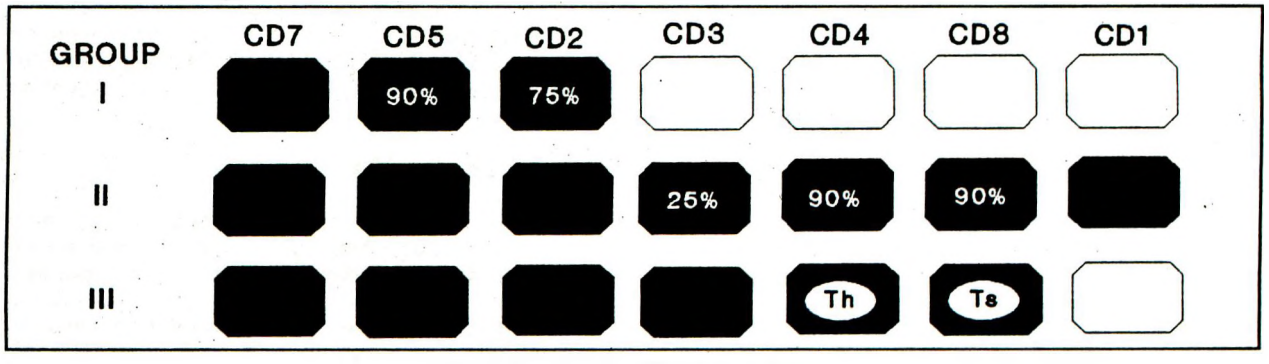

Fig 7: Foon's classification of T-ALL

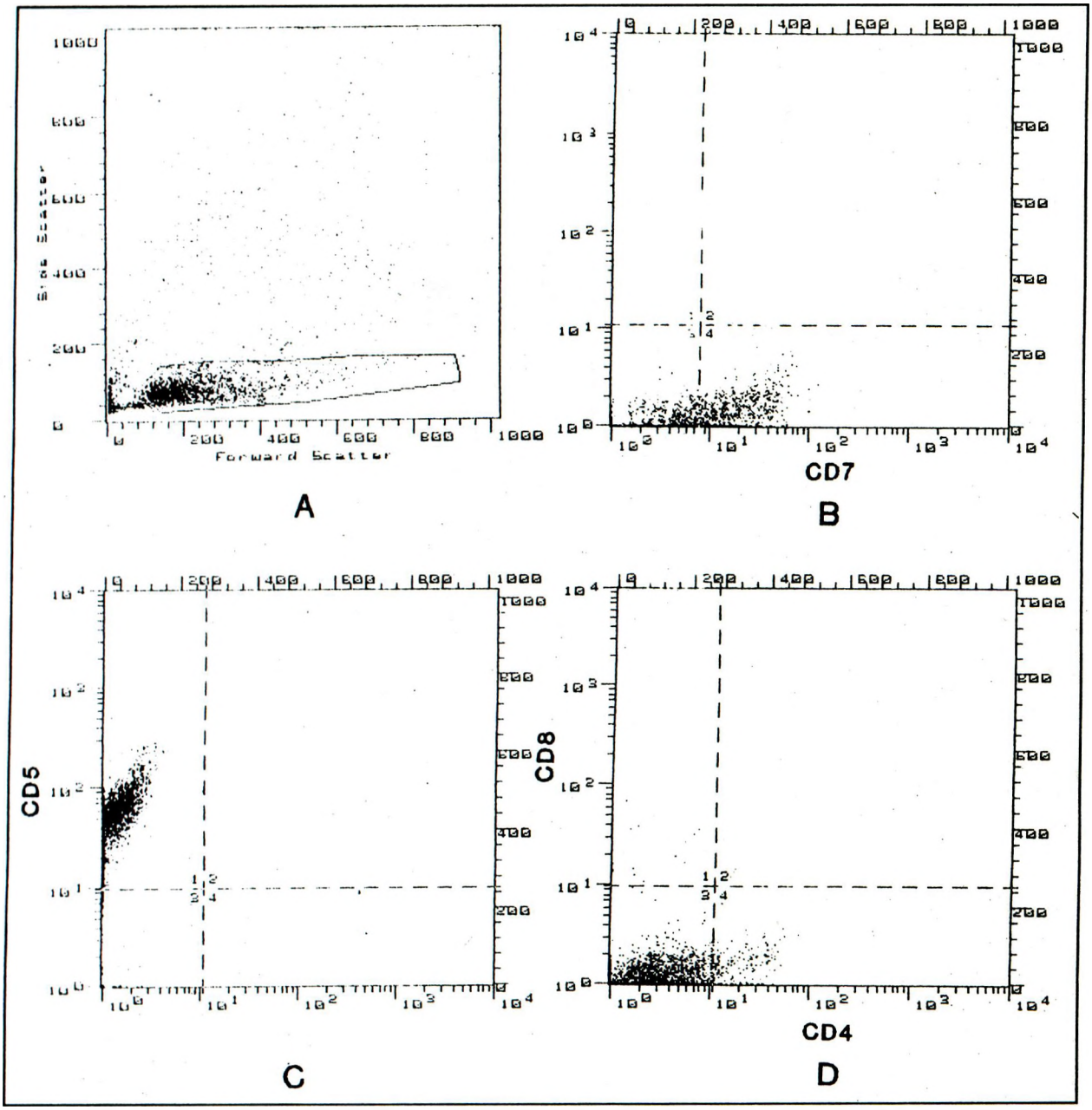

Fig 8: T-ALL: A: gated cells B: majority of cells are CD7 (+) C: CD5 (+) D: CD4 (-) CD8 (-) The absence of these markers confirms immaturity.

Acute nonlymphoblastic leukemia (ANLL) phenotypes:

The immunologic classification is not clear cut in myeloid leukemia. The variable expression of myeloid antigens on normal cells makes the interpretations more difficult. Nevertheless the abundant presence of myeloid antigens in the peripheral blood should definitely suggest a myeloid origin. If more than 20% of cells react with an antibody the reaction is considered to be positive. ANLL is subdivided into seven groups; from M1 to M7. CD33 is present in all types except M6 (Erythroleukemia). CD61 is specifically expressed on megakaryocytic leukemia (M7) cells. The absence of HLADR on the surface of promyelocytic leukemia (M3) is characteristic. CD14 is particularly present on M4 and M5 blasts. M1and M2 blasts are positive for HLA-DR and CD33 but not the other myeloid antigens (Figure 9).

Mixed Leukemia

Routine use of immunophenotyping not only improved the diagnosis and classification of leukemia but also added new dimensions to leukemogenesis and origins of blasts. It has been reported by Hurvitz et al that 78% of leukemic blasts do not possess the same combination of surface antigens with their normal counterparts (11).

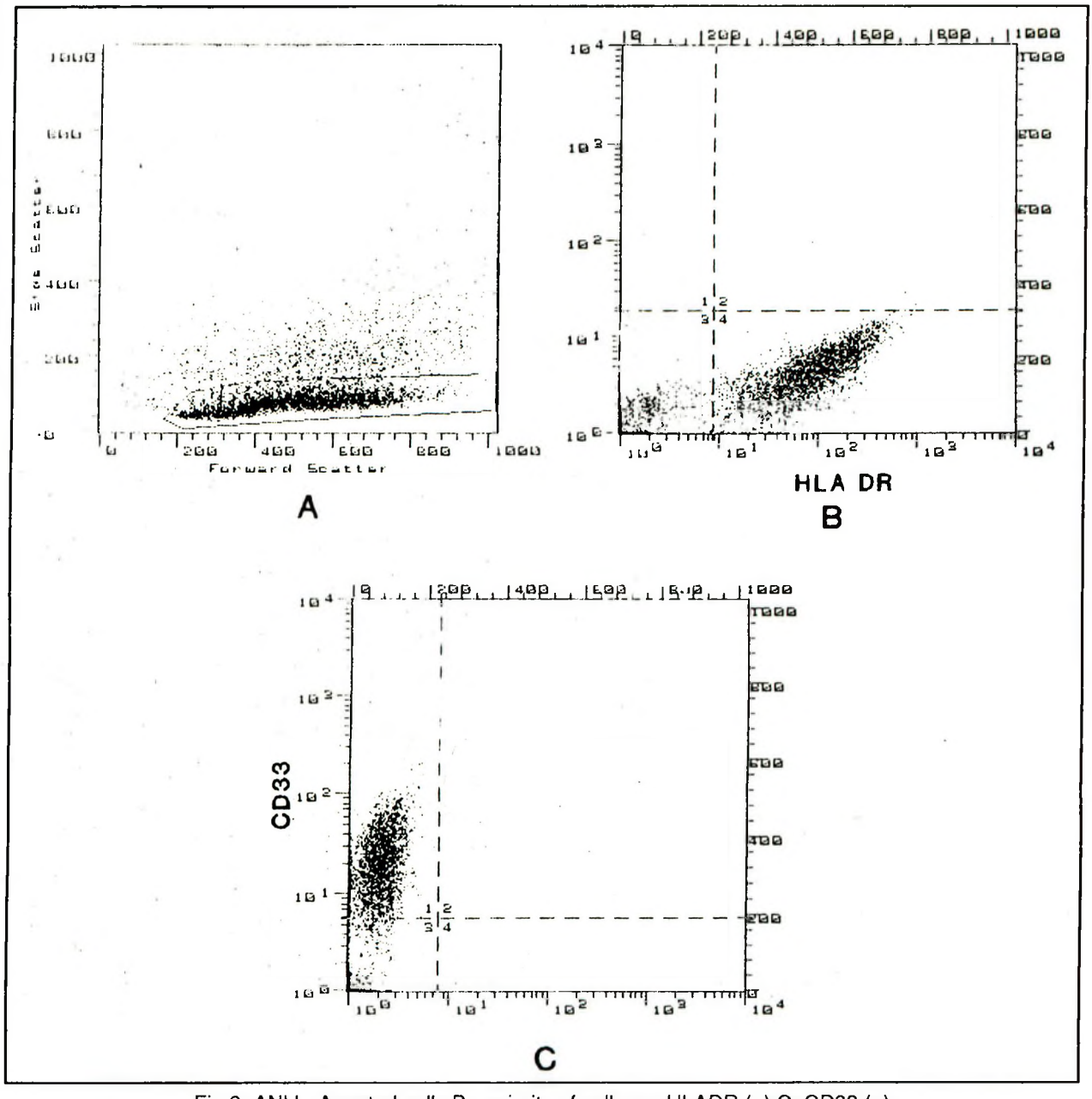

Fig 9: ANLL: A: gated cells B: majority of cells are HLADR (+) C: CD33 (+)

For instance CD10 is the first appearing surface antigen even before CD19 in normal B cell ontogeny (2,6). CD10 (+) CD19 (–) counterparts of normal B cell precursors does not exist in leukemia. This property of blasts is called developmental asynchrony which brings the idea that leukemic blasts are not produced simply by monoclonal proliferation of arrested immature cells. They possess a distinct combination of surface antigens suggesting a different commitment of proliferation. Consistently leukemic blasts do not respond normally to hematopoietic growth factors in in vitro conditions whereas normal immature cells differentiate terminally (12).

Another feature of leukemic blasts is lineage infidelity or lineage promiscuity, i.e some blasts may possess both myeloid and lymphoid antigens. This may be due to the proliferation of cells of an unknown developmental stage (promiscuity) or aberrant gene expression (infidelity).

The most prevalent mixed cases show association of T cell antigens with myeloid antigens as is shown in figure 10. The phenotype of blasts is CD7 (+) HLADR (+) CD33 (+). CD7 is a pan-T cells antigen. HLADR is positive on cells of myeloid and B cell origin. CD33 is present on all myeloid cells. The leukemic blasts possess both T and myeloid cell antigens.

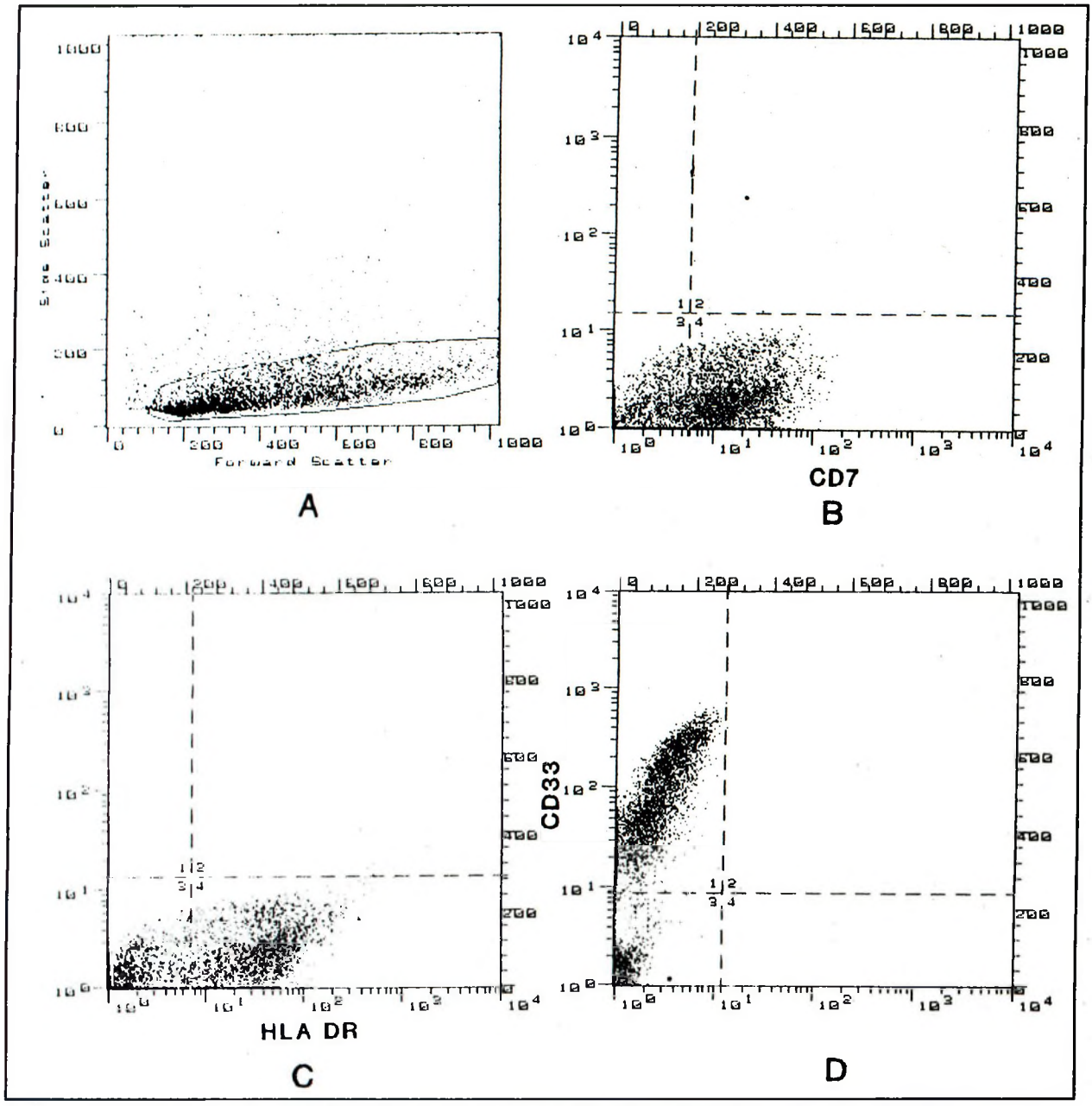

Fig 10: Mixed ANLL/ALL: A: gated cells B: CD7 (+) C: HLADR (+) D: CD33 (+)

CD7 (+) T-ALL may represent malignant counterpart of the multilineage stem cell (13).

The effect of hematopoietic growth factors on CD7 (+) T cell precursors has been studied in in vitro conditions. In the presence of IL-3 and GM=CSF the cells differentiated to the normal phagocytic cells whereas IL-2 induced cytotoxic T Leukemia cell development. When there is no growth factor the cells differentiated to mixed lineage blasts carrying CD7, CD33 and CD13 (Figure 11).

This experiment suggests that the development of mixed lineage blasts may be due to the deficiency of hematopoietic growth factors at the right time and place.

Interpretation

Since there is no leukemia specific antigen; the interpretation depends on "abnormal" amounts and combinations of "normal" surface antigens. Many of the defined surface antigens are shared by both precursor and mature cells of the same and/or different lineages. The combined use of Mabs exclusively confined to one lineage and a maturational stage could solve the problem.

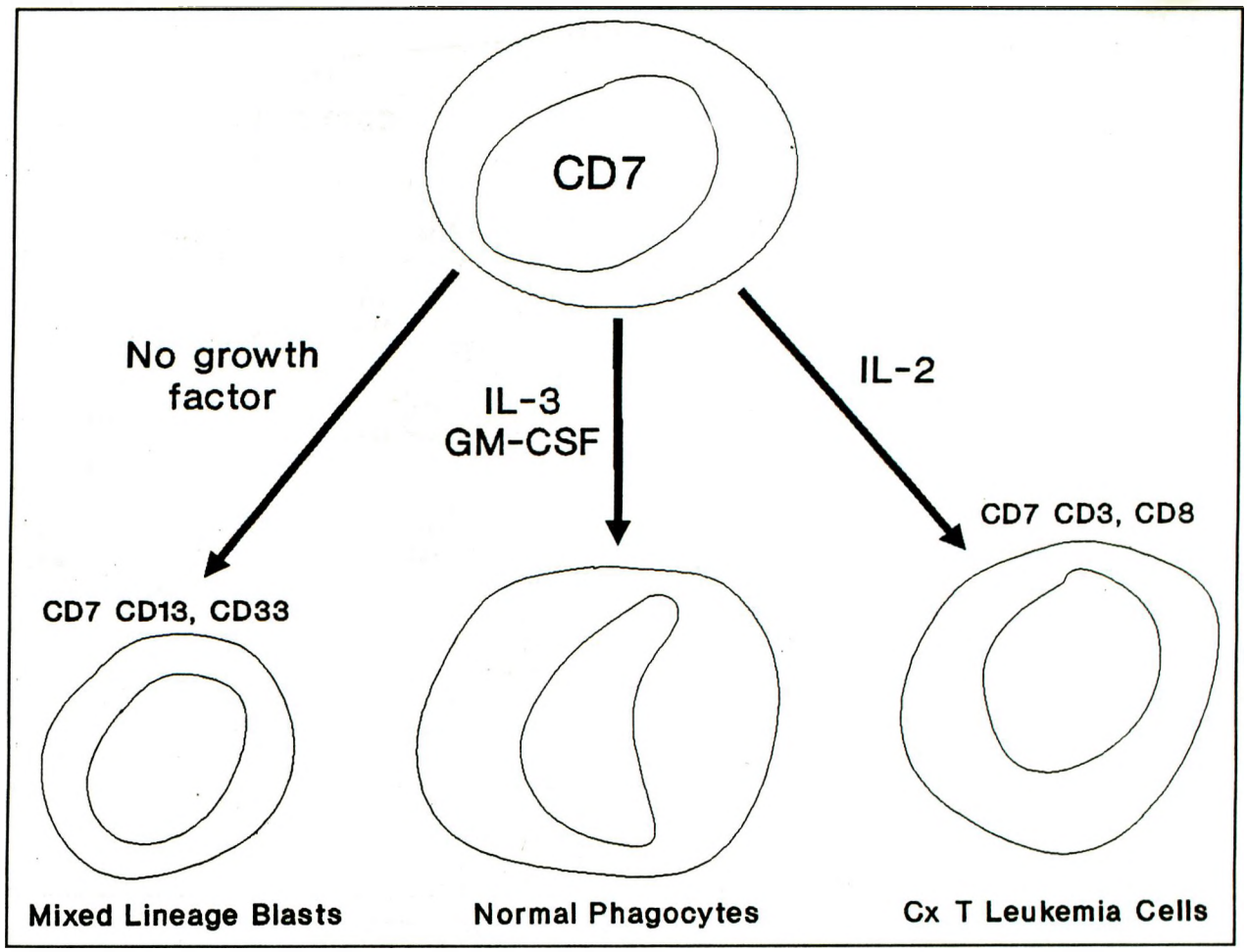

Fig 11: Proposed interaction between multilineage stem cell and hematopoietic growth factors

Thus immonophenotyping is performed by Mab panels in order to decide the cellular origin of the blasts and to assign maturational step. Sequential application of Mabs will provide information for accurate classification.

A leukemia diagnosis flowchart is proposed in Figure 12.

In conclusion, diagnosis of hematological malignancies can be made with one or more of the following findings:

1 - Abundant proliferation of a population possessing mature surface antigens (monoclonality).
2 - Presence of populations expressing immature surface antigens.
3 - Abnormal combination of surface antigens (developmental asynchrony).
4 - Coexpression of surface antigens of different cell lineages (lineage infidelity or promiscuity).

Immunophenotyping should always be combined with clinical, morphologic and cytochemical findings.

Leukemia immunophenotyping provides :

a. the determination of the cellular origin of blasts
b. the diagnosis and classification of leukemia along with morphologic and cytochemical criteria
c. the detection of minimal residual disease
d. the selection of therapy type
e. the detection of relapses before clinical findings
f. the discrimination of reactive lymphocytes from malignant lymphoblasts.

Thus, immunophenotyping becomes an indispensable tool in leukemia management. Routine application of immunophenotyping and data accumulation after long term will shed light into the leukemogenesis, add new criteria to predict prognosis and improve morphologic diagnosis by further evaluation of blasts with composite phenotypes under light microscopy.

Acknowledgement

The author wishes to thank to Duzen Laboratories for providing FACscan analysis, to Ayşe Kayaalp for her technical assistance, to Tolga F. Köroğlu for the preparation of figures and to Serpil Rahin for typing the manuscript.

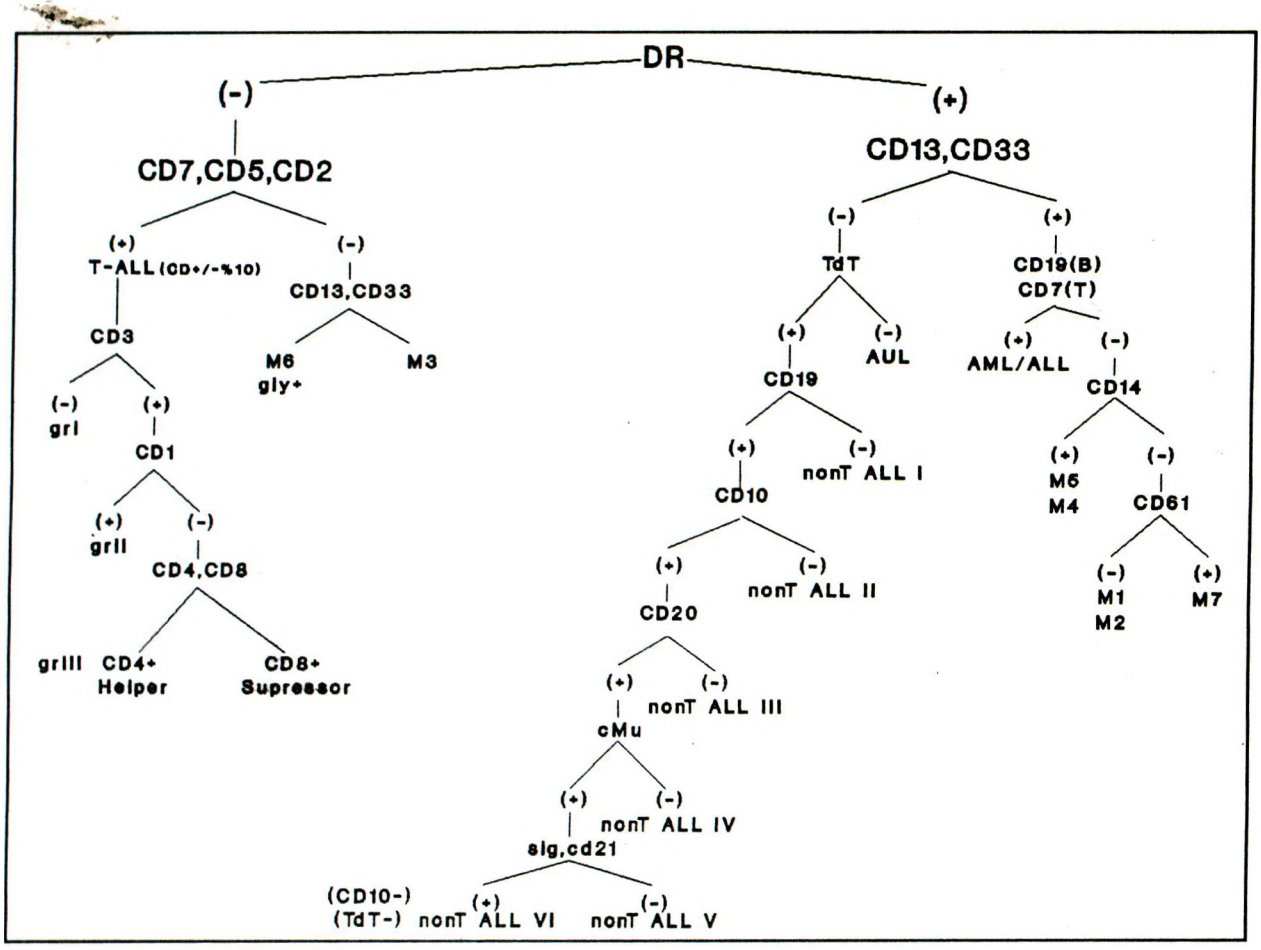

Fig 12: A flowchart for acute leukemia diagnosis

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