THE ROLE OF MONOCLONAL ANTIBODIES IN THE DIAGNOSIS OF ACUTE LEUKAEMIA.

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Thesis submitted in fulfilment of the requirements for the Masters Diploma in Medical Technology in the School of Life Sciences at the Cape Technikon.

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Dedicated to the memory of my parents.

I declare that this thesis is my own work. It is being submitted for the Masters Diploma in Medical Technology, to the Cape Technikon, Cape Town. It has not been submitted before for any diploma or examination at any other Technikon. The work was carried out in the Haematology Department, Groote Schuur Hospital. The opinions and conclusions drawn are not necessarily those of the Cape Technikon.

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10th December 1990

Date

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SUMMARY

Eighty six patients with acute leukaemia were studied using morphological, cytochemical and immunological techniques. The acute leukaemias were subdivided using the French-American-British (FAB) classification. The immunophenotyping studies were compared with the morphological classification to assess their contribution to the diagnosis. Acute non-lymphoblastic leukaemia (ANLL) was diagnosed on the basis of morphology and cytochemical criteria. In addition this group of patients was studied with antibodies directed against myelomonocytic antigens. However, no further clinically useful information was obtained. Patients whose blasts did not stain with Sudan black or myeloperoxidase were considered to have acute lymphoblastic leukaemia (ALL). After assessment with monoclonal antibodies directed against epitopes expressed on cells from the lymphoid lineage, these patients were subgrouped into non-T-ALL, common-ALL, B-ALL, T-ALL and lymphoblastic lymphoma categories. This study confirmed the value of monoclonal antibodies for accurately assigning lineage to the acute leukaemias and particularly in those situations where conventional morphological criteria and cytochemical markers are inconclusive.

OPSOMMING

Ses en tagtig pasiënte met akute leukemie is met behulp van morfologiese, sitochemiese en immunologiese tegnieke ondersoek. Die akute leukemieë is deur die Frans-Amerikaanse-Britse (FAB) - klassifikasie-sisteem onderverdeel. Die immunofenotipiese studies is met die morfologiese klassifikasie vergelyk om hul bydrae tot die diagnose te bepaal. Akute nie-limfoblastiese leukemie (ANLL) is gediagnoseer op die basis van morfologie en sitochemiese kriteria. Verder is hierdie groep pasiënte met behulp van teenliggame gerig teen miëlomonositiese antigene bestudeer. Geen verdere kliniese inligting is verkry nie. Pasiënte waarvan die blaste nie deur Sudanswart of miëloperoksidase gekleur is nie, is beskou as akute limfoblastiese leukemie (ALL) gevalle. Na bepaling met monoklonale teenliggame gerig teen epitope uitgedruk op selle van die limfoïede lyn, is hierdie pasiënte gesubgroepeer in nie-T-ALL, algemene-ALL, B-ALL, T-ALL en limfoblastiese limfoomkategorieë. Hierdie studie het die waarde van monoklonale teenliggame vir die akurate toekenning van die lyn tot akute leukemieë bevestig en in besonder tot situasies waar konvensionele morfologiese kriteria en sitologiese merkers onoortuigend is.

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"The cytologic aspects of the blood in acute leukaemia are of great interest."

Archives of Internal Medicine, January, 1934.

"Morphology is more an art than a science and just as the murmur of mitral stenosis is better characterized by listening to it than hearing descriptions of it, the morphologic distinction between lymphoblasts and myeloblasts is better seen than described."

Medicine 41, 1962.

" ... only by a combination of all available methods of study that an acceptable opinion concerning cell type may be established."

Archives of Internal Medicine, 1934.

CHAPTER 1.

INTRODUCTION

1 INTRODUCTION

Acute leukaemia has traditionally been classified by morphological and cytochemical criteria. This approach was refined by a French-American-British (FAB) working group that subdivided the myeloid and lymphoid variants in an attempt to unify terminology. Subsequently, these recommendations were extended to include megakaryoblastic leukaemia on the basis of ultrastructural cytochemistry and immunological techniques. Diagnostic criteria have been expanded by the use of monoclonal antibodies, which recognise epitopes expressed on the surface of leukaemic blasts and define different lineages. The correlation between these two broadly different approaches has been the subject of a number of studies (San Miguel, et al, 1986; Andreason, et al, 1986; Browman, et al, 1986; Foon, et al, 1986). Furthermore, attempts to develop reliable correlations between FAB classification and immunologic membrane markers have recently been complemented by addition of cytogenetic data giving rise to the preferred morphologic-immunologic-cytogenetic or MIC classification (First MIC Cooperative Study Group, 1986; Second MIC Cooperative Study Group, 1988). It is clear that a multifaceted approach is important in achieving a more precise identification of cell origin and ultimately appropriate treatment for the patient. To this end the role of monoclonal antibodies was studied to assess its role in complementing traditional cell morphology.

1.1 HISTORICAL BACKGROUND

1.1.1 Early Reports of Leukaemia

In 1845 Bennett and Virchow (Necheles, 1979) independently described the first two cases of leukaemia. The disease was named leukaemia by Virchow, since the increased number of white cells gave blood a whitish colour.

He classified the leukaemias into two groups using clinical criteria of splenomegaly and lymphadenopathy. Today we recognise these two groups as chronic myeloid leukaemia and chronic lymphoid leukaemia (Necheles, 1979).

In 1857, Friedereich (Forkner, 1934) reported a case of leukaemia which appeared to run an acute course. This was the first recording of acute leukaemia (AL) as a separate entity. In 1895 Fraenkel (Forkner, 1934), after examining the morphology of leucocytes in patients said to be suffering from AL, drew the conclusion that all leukaemias were lymphoid, as the cells resembled "lymphocytes". Five years later, in 1900, Naegeli (Forkner, 1934) described the myeloblast, thus introducing for the first time the possibility of classifying leukaemias on the basis of cytology.

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John Auer (1906) described rod structures in the large granular "lymphocytes" of a patient suffering from acute leukaemia. The Auer rods, as we know them today, stained a pale red colour. The large granular "lymphocytes" in which these rods were demonstrated, were described as having a delicate nuclear chromatin network with no coarse chromatin threads or clumps. The nuclei were round or oval with a few cells showing nuclear indentation. There were one to three clearly defined nucleoli and the cytoplasm stained pale blue. This was the description of a typical myeloblastic leukaemia, although Auer did not realise this, naming the entity acute lymphoblastic leukaemia.

Acute monocytic leukaemia was described by Reshad and Shilling-Torgau (Forkner, 1934) in 1913, and the following year Hirschfeld (Necheles, 1979) noted the involvement of the erythroid series in the leukaemic process.

1.1.2 Cytology

In 1879, Ehrlich became the first to classify granulocytes into the three types we know today (Clark and Kasten, 1983). When, however, abnormal mononuclear cells were found in the circulation, as in AL, the origin of these cells was not clear and the finer cytologic features of "blast" cells began to play a role in the differentiation of acute leukaemia. Forkner (1934) described myeloblasts as having large round or oval nuclei, slightly indented, containing several nucleoli and having basophilic cytoplasm.

Lymphoblasts were monomorphic with less cytoplasm and large nuclei with a diffuse chromatin pattern. Monoblasts were large cells which "tend to have a complicated shape", probably referring to the marked folding that may be seen in the monocytic series. The cytoplasm was more abundant and contained dustlike granules.

1.1.3 Classification

Initially, leukaemia was differentiated by clinical features alone. It was noted that marked swelling and ulceration of the mucous membranes, especially in the mouth around the gums was found in acute monocytic leukaemia (AMoL), but was an infrequent feature in other acute non-lymphoblastic leukaemias (ANLL) and acute lymphoblastic leukaemias (ALL). Other clinical signs and symptoms taken into consideration and used to assist in the differentiation of the acute leukaemias, were spleen, liver and lymph node enlargement. The variable clinical signs, however, made it very difficult to divide the disease into specific types (Forkner, 1934). A further distinction was made clinically when it was realised that leukaemia could be divided into two groups, acute and chronic, based on the natural history of the disease. A definition by The Committee for the Classification of the Nomenclature of the Cells and Diseases of the Blood and Blood-forming organs (1950) reads: "Acute leukaemias are defined as those leukaemias with an expected duration of life of three months or less from the onset of the

first symptom". If the patient lived longer than three months the type of leukaemia was said to be chronic. The acute leukaemias were further characterised by the presence of undifferentiated cells in the peripheral blood, while the chronic group showed more mature forms of the predominant cell type.

An attempt to classify the acute form of the disease on the basis of age was also considered, as children tended to have the lymphoblastic type and a more favourable prognosis than adults who, more frequently, had the myeloblastic type. The exceptions which occurred in the adult-child differentiation rendered this approach unsatisfactory.

The initial haematologic attempts at differentiation were made on the basis of morphologic description of the cells in the peripheral blood. The identification of the immature mononuclear cells was hampered, however, by the large variation in respect of size, shape, number of nucleoli in the nucleus and inclusions in the cytoplasm.

Downey (1938) (Watkins and Hall, 1940) described two types of monocytic leukaemia. The cells of the one had cellular characteristics of myeloblasts and monoblasts. Naegeli (Watkins and Hall, 1940), in support of Downey's theory thus postulated that the origin of monocytic cells was myeloid, a variant of acute myeloid leukaemia or acute non-lymphoblastic leukaemia (ANLL). The morphology of the second type of monocytic leukaemia,

appeared more primitive and showed obvious "grooving" of the nucleus throughout the series.

Many of the early classifications involved detailed cytologic studies and were based on histologic tissue sections. It was established that leukaemic cells infiltrated normal haematopoietic and other tissue and disturbed the normal architecture of organs such as the spleen and liver. Using the oxidase reaction (Schultze, 1909) (Forkner, 1934) the histopathologist was able to differentiate myeloblasts which were oxidase positive from lymphoblasts, which were oxidase negative. The histologic differentiation became difficult when monoblastic leukaemia was discovered, as the oxidase reaction could be variable in these cells. It was found, however, by careful microscopic examination of the monocytic cells, in which the oxidase reaction was positive, that the granules were fewer and smaller than those of the myeloid series (Forkner, 1934). The introduction of the oxidase reaction by Schultze may be the first description of the distinction of cell types by a histochemical staining pattern. The introduction and use of peroxidase by Graham (Hayhoe, et al, 1964) in 1916 was further evidence that the undifferentiated blast cells of the acute leukaemias were not homogeneous.

Paul Ehrlich's application of Aniline dyes to the staining of tissues and blood smears formed the basis of blood stains (Clark and Kasten, 1983). In 1877 he described the important difference between acid (eosin) and basic (methylene blue) dyes for staining purposes. He reported that the acid dyes preferentially stained erythrocytes and eosinophilic granules and the basic dyes coloured the nuclei, cytoplasm and basophilic granules. Two years later he prepared a neutral stain, which stained the various components of the cells, at the same time, in contrasting colours. It was using this stain that he discovered the violet granules in the neutrophil. Nine years elapsed before Arenzinsky (1888) combined eosin and methylene blue as a blood and malarial stain, which led through Plehn (1890) and Romanowsky (1891) to Jenner, May-Grunwald, Leishman, Wright and Giemsa in 1901-1902 to the currently used blood stains (Clark and Kasten, 1983). The delicate distinctions in shades of staining and the differential staining of granules depends on the combinations of dyes - methylene blue and eosin and H^+ ion concentration. A pH of 6.8 is recommended for general routine smears (Clark and Kasten, 1983; Dacie and Lewis, 1975). Distinctive cytological criteria belonging to specific primitive leukaemic cells were identified when Romanowsky stains were used. Distinguishing features included Auer rods, nuclear cytoplasmic ratio, nuclear chromatin pattern and number of nucleoli present (Table 1).

CELLULAR FEATURE	MYELOBLASTS	LYMPHOBLASTS	MONOBLASTS
Auer rods	+	-	+
N/C Ratio	variable	high	low
Nuclear chromatin	fine	coarse	open network
Nucleoli (number)	higher than lymphoblasts	lower than myeloblasts	lower than myeloblasts

Table 1. Morphological features

Following these criteria, certain cells could not be identified and assigned to a specific lineage and the "unclassified", "undifferentiated", "haemocytoblastic" or "stem cell" group remained. At this time there was no uniformity, as the views and interpretation of the stained smears varied greatly. Phase-contrast microscopy, where viable cells were examined, was another method used in an attempt to separate the types of leukaemic cells. The motility of the cells and movement of intracellular components were observed and structures such as Auer rods and nucleoli were more conspicuous than on stained smears. The cells were divided into three groups, myeloblasts, lymphoblasts and monoblasts, with no evident separation of undifferentiated cells. This approach did not, however, contribute any further to delineation of the cell types, although it seemed to correlate with the findings on Romanowsky stained films.

Morphology and cytochemical reactions were first studied systematically by Hayhoe, et al, (1964), in an attempt to define criteria by which the cell types could be assigned lineage. This study provided a substantial source of information which in many ways prepared the way for the classification proposed by the French-American-British (FAB) group (Bennett, et al, 1976).

Leukaemic blasts may be indistinguishable by morphological criteria alone. Historically, blast cells which are negative when stained with Sudan black or myeloperoxidase are termed lymphoblasts, as they show no myeloid differentiation. But their lack of myeloid expression in terms of the above stains does not prove that they are lymphoblasts.

The detection of immunologically distinct lymphoid cell subgroups and their categorization as T and B subtypes on the basis of surface membrane characteristics led to the application of cell markers to the classification of acute lymphoblastic leukaemias. The significance of the early attempts to subtype the ALL group was immediately evident (Thierfelder, et al, 1977), as the "undifferentiated" acute leukaemias could be immunologically identified as lymphoblasts.

1.2 Literature Review

Before proceeding with a review of the literature, it is appropriate to discuss briefly the nomenclature used for the monoclonal antibodies. The expanded range of monoclonal antibodies may be confusing and four international workshops on Human Leucocyte Differentiation Antigens have been held to establish an accepted nomenclature. The aim was to test the large numbers of antibodies which had emerged from rapid production and to target their cell specificity. The First Workshop dealt with antibodies directed at T-cells, the Second with B-cell differentiation, the Third (Oxford, 1986) with myelo-monocytic antibodies. Groups of monoclonal antibodies, which share some characteristics, were identified and termed "clusters".The clusters of differentiation (CD) nomenclature arose from this and avoids the confusion of monoclonal antibody jargon (Leucocyte Typing III, 1986). The Fourth Workshop was held in Vienna in February 1989 and reported that two trends seem to be emerging. First, the functional importance of the non-lineage restricted surface antigens present on human leucocytes is being appreciated. Second, molecular cloning and sequencing of genes coding for surface molecules, together with the use of monoclonal antibodies has had a substantial effect in the development of this field (Knapp, et al, 1989).

In 1976 a morphological classification was proposed by a French-American-British (FAB) co-operative study group, (Bennett, et al, 1976) which has, despite criticisms (Bloomfield and Brunning, 1985; Hayhoe, 1988; Bain, 1990) proved more durable than many of its predecessors. This nomenclature attempted to provide a "common language" for haematologists around the world, so that there could be a form of standardization of reporting of the acute leukaemias. The classification takes into consideration the morphology of the blasts and certain cytochemical reactions. The acute leukaemias are divided into two broad groups and then subdivided further according to morphology in the lymphoblastic group, and cell maturation in the non-lymphoblastic group. By this original classification, the lymphoblastic group was divided into 3 types L1, L2 and L3 and the non-lymphoblastic group consisted of 6 types M1, through M6. Acute megakaryoblastic leukaemia, M7, was added to the FAB classification in 1985 (Bennett, et al, 1985a). The blast cells of this type of acute leukaemia are very pleomorphic and are negative when stained with Sudan black and myeloperoxidase. By previous FAB criteria these cells would be undifferentiated. The use of immunologic techniques or ultrastructural cytochemistry or both are required to make the diagnosis of M7. This is a digression from the usual diagnostic procedures and acknowledges the restrictions of light microscopy.

Advances in immunology, the use of monoclonal antibodies and immunological techniques have, over the past 15-20 years, allowed the distinction between most of the acute leukaemias and has identified clinically prognostic groups. The first markers used in ALL were sheep erythrocytes (Sheep-E rosettes) (Er) for T cell enumeration and surface membrane immunoglobulin (SMIg) for the identification of B cells (B-ALL <5% of cases).Both these subsets of AL had a poor prognosis. The histocompatibility-related antigen (HLA-DR), although not lineage specific (Catovsky, 1977), also assists in separating T-ALL from non-T, non-B-ALL as it is usually negative in T-ALL and positive in non-T, non-B-ALL. The ALL subgroups were as follows T-ALL: HLA-DR$^-$, Er$^+$, SMIg$^-$; B-ALL: HLA-DR$^+$, Er$^-$, SMIg$^+$ and non-T, non-B-ALL, HLA-DR$^+$, Er$^-$, SMIg$^-$. A further subset was found when the common acute lymphoblastic leukaemia antigen (cALLa or CD10) was identified on a group of patients whose cells were HLA-DR$^+$. This group of patients, HLA-DR$^+$, cALLa$^+$, Er$^-$, SMIg$^-$, tended to have a more favourable prognosis than those patients who were HLA-DR$^+$, but cALLa$^-$, Er$^-$ and SMIg$^-$. The expression of cytoplasmic immunoglobulin (Cμ heavy chain) by a percentage of patients, whose blast cell phenotype was HLA-DR$^+$, cALLA$^+$, Er$^-$, SMIg$^-$, identified a pre-B-ALL subset. It was assumed that the non-T, non-B subset of ALL was of B-lineage because the HLA-DR was positive, but is negative in most T-ALL. When monoclonal antibodies that recognize B-cell associated antigens were

identified, CD19 was found to be most specific and very useful in distinguishing non-T-ALL from T-ALL, as HLA-DR and cALLa may be found on +/-10% of T-ALL. Research in immunoglobulin gene rearrangement has provided further evidence that non-T, non-B-ALL are of B-cell lineage (Korsmeyer, et al, 1981; Nadler, et al, 1984). Recent subdivision of ALL is two broad groups, non-T-ALL and T-ALL.

The non-T group is divided into subgroups according to the genotype and phenotype (Foon and Todd, 1986).

T-ALL constitutes 15-25% of ALL. The patients are predominantly male, who present with high white cell counts and variable involvement of mediastinum and lymph nodes (Sobol, et al, 1985; Knowles, 1986). The cellular morphology is heterogeneous (Knowles, 1986). T-ALL was first identified by rosetting with sheep erythrocytes. The development of monoclonal antibodies against T-cell specific antigens has permitted definitions of subsets of T-cells and the intrathymic separation of stages of development of the thymocyte. Three stages have been recognized: early or stage I, common or stage II and mature or stage III. The early thymocyte expresses CD71 and CD38, at the common stage they lose CD71, but retain CD38 and simultaneously express CD1, CD4 and CD8. The mature thymocyte loses CD1 activity, retains CD38, acquires CD3 and CD5 and segregates into subsets expressing the phenotype $CD38^+$, $CD3^+$, $CD5^+$,$CD4^+$ (helper) and $CD38^+$, $CD3^+$, $CD5^+$, $CD8^+$ (suppressor).

As the thymocytes pass into the peripheral circulation, they lose CD38 activity (Reinherz, 1980). This scheme of intrathymic differentiation is supported by Koziner, et al,(1982), except for the expression of CD5, which is reported to be present at the early thymocyte stage and retained throughout development. The phenotypic expression of T-ALL is heterogeneous and it is not always possible to fit the phenotypes obtained into one stage (Sobol, et al, 1985; Knowles, 1986).

The monoclonal antibody, CD2, directed against the E-rosette receptor is reported to be more sensitive than the E-rosette technique (Sobol, et al, 1985; Wain and Borowitz, 1987). The pan T antibody, CD7, is the most sensitive marker of T-ALL (Vodinelich, 1983; Wain and Borowitz, 1987). It is important to note that CD7 is not unique to T-cells, as it has been found in some myeloid leukaemias (Vodinelich, 1983; Chan, et al, 1985; Foon and Todd, 1986; Pombo de Oliviera, et al, 1987).

The histocompatibility-related antigen has been found in some cases of T-ALL (Sobol, et al, 1985). The antibody CD10 or cALLa has also been discovered in T-ALL (Sobol, et al, 1985; Greaves, et al, 1983a; Chan, et al, 1985).

Terminal deoxynucleotidyl transferase (TdT) is a nuclear enzyme that catalyses the polymerization of deoxynucleoside triphosphates without the use of a template (Bollum, 1979). Normal lymphocytes do not contain TdT, but it occurs normally in a small number of cells in the bone marrow and in high concentrations in thymocytes (Coleman, et al, 1974).

It was initially considered to be an important marker in differentiating ALL from ANLL, as it was found in high concentrations in ALL. It has been found however, that TdT is an early differentiation marker (Gale and Bassat, 1987; Bradstock, et al, 1981b; Cuttner, et al, 1984) and as a single marker is not helpful in deciding lineage, since it has been demonstrated in cases of ANLL (Coleman, et al, 1974; Catovsky, et al, 1981b; Bradstock, et al, 1981a; 1983; Drexler, et al, 1986; Parreira, et al, 1988).

In ALL therefore, the use of immunological techniques has permitted the definition of B and T and non-T subtypes. This has led to the recognition of clinically relevant groups, which were previously indistinguishable (Foon and Todd, 1986).

ANLL, however, is still diagnosed by morphological and cytochemical features. Anti-myelomonocytic antibodies directed at specified antigenic epitopes on the maturing myeloid precursor cells were not available in the past, but now antibodies are available directed against specific antigens on granulocytic, monocytic, erythrocytic and thrombocytic

series (Knapp, et al, 1981; Robak and Goldman, 1985). They are not leukaemia specific, but react with granulocyte and monocyte - related antigens (Foon and Todd, 1986). Marked heterogeneity in surface marker expression in ANLL was found in some studies (Pessano, et al, 1984; Ost, et al, 1985; Drexler, 1987). Attempts at correlation with the FAB classification have resulted in diverse conclusions. Pessano, et al, (1984) did not find any correlation with the morphological subgroups, while patterns of reactivity using myelomonocytic antibodies corresponding to FAB myeloid subclassifications could be observed by others (Neame, et al, 1986; Griffen, et al, 1986; Drexler, 1987).

Monoclonal antibodies against erythroid cells (Greaves, et al, 1983b) and megakaryocytic cells (Vainchenker, et al, 1982) have also been generated.

The aim of the diagnostic laboratory is to arrive at an accurate diagnosis of acute leukaemia in the shortest possible time. This can best be achieved by a systematic approach integrating morphological and cytochemical examinations with immunological analyses (del Canizo, et al, 1987). Primary and secondary screening panels of antibodies which allow accurate identification of lineage may also be useful (Chan, et al, 1985; Neame, et al, 1986; Wain and Borowitz, 1987).

CHAPTER 2.

MATERIALS AND METHODS

2 MATERIALS AND METHODS

The studies carried out on samples from patients with acute leukaemia included full blood counts, morphological assessments, cytochemical stains and immunological membrane markers.

2.1 Patients

Over a period of 3 years, samples of peripheral blood (PB) and/or bone marrow (BM) from 80 patients with acute leukaemia (AL) were studied prior to chemotherapy. Six patients suffering from chronic myelogenous leukaemia (CML) in blastic transformation (BT) were also investigated.

2.2 Control Samples

Peripheral blood obtained from normal adult volunteers was tested with each run of immunological studies to provide the normal reference range for lymphoid and other membrane markers, and appropriate positive controls were used for the cytochemical stains.

2.3 Full Blood Counts

Ethylenediaminetetraacetic acid (EDTA) anticoagulated venous blood was collected for full blood count on the Coulter Counter Model S plus II (Rowan, et al, 1979).

2.4 Morphology

Peripheral blood smears were prepared on a miniprep and stained in a Haemastainer, Automatic Slide Stainer (Geometric Data Company) using Wright's stain. May Grunewald Giemsa stain was used for the routine staining of the BM smears. Differential counts were performed and the acute leukaemias were subdivided morphologically according to the French-American-British (FAB) classification (Bennett, et al, 1976). In cases, where BM aspirations were not successful and therefore smears not available for assessment of morphology, no FAB subtype could be assigned.

2.5 Cytochemistry

Cytochemical stains included Sudan black B, myeloperoxidase, acid phosphatase and non specific esterase, according to the recommended methodology of the International Committee for Standardization in Haematology (ICSH) (Shibata, et al, 1985). A result of >3% positive blasts for Sudan black B and myeloperoxidase was considered diagnostic of ANLL and where >20% blasts showed positivity in the non specific esterase, the leukaemia was regarded as having monocytic characteristics (Bennett, et al, 1985b).

Smears for Sudan black (Sheehan, 1939;, Shibata, et al, 1985) were fixed in 10% formal ethanol for 30 seconds, allowed to air dry, rinsed in distilled water and immersed in Sudan black working solution (Appendix) at room temperature (RT) for 60 minutes. After incubation the smears were differentiated in 70% alcohol, rinsed in distilled water and allowed to air dry. They were counterstained with 0.1% safranin for 60 seconds, allowed to drain and air dry. The blood films were assessed and the data presented as a percentage of the average number of blasts counted.

Smears for peroxidase (Shibata, et al, 1985) were fixed in 10% formal ethanol for 30 seconds, allowed to air dry, rinsed in distilled water and incubated in working solution (Appendix) for 10 minutes, after which they were rinsed in distilled water and allowed to air dry. Excess powder was wiped off the slides and the smears counterstained in Harris' haematoxylin for 2 minutes, allowed to blue in running tap water for 10 minutes, dried and then examined microscopically for positivity in the blast cells. The result was reported as a percentage of the average number of blasts counted.

Smears for acid phosphatase using naphthol ASB1 phosphoric acid No 2550 (Sigma) were fixed in acid buffered acetone pH4.2 for 60 seconds, rinsed in several changes of distilled water and incubated in the working solution (Appendix) at 37°C for 60 minutes.

Smears were then rinsed in distilled water, counterstained in Harris' haematoxylin for 2 minutes, blued in running tap water for 10 minutes, then air dried. The blood films were assessed microscopically and the findings reported as a percentage of total blasts counted.

Smears for non specific esterase (Shibata, et al, 1985), using alpha naphthyl butyrate (Sigma), were fixed in buffered acetone for 30 seconds, washed in distilled water, stained in the working solution (Appendix) for 45 minutes and rinsed in distilled water. They were then counterstained in methyl green for 2 minutes, rinsed in distilled water and allowed to dry before microscopic assessment. The observations were recorded as a percentage of total blasts.

2.6 Serum Muramidase

Serum muramidase (Sewell, 1972) was performed on 45 of the 80 patients with acute leukaemia. An aliquot, 0.2ml of serum or standard was added to 2.0ml micrococcus solution (Appendix) and a stop watch started. Two readings were taken at 600nm, one at 30 seconds and the second at 180 seconds. The optical density change between the two readings was calculated and the amount of muramidase estimated by comparison against a reference standard curve (Appendix). A result of >3 times the normal range was considered significant (Bennett, et al, 1985b). The normal reference range is 5 - 15 μg/ml.

2.7 IMMUNOLOGICAL STUDIES

2.7.1 Monoclonal Antibodies

Details of the monoclonal antibodies used in this study are given in table 2.

Antigen		Main cellular distribution	Commercial source
Goat antiserum to human total immunoglobulins; fluorescein conjugated			
$F(ab')^2$ fragment antiserum		B-cell surface membrane immunoglobin	Iepsa (Pty) Ltd
TdT		Early lymphoid progenitors.	Supertechs
Class II (HLA-DR)		B cells, monocytes, activated T cells.	Ortho
OKT6	CD1a	Cortical thymocytes	Ortho
OKT11	CD2	T cells, Sheep erythrocyte receptor.	Ortho
OKT3	CD3	T cells	Ortho
OKT4	CD4	Helper T cells	Ortho
OKT8	CD8	Suppressor T/cytotoxic cells	Ortho
Leu9	CD7	T cells	Bectin Dickinson
B4	CD19	B cells	Coulter
J5, OKB-cAlla	CD10	2% of cells in normal bone marrow and in foetal liver	Coulter, Ortho
MO1	CD11b	Granulocytes, monocytes, null cells	Coulter
MY7	CD13	Granulocytes, monocytes	Coulter
MO2, MY4	CD14	Monocytes	Coulter
MY9	CD33	Early myeloid progenitors	Coulter
AN51, gp1b	CD42	Megakaryocytes, platelets	M.F. Greaves
R10		Erythroid cells	M.F. Greaves

Table 2 Panel of Monoclonal Antibodies Used

2.7.2 Interpretation of Marker Results

The interpretation of the results of immunophenotyping in ALL was based on criteria for the definition of ALL subtypes (Greaves, et al, 1985) (Table 3). A result of >20% positive cells with anti-myeloid antibodies was considered significant (Foon and Todd, 1986; Drexler, 1987). TdT was considered positive if 10% or more of the blast cells were reactive (Drexler, et al, 1986).

Definition of ALL subtypes

(1) $cALL^+$ DR^+ T(WT1/Leu9) - $SmIg^-$ Tdt^+ = common ALL subset of "pre-B" ALL

(2) $cALL^-$ DR^+ T^- $SmIg^-$ TdT^+ = null ALL subset of "pre-B" ALL

(3) $cALL^+$ or $cALL^-$ DR^- T^+ $SmIg^-$ TdT^+ = T (or pre-T) ALL

(4) $cALL^-$ DR^+ T^- $SmIg^-$ (Kappa or Lambda) TdT^- = B-ALL (or disseminated lymphoma)

Table 3 Suggested Immunophenotyping Classification of ALL

Notes

1. About 5% of T, common or null ALL may be TdT^-.
2. If DR^-/T^- or DR^+/T^+, score as unclassifiable.
3. >10% $cALL^+$ cells places DR^- ALL in common subtype. About 15% of T-ALL are $cALL^+$.
4. Approximately 5% of AML are TDT^+. Score as TDT^+ AML provided haematological diagnosis is AML and MY9 is positive; otherwise score as unclassifiable acute leukaemia.
5. If incomplete data, eg. DR or cALL antigen or Ig not tested, then score as uncertain.
6. All ALL should be $MY9^-$.

7. Criteria for scoring DR as positive are:

   a) If blasts are >75% then >25% DR^+ cells = DR^+ phenotype.

   b) If blasts are <75% then sum of % blasts + % DR^+ must be 110 to score as DR^+ phenotype, similarly, for scoring WT1/T as positive.

8. Alternative or additional criteria for scoring positively can be derived from "double" or simultaneous staining.

   eg. DR+ TdT : If >25% of TdT^+ cells are DR^+ then score leukaemia as DR^+.

   T+ TdT : If > 25% of TdT^+ cells are T^+ then score leukaemia as T^+.

9. Other T cell markers : E/T11, T1 or T6 positively overrides WT1, i.e. DR^- $E/T11^+$ or $T1^+$ or ($T6^+$) $WT1^-$ TdT^+ score as T-ALL.

   Scoring threshold for T11 and T1 as for Wt1/T above. Scoring threshold for T6 >10% (irrespective of blast cell %).

10. DR^+/TdT^-, other marker negative: score as null ALL if MY9 is negative and/or diagnosis is clearly ALL.

11. All markers negative : test with anti-glycophorin for "cryptic" erythroleukaemia.

12. If DR not assessed but WT1 plus one other T cell marker, eg. T11, are strongly positive then score as T.

13. If conflict between immunophenotype and haematological diagnosis, eg. ALL vs AML, then score as uncertain type of acute leukaemia, for later review.

14. Note that immunophenotype can shift in relapse, eg. ALL ... null ALL, and some subsets are more likely to relapse than others. Therefore subset phenotypes will be entered into a separate relapse category unless they are tested at diagnosis or before first complete remission.

Source: Leukaemia Research, 1985 9(6), 715-733

2.7.3 Preparation of Cells

Heparinized venous blood or BM samples were collected and investigations carried out within 24 hours. Occasional specimens not tested on the day of collection were stored at 4°C and cell viability ascertained by Trypan blue exclusion. Immunological studies were performed on blasts separated from the heparinized whole blood by centrifugation (1800 rpm) on a Ficoll-hypaque density gradient (Lymphoprep: 1.077 ± 0.001 g/ml) at 20°C for 30 minutes. The mononuclear cell layer was harvested and washed 3 times in Hank's balanced salt solution (HBSS : Sigma Chemical Co. St Louis, Missouri, USA). Three cell suspension media were used. Two were concentrations of foetal calf serum (FCS) (State Vaccine Laboratory, Pinelands, Cape, South Africa) in HBSS and a third was HBSS without FCS. An aliquot of mononuclear cells was reconstituted with 50% FCS : HBSS to a final concentration of 5 x 10^9/L. A second aliquot of mononuclear cells was reconstituted, with 5% FCS : HBSS buffered with 0.25M Hepes, to a final concentration of 10 x 10^9/L. A cell concentration of 2 x 10^9/L was made in HBSS without FCS. The interface of all samples studied had over 50% blast cells and the majority had more than 80%. Terminal deoxynucleotidyl transferase (TdT) was assessed on only 48 of the 86 cases in the study owing to the periodic non-availability of the primary antibody.

For the E rosette test (Er) (Catovsky, 1981a), equal volumes (200 μl) of cell suspension in 50% FCS : HBSS and 2 - 5% concentration of sheep RBCs (S-RBCs) (Provincial Animal Centre : Delft, Kuils River) in 50% FCS : HBSS suspension medium, were incubated at 37°C for 30 minutes. After centrifugation at 700 rpm at RT for 5 minutes, the cells were gently re-suspended by tapping the tube and left at 4°C overnight or on the bench at RT for 60 minutes. Wet preparations were assessed microscopically and the rosettes (minimum of 3 S-RBCs attached to a blast/lymphocyte constituted a rosette) counted and the result expressed as a percentage of total blasts.

A 500μl volume of cells suspended in 50% FCS : HBSS was incubated with 100μl anti human immunoglobulin (goat anitserum to human total immunoglobulins conjugated with fluorescein isothiocyanate [FITC]) at 4°C overnight for surface membrane immunoglobulin (SMIg) estimation (Catovsky, 1981a). The cells were washed twice in HBSS, then examined microscopically (Nikon optiphot with episcopic fluorescence attachment and a high power mercury vapour lamp). The positive cells were enumerated and the result reported as a percentage of total blasts.

Using the 5% FCS : HBSS cell suspension, an indirect immunofluorescence technique (Catovsky, 1981a) was employed, where 50μl volumes of cell suspension were allowed to react with 5μl aliquots of primary antibody (Table 2) at 4°C for 30 minutes. After washing twice in the suspension medium, 5μl of a secondary antibody, goat antimouse, conjugated with FITC (GAM-FITC : Coulter Electronics Ltd) was added and incubated at 4°C for 30 minutes. The cells were again washed twice and then assessed on a fluorescent microscope. The fluorescing cells were counted and reported as a percentage of total blasts.

Smears were prepared for TdT, cytoplasmic immunoglobulin (Cμ chain) and cytoplasmic CD3 (cCD3) evaluation, in a Shandon Elliott Cytospin. One hundred microlitres per cup of cell suspension in HBSS without FCS added was centrifuged at 1000 rpm for 5 minutes and allowed to dry overnight. Smears for TdT were fixed in cold methanol (analar grade, Merck, Germany) at 4°C for 30 minutes, transferred to a moist chamber, washed twice in phosphate buffered saline pH 7.4 (PBS) for 5 minutes, stained with mouse anti-TdT at RT for 30 minutes and washed again twice in PBS. The smears were then stained with goat anti-mouse FITC at RT for 30 minutes, finally washed in PBS, mounted with a drop of FA mounting fluid pH 7.2 (Difco Laboratories, Detroit, Michigan, USA) and observed for nuclear positivity. The percentage of positive blasts was reported.

Smears for CyIgM (Han,et al, 1982) were fixed in cold methanol for 30 minutes, washed twice in PBS and CyIgM FITC (Fluorescein conjugated IgG fraction Goat Anti-human IgM-μ chain specific. Cappel Laboratories) added to the smears and incubated in a moist chamber at RT for 30 minutes. The smears were then washed twice in PBS, mounted with a drop of FA mounting medium (see TdT method) and intracytoplasmic positivity evaluated microscopically and expressed as a percentage of total blasts.

Smears for cCD3 (Campana, et al, 1987) were fixed in cold methanol at 4°C for 30 minutes, transferred to a moist chamber, washed twice in PBS for 5 minutes, stained with CD3 at RT for 30 minutes and washed again twice in PBS. The smears were then stained with goat anti-mouse FITC at RT for 30 minutes, finally washed in PBS, mounted with a drop of FA mounting medium (see TdT method) and observed for cytoplasmic positivity.

CHAPTER 3.

RESULTS

3 RESULTS

A total of 86 patients were studied using morphological, cytochemical and immunological techniques. Twenty nine patients were found to have tumours of a lymphoid nature, 39 were of non lymphoid origin and 6 were chronic myelogenous leukaemia (CML) in blastic transformation (BT). Twelve could not be categorized morphologically by the FAB criteria as bone marrow (BM) aspirates were not obtained on these patients.

3.1 Morphology

Twenty nine patients had lymphoid tumours. Twenty seven were ALL and were classified by the FAB classification as follows : L1:18, L2:8, L3:1. Two patients had lymphoblastic lymphoma with spill into the peripheral blood and bone marrow. Thirty nine patients had non lymphoid leukaemias and were grouped by the FAB criteria, as follows: M1:19, M2:8, M3:5, M5a:6 and M5b:1. Auer rods were noted in 10 patients, 3 in M1, 3 in M2, 3 in M3 and 1 in M5b groups.

3.2 Cytochemistry

In the lymphoblastic leukaemias, the stains for Sudan black B (SB) and myeloperoxidase (MPO) (1 patient not tested) were negative. The acid phosphatase (AP) (1 patient not tested) showed "polar dot" positivity of 20% or more in 4 cases. In the non lymphoblastic leukaemias, blasts from 36 out of 39 patients stained positively with SB or MPO. Blast cells from 3 patients

were negative when the above stains were applied, but Auer rods were present in 1 case and the blast cells from 2 cases were strongly positive when stained with non specific esterase (NSE), thus confirming their monocytic nature. In the group of CML patients in BT, the blasts of 4 out of 6 patients were positively stained with SB or MPO and one of these cases showed the presence of Auer rods. Two patients were negative for both stains. The blasts from one of these patients stained with periodic acid Schiff stain (PAS). In the group of 12 patients who could not be assigned an FAB subtype, owing to failure to aspirate bone marrow, 5 showed minimal positivity with SB or MPO (≤6%), 1 case was strongly positive with SB and 6 cases were negative for both stains. Auer rods were noted in 1 case only.

3.3 Serum Muramidase

Serum muramidase was performed on 45 of the 80 cases of acute leukaemia. Only 3 of the cases (FAB M1:1, M5:2) tested showed a significant increase ie: >45μg/ml. (>3 times the upper limit of the normal range) (Bennett, et al, 1985b).

3.4 Immunological Studies

3.4.1 Acute Lymphoblastic Leukaemia

Of the 29 patients who were classified morphologically as lymphoblastic, 20 were subtyped immunologically as follows :

non-T-ALL:4, common-ALL:8, T-ALL:7 and B-ALL:1 (Table 4). The detailed immunophenotyping results of this study of ALL patients appear in the appendix. The T-ALL results have been extracted and are shown separately (Appendix). Nine patients could not be subtyped. Of these 9 patients, 2 were unclassifiable. One patient was found to have conflicting marker results and one patient was reactive with only one of the antibodies tested. Two of the nine patients were classified as acute leukaemia of uncertain origin as marker studies were incomplete, while the remaining five patients marked with an anti-myeloid antibody, CD13 or CD33. The detailed phenotyping results are set out in a separate table (Appendix).

FAB	non T-ALL	Common-ALL	T-ALL	B-ALL
L1	3	7	5	
L2	1	1		
L3				1
LL			2	

Table 4 FAB classification and immunologic subtypes of ALL (total 20)

3.4.2. Terminal deoxynucleotidyl transferase (TdT)

Of the 20 cases which were subtyped immunologically, 11 were tested for TdT, all of which showed >60% positive cells, except in B-ALL where 1 patient was negative and 1 case of T-lymphoblastic lymphoma, which had 13% positive cells.

3.4.3 Acute Non-Lymphoblastic Leukaemia

It is of note that while no uniform pattern emerged, CD13 and CD33 were found to be the most consistently positive, whereas CD14 was expressed by the leukaemias with a monocytic component. Of the 22 patients tested for TdT, 7 had positivity. Two showed 10% and 25% positive blasts, while 5 had >50% blasts expressing positive nuclear staining. A summary of the immunological marker studies is presented (Table 5), while detailed phenotyping results of each FAB subgroup are tabulated separately in the appendix.

FAB	TdT *	HLA-DR	CD10	SMIg	CD2	CD7	CD11b	CD13	CD14 †	CD14 ‡	CD33
M1	2/11	13/16	4/18	2/18	2/17	6/19	4/17	9/18	1/17	5/17	8/19
M2	2/5	5/8	0/8	0/8	1/4	0/8	1/7	5/7	0/7	1/7	6/8
M3	NT	0/5	1/5	0/5	0/5	0/5	1/5	5/5	0/5	1/5	5/5
M5	2/6	7/7	2/7	4/7	2/5	3/6	4/7	4/5	2/7	4/5	6/7

Table 5. Summary of ANLL Phenotyping results.
† Number of cases showing >20% positive blasts
* Number of cases showing ≥ 10%

3.4.4 Chronic Myeloid Leukaemia in Blastic Transformation

Anti-myeloid antibodies were positive in 5 out of the 6 patients. The blasts of 1 patient were not reactive with any antibodies tested, except HLA-DR. In the only patient tested for TdT, positivity was noted in 86% of the blasts.

3.4.5 Morphologically Uncharacterized Acute Leukaemia

This group consists of 12 patients, all of whom had unsuccessful BM aspirates and could not be FAB classified. The phenotyping on peripheral blood revealed the following results : common-ALL 1, non-T-ALL 1, T-ALL 1, B-ALL 1, acute megakaryoblastic leukaemia 1 ($AN51^+$), and ANLL 7. Of the 7 ANLL patients 1 was not reactive with anti-myeloid antibodies, and the cytochemical reactions for Sudan black and myeloperoxidase were minimally positive at 4%, however Auer rods were noted on the peripheral blood smears. Two of the 7 ANLL patients were also positive with CD7. Detailed surface marker results appear in the appendix.

3.4.6. Ethnic Analysis

For ethnic analysis, those patients with CML and unclassifiable leukaemia and those individuals who could not be characterized morphologically were excluded. Of the 39 individuals with ANLL, 10 were black (5 male and 5 female patients) 12 of mixed ancestry, (9 male and 3 female patients) and 17 white, (7 male and 10 female patients). Among the 29 individuals with ALL, there were 2 black males and 4 black female patients, 9 male and 6 female patients of mixed ancestry and 6 male and 2 white female patients.

When the groups were further analysed for age, division into gender was not possible because the numbers were too small. Students t-test was used for comparing sample means, assuming reasonably normal distribution. A p-value of <0.05 was deemed significant. For ANLL, mean ages for white, mixed ancestry and black groups were respectively 53 years, 32.5 years and 30.9 years. These differences were significant when white patients were compared with black (p=0,0003) and patients of mixed ancestry (p=0,0040), but not significant when the patients of mixed ancestry were compared with the black group (p=0.7694).

For ALL the mean age for white patients was 44.6 years, patients of mixed ancestry 40.2 years and black patients 19.5 years. The difference was not significant when whites were compared with patients of mixed ancestry (p=0.6535). However, the difference was significant when whites were compared with black patients (p=0.0152) and when patients of mixed ancestry were compared with blacks (p=0,0048) (Table 6). Analysis of the present series for difference in ethnic groups showed a significantly higher mean age for whites in the ANLL group. Analysis of the 1985 census (Central Statistical Services, 1986) in the Western Cape showed a higher proportion of people over the age of 55 years in the white group compared with other populations.

	White		Mixed Ancestry		Black	
	No.	Mean age(yrs)	No.	Mean age(yrs)	No.	Mean age(yrs)
ANLL	17	53	12	32.5	10	30.9
ALL	8	44.6	15	40.2	6	19.5

Table 6 Age distribution of the individuals with acute leukaemia in the various racial groups

The mean age for whites was significantly higher than that for individuals of mixed ancestry (p=0,0040) and blacks (p=0,0003) in ANLL. In ALL the mean age was significant when those of mixed ancestry were compared with blacks (p=0,0048) and when whites were compared with blacks (p=0,0152).

CHAPTER 4.

DISCUSSION

4 DISCUSSION

Treatment programmes for patients with acute leukaemia are determined by a number of prognostic factors, of which tumour origin is of particular importance. Accordingly, attempts have been made to refine diagnostic accuracy by supplementing morphological assessment with cytochemical markers and, more recently, the addition of monoclonal antibodies. The use of immunological membrane markers to analyse cell surface characteristics has led to important insights into the cellular origins of leukaemia and revealed the heterogeneity of the disease.

4.1 Acute Lymphoblastic Leukaemia

It was possible to clearly subgroup 20 of the 29 morphologically classified acute lymphoblastic leukaemias, into acute lymphoblastic subentities (table 4).

Obvious subdivision was not feasible in 4 of the remaining 9 patients. Two cases, in which the marker studies were incomplete, failed to meet the criteria for suggested phenotypic classification (table 3) and were termed AL of uncertain origin. Two cases were unclassifiable. The blasts of case 41 marked only with CD2 (91%). Other T-cell antibodies CD7, CD1, CD3, CD4, CD8 and the anti-myeloid antibody CD33 were all negative. It is of interest that the sheep E-rosette receptor, by the rosetting technique, was also negative (6%). The expression of CD2 has been documented in ANLL, usually in association with

anti-myeloid antibodies and lymphoid affiliated antibodies CD7, CD10 and TdT (Bradstock, et al, 1989). This case did not show co-expression of any of the above antibodies.

The marker profile of case 14, TdT^-, $HLA\text{-}DR^+$, $CD2^+$, $CD7^-$, $CD19^-$, $CD10^+$ and $CD33^-$, shows conflicting results, in that CD2 and CD10 were both positive. Common-ALL could be concluded from the $HLA\text{-}DR^+$ and $CD10^+$, even in the event of TdT^-, (Janossy, et al, 1980) but CD19 was also not expressed. The $CD2^+$ was not supported by the pan T cell marker, CD7 and the T-cell antibodies CD1, CD3, CD4 and CD8 were also all negative. A final conclusion was not reached.

The remaining 5 cases had either $CD13^+$ or $CD33^+$ (Table 7).

Case	TdT	HLA-DR	CD2	CD7	CD10	CD13	CD33
19	70	55	21	NT	<1	NT	65
49	NT	64	9	68	9	33	3
50	NT	<1	10	65	<1	<1	64
72	15	<1	<1	85	71	<1	50
83	<1	49	13	11	<1	55	11

Table 7 ALL expressing $CD13^+/CD33^+$ blasts

Two of these 5 patients (cases 19 and 83), had myeloid morphological features, but failed to stain with Sudan black or myeloperoxidase thereby making it impossible to classify them as myeloid by the FAB classification. These patients were positive with the myeloid antibodies CD13 or CD33. It is interesting that the immunological markers tended to support the morphological observations. Case 72 was more complex and is discussed in detail. Three possibilities are considered: Firstly, T-ALL. The cytochemical stains SB and MPO were both negative, but the AP was positive (66%) showing the localized staining pattern, a feature of T-cells. The most sensitive T-cell marker CD7, was also expressed in 85% of the blasts, however, the cytoplasmic CD3 (cCD3) was negative. Usually the presence of cCD3 is confirmatory of T-cell origin (Campana, et al, 1987). In addition all the other T-cell markers CD1, CD3, CD4, CD8 were negative, and the minimally positive TdT is not in keeping with T-ALL, although CD10 may be expressed in a percentage of T-ALLs (Greaves, et al, 1983a; Janossy, et al, 1980). There is no explanation for presence of CD33.

Secondly, "hybrid" acute leukaemia. This case did not meet the requirements listed in the criteria for hybrid acute leukaemia (Gale and Bassat, 1987), where the authors take cytochemical staining, immunological reactions and T-cell receptor gene rearrangement into consideration.

Thirdly, TdT^+ ANLL with co-expression of $CD10^+$. It has been reported that CD33 (MY9) can be found on myeloblasts, but tested lymphoid leukaemic cells were completely unreactive (Leucocyte Typing III, 1987). This implies that if CD33 is found on leukaemic blasts, these cells are of myeloid origin. Cases where blasts in ANLL react with "lymphoid" antibodies most often include TdT, CD7 and CD10 (Bradstock, et al, 1989). In addition to CD33, the blasts of this patient reacted with all three antibodies, TdT, CD7 and CD10, therefore exhibiting substantially more cytochemical and immunological evidence for a lymphoid tumour. Perhaps in this situation molecular biological techniques would be more helpful.

4.2 T-Acute Lymphoblastic Leukaemia

In this study all the patients suffering from T-ALL were males (5 coloured, 2 black) and had a mean age of 19.0 years (n= 7). The heterogeneity of the disease is striking. The clinical presentation varied, lymphadenopathy being most common (Table 8).

Case	Mediastinal Mass	Lymphadenopathy	Splenomegaly	Hepatomegaly
1	+	+		+
21		+		
23	+		+	+
34		+		
39		+		+
57		+		
86				+

Table 8 Clinical findings in T-ALL

The total leucocyte counts also varied markedly, ranging from 6.0 x 10^9/L - 838 x 10^9/L and 5 of the 7 had blast counts of >60%. The morphological assessment was L1 for 5 and 2 had lymphoblastic lymphoma with spill into the peripheral blood. Inconsistent cytochemical staining with acid phosphatase was noted in this small group of patients (Table 9).

Case	AP	TdT	CD7	CD1	CD2	CD3	CD4	CD8
1	39	61	81	1	1	NT	1	1
21	20	75	79	<1	NT	32	13	17
23	36	NT	89	<1	95	93	5	3
34	<1	70	81	<1	52	57	13	36
39	7	13	73	1	74	46	27	34
57	87	NT	91	<1	85	1	5	5
86	<1	60	82	<1	27	26	12	10

Table 9 Heterogeneity in acid phosphatase and phenotypic expression in T-ALL

This stain is regarded as being a diagnostic feature of T-ALL, showing localized positive granules (Catovsky, et al, 1974; Brouet, et al, 1976; Catovsky, et al, 1978). The results in this study show 3 cases with >30% positive blasts and 4 cases with positive staining ranging from <1% (2 cases) to 20%. Acid phosphatase did not appear to be a useful indicator, as the blasts in less than 50% of the cases were convincingly reactive. However, the T-lineage was substantiated by marker analyses (Thierfelder, et al, 1977). CD7 was the marker most consistently positive (all cases >70%). These findings are in accordance with the findings of other workers (Vodinelich, et al, 1983; Foon and Todd, 1986). It is important to bear in mind, however, that CD7 is not unique to T-cells and may be expressed by a percentage of ANLLs (Vodinelich, et al, 1983; Foon and Todd, 1986).

TdT was ≥ 60% in 4 of the 5 cases tested, whereas one case had only 13% positive blasts. Other T-cell antibodies were variably expressed and it proved difficult to "classify" the individual cases according to the intrathymic differentiation described by Reinherz, et al,(1980) (Table 9). These findings are consistent with earlier reports (Knowles, et al, 1982; Koziner, et al, 1982; Knowles, 1986).

4.3 Acute Non-Lymphoblastic Leukaemia

The most consistent markers for ANLL were CD13 and CD33. When the antibodies were assessed individually on the same sample, the incidence of $CD13^+$ and $CD33^+$ was 22 (63%) and 24 (69%) respectively with a combined positivity of 25 out of 35 cases (71%). However, the blasts from 10 patients (29%) failed to express any of the anti-myeloid antibodies. Eight of these were FAB M1, 1 of which had 25% blasts with positive staining TdT. The remaining 2 were FAB M2 and M5.

HLA-DR was variably expressed by the blasts in the FAB M1 and M2 subgroups, but was present on all the leukaemic cells of the FAB M5 type. This antigen failed to be expressed on the cells of the FAB M3 group of patients. This latter subgroup of ANLL however showed a consistent phenotypic pattern $HLA\text{-}DR^-$, $CD13^+$, $CD33^+$ (Van der Reijden, et al, 1983).

Of five cases classified as FAB M1, 4 had blasts with monocytic features. The NSE on all five patients was negative and the serum muramidase was within the normal range in all but 1 patient, who had a significant increase (127μg/ml), but the blasts from this patient did not exhibit monocytic features. The phenotyping results in these cases suggested that there was a monocytic element, by the presence of $CD14^{+}$ (>20%) cells in addition to other anti-myeloid antibodies (Table 10).

Case	NSE	SE.MUR.	CD11b	CD14 +	CD14 ++	CD13	CD33	HLA-DR
10	<1	127	4	1	31	42	7	93
70	<1	5	89	14	83	83	81	77
79	<1	4	85	62	74	70	72	70
82	<1	4	44	9	43	41	57	61
90	14	2	31	3	35	48	35	37

Table 10 Phenotyping of FAB M1 patients, showing increase in CD14 + blasts

CD14 identifies the majority of ANLL patients classified as FAB M4 and M5, for it is not expressed frequently on the other ANLL subtypes (Griffen, et al, 1981). Most of these patients had the morphological features, but lacked the confirmatory tests, such as positive NSE or increased serum muramidase, to classify them as monocytic subgroups. However, the use of monoclonal

antibodies revealed their monocytic nature. In a study by Scott et al, (1988), it was found that, when using alpha naphthyl acetate esterase (ANAE), variable or absent reactions were noted in both leukaemic and non malignant disorders. Drexler, (1987) suggests that the immunophenotype reflects a somewhat different view of the status of cell differentiation, than do morphology and cytochemistry. This suggestion may offer a possible explanation for some of the discrepancies we are confronted with. The antibodies MO2 and MY4 are both designated CD14. This study shows a difference in results between the two antibodies, MY4 staining a higher number of cells (Table 10). Although this is a small study, the experience is similar to reports in the literature (Neame, et al, 1986).

Among the ANLL group of patients, there were 5 whose leukaemic cells marked with CD11, CD13, CD14 CD33 and in addition were $CD2^+$ (4), $CD7^+$ (5) and $CD10^+$ (3). Malignant monocytes have been reported to be activated cells, which have enhanced binding ability via Fc receptors, and the lymphoid associated positive markers were considered false positives as a result of non specific binding by these receptors, rather than lineage infidelity (Lawlor, et al, 1986; Greaves, et al, 1986).

Two out of 38 cases (5.2%) had $CD7^+$ blasts. This T-lineage associated antibody is well described in ANLL (5% - 10% of cases) (Vodinelich, et al, 1983; Chan, et al, 1985; Greaves, et al, 1986). All 3 cases were diagnosed on the basis of cytochemical stains (SB^+ MPO^+). Phenotypically they were TdT^- and $HLA\text{-}DR^-$, but lacked reactivity with CD13 or CD33. The usual T-phenotype is TdT^+, $HLA\text{-}DR^-$, $CD7^+$, however TdT^+ blasts are also found on a percentage of ANLLs, so it is very important to distinguish between T-ALL and $CD7^+$ ANLL by careful interpretation of results.

The incidence of TdT^+ ANLL in this study is 30%. This figure is higher than those reported in the literature (13%, Drexler, et al, 1986; 22.5%, Parreira, et al, 1988; 22.1%, Bradstock, et al, 1989). TdT is a DNA polymerase which has a role in the development of immunoglobulin diversity during heavy chain gene rearrangement, although, its biological role is still not completely understood (Stryer, 1988). Whether the aberrant expression is a result of "lineage infidelity", or is a separate subentity of leukaemia involving very immature cells, remains unclear at present. In the diagnostic laboratory the importance lies in the precise identification of these cases, as patients may require different clinical management.

In the ANLL group, the blasts of three patients had lymphoid features, but were positive when stained with Sudan black. By adhering strictly to the FAB criteria these cases were classified as FAB M1. The markers were, c-ALL 1, T-ALL 1 and one patient was only HLA-DR$^+$ and CD22$^+$. Sudan black positivity has been reported in ALL (Tricot, et al, 1982). It is interesting that, as in the ALL group, showing similar findings, such as discordant morphology and cytochemical staining the immunological markers tended to support the morphological observations.

4.4 Chronic Myeloid Leukaemia

The value in performing immunological markers on patients with chronic myeloid leukaemia in blastic transformation (CML-BT) is to identify those who may have lymphoid transformation, as it may be possible to induce remission in this group. The patients in this study were all myeloid.

4.5 Morphologically uncharacterized acute leukaemia

Case No. 11 was shown to be an example of megakaryoblastic leukaemia with a positive marker for platelet glycoprotein receptor CD42 (GP1b).

CHAPTER 5.

CONCLUSION

5 CONCLUSION

The most important function in the diagnosis of acute leukaemia is to distinguish between ALL and ANLL. Each laboratory will have a different level of sophistication and this will govern the choice of system for routine phenotyping. The several systems available today include flow cytometry, immunofluorescence by fluorescent microscopy and immunocytochemistry (Sandhaus, et al, 1984; Cordell, et al, 1984; Hsu, et al,1981; Erber, et al, 1986; Krause, et al, 1988). Irrespective of the system chosen it is necessary to select antibodies carefully. The expanded range of monoclonal antibodies may be confusing and the use of accepted antibodies with cluster designation have much to recommend them. The CD nomenclature is also a recognized basis for reporting results.

A screening panel of antibodies, which will identify lineage accurately, is useful. Based on the results of the screen, testing will be stopped or further testing or selected testing may be carried out (Figure 1). The best method for AL typing is by using a combination of morphology, cytochemical stains and immunophenotyping.

In those individuals where leukaemic blasts are clearly of non-lymphoid origin on the basis of morphology and cytochemical stains, monoclonal antibodies add little further diagnostic information. Nevertheless, continued effort to refine diagnosis should be made and immunophenotyping correlated with morphology

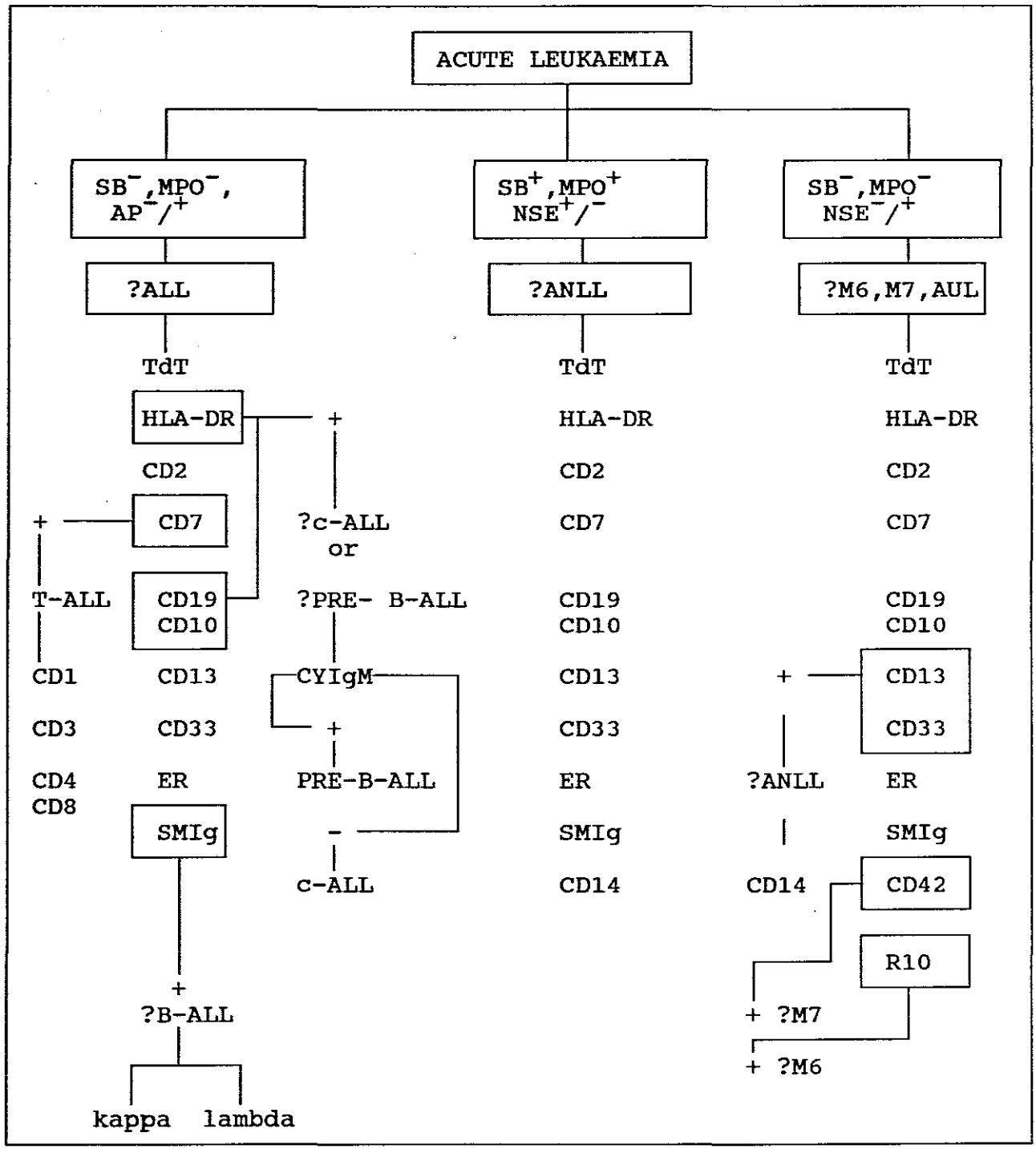

Figure 1. Diagnosis of acute leukaemia: showing selection of antibodies

and cytogenetics (Keating, et al, 1987). In patients where cytochemical reactions are negative or equivocal, the use of TdT and monoclonal antibodies has clearly emerged as of diagnostic value in recognizing lymphoid (Jacobs, 1985) and non-lymphoid tumours (Bennett, et al, 1985a). It can be concluded that judicious use of monoclonal antibodies is an integral part of the diagnostic evaluation of patients with leukaemia. Immunophenotyping has clearly improved diagnosis that was originally based on morphology and cytochemical stains, but represents only one further refinement, en route to the increasing use of molecular biological techniques that now include cytogenetic studies and immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements. Comprehensive classification of tumours is necessary to understand leukaemogenesis better and, at the same time, recognize those prognostic factors that have *significance for response to available treatment.*

APPENDIX

APPENDIX

Key to Phenotyping Tables

TdT	Terminal deoxynucleotidyl transferase
SMIg	Surface membrane immunoglobulin
ER	Sheep erythrocyte rosettes
NT	Not tested
AN5I	GP1b
RIO	Glycophorin A
FAB	French-American-British
┼	CD14 = MO2
╪	CD14 = MY4
L	Lymphoid
M	Myeloid

Case No.	Serum Muramidase (μg/ml)
1	12
3	18
5	15
6	30
10	127
12	20
17	4
18	2
19	21
21	6
22	2
23	3
26	6
27	27
28	1
29	57
30	3
33	3
34	2
36	5
38	10
39	4
40	5

Case No.	Serum Muramidase (μg/ml)
42	3
45	1
46	4
48	2
50	4
54	9
56	3
57	2
58	6
62	5
63	400
70	5
71	1
72	3
81	2
82	4
83	2
84	3
85	6
86	7
87	1
90	2

Serum Muramidase Results

Case	TdT	HLA-DR	CD19	CD22	CD10	SMIg	Kappa	Lambda	ER	CD2	CD7	CD13	CD33
1	61	2	NT	NT	1	1	NT	NT	10	1	81	<1	<1
7	70	74	NT	NT	78	<1	NT	NT	<1	1	1	NT	<1
8	NT	NT	NT	NT	92	3	NT	NT	1	2	1	NT	NT
14	<1	69	<1	<1	36	<1	NT	NT	60	58	11	NT	<1
15	NT	71	NT	NT	2	<1	NT	NT	1	<1	4	NT	<1
19	70	55	13	NT	5	58	38	20	<1	21	NT	NT	65
20	90	40	70	NT	<1	1	NT	NT	12	12	10	NT	<1
21	75	25	NT	NT	<1	4	NT	NT	61	NT	79	NT	<1
22	85	62	65	NT	87	<1	NT	NT	2	1	7	NT	<1
23	NT	<1	<1	NT	<1	<1	NT	NT	97	95	89	NT	<1
28	80	68	82	NT	75	1	NT	NT	3	1	<1	NT	<1
34	70	<1	<1	NT	<1	NT	NT	NT	NT	52	81	NT	<1
37	NT	52	NT	NT	52	2	NT	NT	10	15	12	NT	<1
39	13	14	NT	1	1	1	NT	NT	56	74	73	NT	<1

Phenotyping Results : Acute Lymphoblastic Leukaemia

Phenotyping Results continued

Case	TdT	HLA-DR	CD19	CD22	CD10	SMIg	Kappa	Lambda	ER	CD2	CD7	CD13	CD33
41	<1	25	NT	4	10	2	NT	NT	6	91	2	NT	<1
44	NT	78	NT	88	82	<1	NT	NT	<1	<1	<1	NT	NT
47	NT	65	NT	93	83	<1	NT	NT	3	4	3	NT	<1
49	NT	64	NT	6	9	2	NT	NT	1	9	68	33	3
50	NT	<1	NT	4	<1	1	NT	NT	6	10	65	<1	64
56	NT	90	NT	1	3	1	NT	NT	6	2	1	<1	<1
57	NT	1	NT	<1	<1	<1	NT	NT	93	85	91	<1	<1
60	NT	58	NT	67	7	1	NT	NT	20	19	23	4	7
68	NT	68	NT	81	1	<1	NT	NT	13	16	20	NT	8
71	NT	66	NT	45	74	3	NT	NT	5	1	<1	NT	<1
72	15	<1	NT	<1	71	<1	NT	NT	<1	<1	85	<1	51
81	<1	84	62	NT	5	78	1	77	13	13	7	<1	<1
83	<1	49	2	NT	<1	<1	15	NT	NT	13	11	55	11
85	65	89	71	NT	23	<1	NT	NT	6	3	7	10	7
86	60	<1	<1	NT	<1	<1	NT	NT	10	27	82	5	<1

Phenotyping Results : Acute Lymphoblastic Leukaemia

Case	TdT	HLA-DR	CD19	CD22	CD10	SMIg	ER	CD2	CD7	CD1	CD3	CD4	CD8	CD13	CD33
1	61	2	NT	NT	1	1	10	1	81	1	NT	1	1	<1	<1
21	75	25	NT	NT	<1	4	61	NT	79	<1	32	13	17	NT	<1
23	NT	<1	<1	NT	<1	<1	97	95	89	<1	93	5	3	NT	<1
34	70	<1	<1	NT	<1	NT	NT	52	81	<1	57	13	36	NT	<1
39	13	14	NT	1	1	1	56	74	73	1	46	27	34	NT	<1
57	NT	1	NT	<1	<1	<1	93	85	91	<1	1	5	5	<1	<1
86	60	<1	<1	NT	<1	<1	10	27	82	<1	26	12	10	5	<1

Phenotyping Results : Acute T-Acute Lymphoblastic Leukaemia

Case	TdT	HLA-DR	CD19	CD22	CD10	SMIg	ER	CD2	CD7	CD11b	CD13	CD14 +	CD14 +	CD33
2	<1	11	NT	NT	3	16	2	8	3	19	5	1	10	65
4	25	3	NT	NT	<1	2	12	3	9	<1	<1	<1	<1	<1
5	<1	85	NT	NT	<1	3	21	11	27	8	19	1	8	6
6	62	57	NT	NT	79	2	9	11	12	5	8	1	3	4
10	NT	93	NT	NT	<1	2	1	NT	7	4	42	1	31	7
16	NT	2	NT	NT	84	<1	87	NT	83	NT	NT	NT	NT	<1
36	80	13	NT	25	<1	2	3	1	20	<1	28	<1	1	22
40	<1	68	NT	1	2	1	13	20	18	4	<1	1	2	1
43	NT	89	NT	4	4	<1	1	3	4	5	11	1	16	63
45	NT	<1	NT	<1	<1	<1	2	1	1	<1	24	<1	9	78
59	NT	78	NT	<1	<1	<1	4	<1	4	3	52	<1	<1	<1
64	NT	73	NT	62	<1	NT	NT	1	1	NT	<1	NT	NT	<1
65	NT	5	NT	3	<1	1	8	6	1	2	3	2	1	1
70	<1	77	NT	70	NT	49	9	70	72	89	83	14	83	81
74	<1	60	NT	<1	<1	1	7	1	43	<1	2	<1	5	2
79	<1	70	NT	NT	75	68	12	70	69	85	70	62	74	72
82	<1	61	<1	NT	24	2	8	15	10	44	41	9	43	57
84	<1	48	1	NT	<1	<1	1	3	47	3	<1	<1	3	19
90	<1	37	<1	NT	<1	<1	3	<1	5	31	48	3	35	35

Phenotyping Results : Acute Non Lymphoblastic Leukaemia - FAB M1

Case	TdT	HLA-DR	CD19	CD22	CD10	SMIg	ER	CD2	CD7	CD11b	CD13	CD14 ┼	CD14 ╪	CD33
3	<1	2	NT	NT	<1	6	12	5	3	1	45	<1	5	21
13	<1	27	NT	NT	4	3	13	NT	5	9	25	3	4	41
30	70	1	1	NT	<1	4	15	NT	13	NT	NT	NT	NT	58
53	NT	67	NT	1	<1	1	14	4	5	16	53	2	10	24
61	NT	75	NT	<1	<1	1	1	2	3	1	17	1	6	20
69	<1	42	NT	14	2	2	2	NT	15	24	50	11	27	31
73	NT	17	NT	41	<1	2	3	NT	4	19	19	<1	1	57
78	10	54	4	NT	10	4	5	21	8	6	43	1	8	20

Phenotyping Results : Acute non lymphoblastic leukaemia - FAB M2

Case	TdT	HLA-DR	CD19	CD22	CD10	SMIg	ER	CD2	CD7	CD11b	CD13	CD14 +	CD14 +	CD33
38	NT	13	NT	1	35	4	<1	7	2	24	77	2	27	49
48	NT	<1	NT	1	2	1	12	8	3	3	36	<1	2	50
54	NT	<1	NT	<1	<1	<1	<1	<1	<1	3	50	<1	5	48
58	NT	<1	NT	<1	<1	2	<1	1	<1	2	47	1	7	53
67	NT	1	NT	<1	<1	<1	3	1	3	1	23	<1	<1	62

Phenotyping Results : Acute non lymphoblastic leukaemia - FAB M3

Case	TdT	HLA-DR	CD19	CD22	CD10	SMIg	ER	CD2	CD7	CD11b	CD13	CD14 ϟ	CD14 ϟ	CD33
12*	<1	36	NT	NT	45	31	14	NT	30	73	50	2	49	47
27	75	84	2	NT	4	52	17	71	29	80	NT	21	NT	58
29	54	69	<1	NT	<1	31	9	NT	<1	69	NT	2	NT	62
62	NT	25	NT	<1	<1	5	8	4	3	13	47	19	29	23
63	<1	77	NT	<1	<1	<1	6	3	5	<1	<1	1	<1	<1
66	<1	67	NT	<1	<1	<1	8	<1	NT	62	43	72	77	24
77	<1	88	<1	NT	80	80	4	79	75	5	86	<1	89	88

Phenotyping Results : Acute non lymphoblastic leukaemia - FAB M5

* Case 12 - FAB M5b

Case	TdT	HLA-DR	CD19	CD22	CD10	SMIg	ER	CD2	CD7	CD11b	CD13	CD14 ∔	CD14 ∔	CD33	AN51	R10
24	NT	<1	<1	NT	<1	1	6	<1	<1	NT	NT	NT	NT	69	2	1
25	NT	4	<1	NT	<1	5	13	1	14	NT	NT	NT	NT	67	<1	<1
32	86	79	<1	NT	<1	<1	4	NT	4	NT	NT	NT	NT	56	<1	<1
52	NT	10	NT	20	9	1	3	7	2	38	69	11	32	23	NT	NT
55	NT	71	NT	4	<1	1	2	4	2	<1	<1	<1	<1	<1	NT	NT
76	NT	1	NT	12	2	4	1	4	1	7	64	<1	1	66	NT	NT

Phenotyping Results : Chronic myeloid leukaemia in blastic transformation

Case	TdT	HLA-DR	CD19	CD22	CD10	SMIg	ER	CD2	CD7	CD11b	CD13	CD14 +	CD14 +	CD33	AN51	R10
11	<1	64	NT	NT	<1	5	14	NT	5	NT	NT	NT	NT	9	60	<1
17	<1	55	13	NT	5	58	<1	21	NT	NT	NT	NT	NT	65	<1	<1
18	40	1	<1	NT	<1	34	<1	NT	<1	NT	NT	NT	NT	75	NT	<1
26	NT	6	2	NT	<1	3	14	12	80	NT	NT	NT	NT	53	<1	<1
31	80	17	<1	NT	<1	<1	<1	NT	<1	<1	NT	<1	NT	60	<1	<1
33	90	<1	<1	NT	<1	2	83	85	99	NT	NT	NT	NT	<1	NT	NT
35	<1	79	4	NT	<1	4	2	12	32	21	12	11	19	13	7	3
42	NT	33	NT	10	15	2	11	8	10	6	57	5	22	21	NT	NT
46	NT	49	NT	79	7	11	16	25	19	NT	NT	NT	NT	<1	NT	NT
51	NT	68	NT	81	59	11	16	19	7	NT	NT	NT	NT	<1	NT	NT
87	95	76	<1	NT	<1	<1	2	2	1	<1	46	<1	<1	<1	NT	NT
89	60	60	17	NT	<1	2	9	15	6	NT	42	NT	NT	5	NT	NT

Phenotyping Results : Morphologically uncharacterized acute leukaemias

REAGENTS USED IN CYTOCHEMICAL STAINS

SUDAN BLACK B

Stock Solutions

A Sudan black B 0.3g

absolute alcohol 100ml

B phenol 16g in 30ml absolute alcohol

$Na_2HPO_4 12H_2O$ 0.3g in 100ml distilled water.

Working Solution

Solution A : 3 parts

Solution B : 2 parts

pH 7.0 or slightly alkaline

PEROXIDASE

Working Solution

O-toluidine/benzidine	250mg
ethanol	6ml
distilled water	4ml
H_2O_2 (20 volumes)	0.02ml

ACID PHOSPHATASE

Working Solution

Mix 0.4ml pararosaniline (Sigma)

0.4ml 4% Sodium nitrite

Allow to react for 3 minutes

Add 29.2ml 0.2M Sodium acetate buffer pH5.2

Add 10ml acid phosphatase substrate (20mg naphthol ASB1 phosphoric acid No. 2550 [Sigma]) in 10ml distilled water.

NON SPECIFIC ESTERASE

Working Solution

Mix 0.1ml pararosaniline (Sigma)

0.1ml 4% Sodium nitrite

Allow to react for 3 minutes

Add 38mls Sörensen's buffer 6.15

Add 2 ml and naphthyl butyrate (Sigma)

MURAMIDASE

Standard muramidase solution 250µg muramidase/ml PBS to give a concentration of 250µg, 125µg, 62.5µg, 31.25µg, 15.6µg/ml. A standard curve is prepared for each new batch of Micrococcus solution.

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