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FLOW CYTOMETRY IN DIAGNOSTIC HAEMATOPATHOLOGY:

Part I : IMMUNOPHENOTYPING IN ACUTE LEUKAEMIAS . CD10 ANTIGEN DENSITY IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKAEMIA.

Part II : DNA PLOIDY ANALYSIS IN ACUTE LEUKAEMIAS. COMPARISONS OF S PHASE.

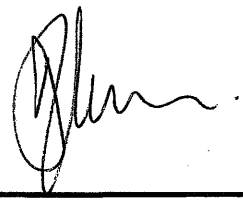
Deborah Kim Glencross

A Research Report Submitted to the Faculty of Medicine, Department of Haematology, University of the Witwatersrand, Johannesburg for the degree of Master of Medicine (in the branch of Haematology).

Johannesburg 1992

DECLARATION

I declare that this research report is my own, unaided work. It is being submitted for the degree of Master of Medicine (in the branch of Haematology) in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any other university.



Deborah Kim Glencross

28th day of APRIL, 1992.

DEDICATION

To KRG, who lives on in my mind and my memories.

CLEARANCE CERTIFICATE

This project has been **approved** by the University of the Witwatersrand, Division of the Deputy Registrar (Research), **COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS** (MEDICAL)
Ref : R14/49 (Registry)

Clearance certificate / **Protocol Number 19/01/91.**

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LIST OF ABBREVIATIONS :

ALL, Acute Lymphoid Leukaemia

AML, Acute Myeloid Leukaemia

cALL, Common Acute Lymphoblastic Leukaemia

CALLA, Common Acute Lymphoblastic Leukaemia Antigen

CD10, Cluster Designation 10

CML, Chronic Myeloid Leukaemia

DI, DNA Index

FAB, French- American- British

FI, Fluorescence Intensity

FCM, Flow Cytometry

ICP, Impulsecytophotometer

nALL, Null Acute Lymphoblastic Leukaemia

MCN, Mean Channel Number

%SPb, peripheral blood S phase percentage

C%SPb, corrected peripheral blood S phase percentage

%SBm, S phase percentage of the bone marrow

T ALL, T cell Acute Lymphoblastic Leukaemia

CHAPTER 1

GENERAL INTRODUCTION AND BACKGROUND

Early Flow Cytometry and the study of DNA.

The historical development of flow cytometry has been reviewed recently (Shapiro,1988,1991). Briefly, flow cytometry was initiated by developments in the field of automated cytology when an aerosol particle counter (evaluated by the US Army during World War II for the detection of air-borne bacteria and spores) was described (Gucker *et al*, 1947). Later during the 1950's, the principles of this aerosol particle counter were applied and modified for blood counters, initially by Crossland-Taylor (1953) and later by Coulter (1956). Although these systems rapidly gained acceptance in routine haematology laboratories, they were only adequate for cell detection and sizing, and not for the detection of malignant or mixed populations. Caspersson (1950) had shown that differences in cellular nucleic acid and protein content during normal and abnormal cell growth could be detected by microspectrophotometric measurement of the absorption of unstained cells in the UV and visible regions of the light spectrum. This was followed during the 1950's by investigation of fluorescent dyes for the identification and quantitation of DNA, especially as an alternative to the Papanicolaou's stain *e.g.* Acid Fuschin, Acridine Yellow and Berbine sulphate. Subsequent development of a scanning microfluorometer by Mellors and Silver (1951), enabled easier detection of cellular DNA fluorescence. Descriptions of acridine orange and other fluorescent DNA dyes followed (Melamed *et al*,1972a and 1972b, 1973, 1974; Darzynkiewicz *et al*,1975; Traganos *et al*,1977; Barlogie *et al*, 1977; Watson and Chambers, 1977; Fried *et al*, 1978), further facilitating and improving the study of DNA.

Antigen Detection by Flow Cytometry.

Fluorescent antibody techniques (Coons *et al*,1941) were applied to the detection of antigen on leukaemic and other cells, initially facilitated by the use of heteroantisera *e.g.* acute leukaemia antisera (Greaves *et al*, 1975). Weak expression of fluorescence on the surface of cells had made these techniques difficult to apply to early flow cytometric systems. The subsequent development of monoclonal antibody production (Kohler and Milstein, 1975), and the direct conjugation of these monoclonal antibodies with specific fluorochromes *viz.* FITC conjugation (Coons and Kaplan, 1950), firmly laid the foundation for modern flow cytometry.

Rapid Cell Spectrophotometer / Cell sorters.

Louis Kamentsky, a computer enthusiast with an interest in cell biology and cytology automation, had collaborated with several cell biologists and pathologists to build the rapid cell spectrophotometer (Kamentsky *et al*, 1965); a computerised optical flow cytometer to detect fluorescence of nucleic acids of cells in free suspension. A syringe pump, added to Kamentsky's original apparatus, created a fluidic cell sorter (Kamentsky and Melamed, 1967), which allowed the selective removal of specific cell populations or particles. The first description of an argon laser as a light source in a flow cytometric system, instead of a mercury arc lamp, was described by van Dilla *et al* (1969). In this paper these authors also described the use of a series of filters and mirrors to focus fluorescently re-emitted laser light, from fluorochrome stained cells, directly onto photomultiplier tubes (PMT). This

latter instrument has subsequently evolved into the modern generation of flow cytometers recognised today.

During the same period, the Fluorescence Activated Cell Sorter (FACS®) was described (Hulett *et al*, 1969), also utilising an argon laser instead of a mercury arc lamp. The method of cell separation utilised in this latter instrument (FACS) was different from Kamentsky's apparatus, and required an ultrasonic vibration, which was applied to the sheath stream (containing the sample stream) to cause droplet formation. Thus a particle of interest, designated by a specific amplitude of fluorescence, could be positioned into a single droplet. By charging and deflecting the droplet, either negatively or positively, between a pair of charged deflection plates, the particle of interest could be sorted. Subsequent collection of the designated droplets would follow.

Commercial Flow Cytometers.

The first commercially available flow cytometers were manufactured by Phywe AG in the early 1970's. The Impulsecytophoto-meter (ICP®), was built utilising a mercury arc lamp to illuminate cells flowing along the optical axis of the microscope objective, which acted as a condenser and a collection lens. The ICP was used widely during this time, by workers in Europe, to study DNA content and S phase fractions of leukaemias (Hillen *et al*,1975).

The first commercially available flow cytometers were the Cytograf® and the Cytofluorograf® instruments (Shapiro, 1991). These latter instruments were built by Louis Kamentsky for Bio/Physics Systems in 1970 and utilised

helium-neon or argon laser light sources. Coulter Electronics followed with the development of their own commercial instruments, and in addition, incorporated 90° light scatter and gating (population selection) facilities, now an integral part of modern flow cytometers.

Multiparameter Flow Cytometry.

Multiple parameter analysis with forward and right-angle light scatter detection, as well as multiple wavelength generation using argon and UV lasers and combinations of dyes with different emission spectra, further widened the spectrum of flow cytometry. Detection of up to eight parameters on a single particle was facilitated by the use of high speed electronics. Practically, this would allow the measurement of various surface and intracellular antigens or proteins, as well as nucleic acid content. The collection of list mode data (storage of the actual sample run on floppy disc for re-analysis at a later stage), with the use of a dedicated minicomputer system, added further dimensions to this technology.

Routine Laboratory Flow Cytometry.

Developments during the 1980's were still, however, largely research orientated. Computerised, multiple beam flow cytometers, equipped with sorting facilities, were suited for research laboratories only. These were large, complex instruments requiring dedicated rooms, power and water cooling systems, as well as the technical expertise of dedicated operators. However, as routine and clinical applications of flow cytometry have

increased, a new generation of flow cytometers has emerged. These flow cytometers are sophisticated instruments which make optimal use of optics and microcomputers for instrument control, data collection and analysis. In addition, they are small, compact and user friendly, and cost no more than the average large piece of benchtop equipment used in a routine haematology laboratory. Such adaptations have finally brought flow cytometry into the realm of routine diagnostic pathology, with broad application and acceptance in the clinical setting.

CHAPTER 2

Part I : IMMUNOPHENOTYPING IN ACUTE LEUKAEMIAS

CD10 ANTIGEN DENSITY IN CHILDHOOD COMMON ACUTE LYMPHOBLASTIC LEUKAEMIA : COMPARISONS OF RACE AND SEX.

SUMMARY

A total of 89 (including 58 children) new patients with FAB defined ALL, referred for immunophenotypic analysis, were studied. Epidemiological analysis showed that cALL is the predominant phenotype found amongst white children. Although T ALL appeared to be truly more common in black than in white children and adults, cALL was more frequently diagnosed than T ALL in black children. A slightly older age distribution was found in the black children with cALL than the comparable white childhood group. A striking deficit of cALL in the black adult group was noted.

Forty one of 42 children with a common phenotype were studied specifically to assess CD10 antigen density. A pattern of segregation was found between males and females and between black and white children, with black males having the lowest mean CD10 density and white females having the highest mean CD10 density pattern. It is hypothesized that low density CD10 antigen density patterns in males and in Blacks could be causally related to poor prognosis.

Details of MANUSCRIPTS and ABSTRACTS from PART I

I. **'CD10 antigen density in childhood acute lymphoblastic leukaemia : comparisons of race and sex'**, covering all aspects of the data presented in Part I, has been submitted as a manuscript to **Leukemia Research** (ref 42/92), and is awaiting the editors reply.

II. Two abstracts have been submitted for presentation at the **32nd Congress of the International Society of Haematology,** to be held in London during **August 1992.**

   i. **CD10 Antigen density evaluation in childhood common ALL: Comparisons of race and sex.**

   ii. **Epidemiology of Acute Lymphoblastic Leukaemia in Johannesburg: Distribution of phenotypes by race, age and sex.**

III. The latter abstracts will also be presented locally at the **32nd Annual Congress of the Federation of S.A. Societies of Pathology,** to be held at the Wild Coast Sun, during **June 1992.**

IV. Dr. Richard Cohn, (co-author on the manuscript submitted to Leukaemia Research) will present the CD10 density data as a poster at the **22nd Congress of the International Society of Paediatric Oncology (ISPO)**, to be held in Hanover, Germany during **October 1992.**

INTRODUCTION

Background.

Since the introduction of heteroantisera for the identification of acute leukaemias (Greaves *et al*, 1975), the subsequent development of monoclonal antibody technology (Kohler and Milstein, 1975), and the refinement of routine flow cytometry instrumentation, many advances have occurred with regard to immunophenotypic characterisation of leukaemias and lymphomas. The introduction by the WHO in 1984 (Shaw, 1987) of the Cluster Differentiation (CD) system to define groups of antibodies according to reactivities on specific target cell sites, further helped to clarify the vast collection of abbreviations used in the literature up until this time and simplified analysis. Extensive reviews of leukaemia and lymphoma immunophenotyping have appeared (Foon and Todd, 1986; Greaves, 1986a; Janossy and Campana, 1988; Janossy *et al*, 1989) as well as reports of normal B cell ontogeny (Loken *et al*, 1987; Uckan, 1990), but are beyond the scope of this review.

Routine immunophenotypic analysis.

Over recent years, the value of routine immunophenotypic analysis in leukaemic patient workup has been widely approved and accepted. Morphological assessment is generally acknowledged as being subjective, even in the eyes of the most experienced reviewer, and cytochemistry, although a more objective parameter, is not always conclusive. The merit and utility of

immunophenotyping in the diagnosis of acute leukaemias lies in the detection of acute leukaemic subgroups, not identified by conventional means *viz.* myeloperoxidase staining. The FAB proposals for the classification of acute leukaemias (Bennett *et al*, 1976) do not consider the existence of mixed or undifferentiated categories. However, entities such as biphenotypic leukaemias and acute leukaemic groups demonstrating aberrant expression of apparently unrelated antigens have been identified (Greaves *et al*, 1986b; Gale and Basset, 1987; Cross *et al*, 1988; Ross *et al*, 1990; Auger *et al*, 1991; Bennett *et al*, 1991; Cantu-Rajnoldi *et al*, 1991).

In addition to the value of identifying such mixed groups, immunophenotypic analysis by flow cytometry has allowed the identification of additional diagnostic and prognostic parameters in acute leukaemias. Catovsky *et al* (1991), through their recent proposals for the assessment of biphenotypic leukaemias, have suggested that surface immunophenotypic analysis is essential for the identification of biphenotypic leukaemias, or those leukaemias where aberrant unrelated antigens are co-expressed. Recently, a relatively new addition to the group of acute leukaemias has been documented (although previously recognised) which cannot be identified routinely without the aid of immunophenotypic analysis *viz.* AML M0 (Bennett *et al*, 1991). Although the presence of a particular antigen may be important for the diagnosis of a particular type of acute leukaemia, the extent of the antigen expression adds a further dimension to the flow cytometric facilitated analysis. For example, low density CALLA/CD10 (CALLA

expression is weak or dim) has been shown to be related to a worse prognosis in childhood common acute lymphoblastic leukaemia (Look *et al*, 1984).

An introduction to CD10.

The biology and characterisation of CD10 has been reviewed recently (LeBien and McCormack, 1989). Briefly, the production of rabbit antisera to CD10 (CALLA) was first described by Greaves *et al*, (1975), in an attempt to generate acute leukaemia specific antibodies. Some reactivity was noted by these authors in normal bone marrow, spleen and fetal liver, as well as in a wide variety of haematological tumours including acute lymphoblastic leukaemia, chronic myeloid leukaemia in blastic transformation, non-Hodgkins lymphomas, Burkitt's lymphomas, and undifferentiated leukaemias (Roberts *et al*, 1978, Greaves *et al* , 1983). The reactivity and expression was noted mainly in B cell lineage acute lymphoblastic leukaemias, identified by immunoglobulin gene rearrangement studies (Korsmeyer *et al*, 1983), as well as in some T acute lymphoblastic leukaemias (Ritz *et al*, 1981). Although the first description of a monoclonal antibody to CD10 was directed toward a murine antigen (Ritz *et al*, 1980), antibodies to the human CD10 antigen followed. This was later confirmed by Greaves *et al*, (1983) to be directed toward the same molecule detected by heteroantisera that were used in studies prior to the development of monoclonal CD10.

Expression of CD10 in normal tissues.

Expression of CD10 in normal regenerating bone marrow was studied by Janossy *et al*, (1979,1980) and Greaves *et al*, (1980b). CD10 positive cells were found to constitute approximately 1% in adult marrows, with higher percentages found in childhood and fetal bone marrows. Expression of the molecule on peripheral blood polymorphonuclear neutrophils was described later independently by Cossman *et al*, (1983) and Braun *et al*, (1983). Neutrophils have been documented to actively produce and stably express CD10 on their surface membranes (McCormack *et al*, 1986).

Molecular characteristics of CD10.

The CD10 molecule has been characterised as a 95 to 100 Kd glycoprotein with 20 to 25% carbohydrate content (Sutherland *et al*, 1978; Newman *et al*, 1981). Interestingly, the molecular mass of CD10, detected by migration through polyacrylamide gels, varies according to the tissue type. Neutrophil CD10 (Cossman *et al*, 1983), has been measured at approximately 110Kd, whereas CD10 from kidney and fibroblasts, have been found to be approximately 90Kd (Metzgar *et al*, 1981; Braun *et al*, 1983). No differences exist between CD10 on neutrophils and the CD10 on leukaemic cells (McCormack *et al*, 1986). Characterisation of CD10 as a neutral endopeptidase (NEP) was described by Letarte *et al*, (1988). NEP can be classified as a metallo-enzyme, using zinc as a co-factor with a broad specificity to cleave alpha amino groups of hydrophobic amino-acids (LeBien and McCormack, 1989). Details of the cDNA molecular cloning were

further outlined by Shipp *et al*, (1988). Several messenger RNA species were identified including two predominant species of 5.7 and 3.7 kb and a low abundance of 4.7, 3.1 and 2.7kb species. Differential mRNA expression between different cell types was noted.

Variation of CD10 expression.

Look *et al*, (1984) described the variation in surface CD10 in acute lymphoblastic leukaemias, and showed that the expression remained constant throughout the leukaemic cell cycle. Several reasons for the variation in CD10 expression have been suggested. Antigenic down modulation of the CD10 antigen after interaction with anti-CD10 antibodies (Ritz *et al*, 1980; Pesando *et al*, 1981) may be responsible. This feature is temperature and dose dependant, and related to the antibody affinity (LeBien *et al*, 1982). The possibility that a particular CD10/NEP substrate may be present in the sera of certain patients, cannot be ruled out (Pesando *et al*, 1981). This substrate is then acted on by the CD10/NEP molecule with subsequent internalisation of the CD10/substrate complex and loss of surface CD10. It was further suggested by the same authors that this modulation may, in addition, be induced by peptide hormones. Komada *et al*, (1986) showed that CD10 is shed or released into the serum of patients with Common ALL. In addition, different epitopes of CD10 have been described which may also contribute to the variation of CD10 noted between leukaemic cells (Matsuzaki *et al*, 1987). Thus CD10 antigen variation may occur on several levels, and if genetic differences cannot account for the variation of

expression, at least there are differences related to functional capabilities of the CD10 moiety.

Prognostic value of CD 10.

Prognostic differences of common acute lymphoblastic leukaemia (cALL) between Black and White children, and between boys and girls has been documented (Crist *et al*, 1989). Heterogeneities with respect to CD10 density are described (Look *et al*, 1984; Kreindler *et al*, 1990; Ross *et al*, 1990), and in one report (Look *et al*, 1984) low density CD10 expression has been recognised as an adverse prognostic factor.

Epidemiology of childhood acute lymphoblastic leukaemia.

It has long been recorded in both Africa and America, that there is a difference in common acute lymphoblastic leukaemia (ALL) in black and white children with respect to incidence and prognosis (Bowman *et al*, 1984; Fleming and Peter, 1984; Reaman *et al*, 1984; Walters *et al*, 1984; Greaves *et al*, 1985; Kalwinsky *et al*, 1985; MacDougall, 1985). Recently a study from another southern african country (Paul *et al*, 1991) has confirmed previous reports (Bowman *et al*, 1984; Kalwinsky *et al*, 1985; MacDougall, 1985) which suggest that the incidence patterns of acute lymphoblastic leukaemia in third world children (in Africa) are evolving and ultimately will reflect patterns seen in both black and white children with acute lymphoblastic leukaemia in the first world.

In this present study of acute lymphoblastic leukaemia in children, in addition to an epidemiological study of the case data, an evaluation of CD10 antigen density was performed in black and white children presenting with common ALL (cALL). An interesting divergence of patterns has been observed between male and female children, and between black and white children.

MATERIALS AND METHODS

Patients.

A total of 89 new cases of ALL referred from the Johannesburg or Baragwanath hospitals for immunophenotypic evaluation, were studied during the period from July 1989 until August 1991. This group included 58 children and 31 adult (>14 years) patients. CD10 density evaluation was performed on 41 of 42 cases of children with cALL. Although the common-ALL antigen (CALLA) is stable within the individual leukaemic cell population in the majority of patients, alterations in CD10 expression have been described in relapse (Greaves *et al*, 1980a), thus warranting the exclusion of one black male child who presented during the period of study, but whose immunophenotype was ascertained only during relapse.

Immunophenotyping.

Pre-treatment heparinised bone marrow aspirates (all childhood cALL cases) or peripheral blood specimens with >80 % blasts were submitted for surface

marker analysis. A panel of monoclonal antibodies were performed as dual fluorescent studies and included CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD33, Kappa/Lambda, HLA-Dr as well as relevant isotypic controls. A phenotype of cALL was defined as the positive surface marker combination of CD10, CD19 and HLA-Dr without evidence of surface membrane immunoglobulin expression. Negative myeloperoxidase and CD33 expression, with positive CD7 expression alone or in combination with CD2, CD3, or CD5 was classified as T cell ALL. During the period of this study, CD13 was not included in the screening panel for the exclusion of acute myeloid leukaemia FAB subtype M0 (Bennett *et al*, 1991). Patients thus presenting with negative myeloperoxidase cytochemistry, negative CD33 expression but positive HLA-Dr expression, in isolation, or in combination with CD7, were classified as undifferentiated.

Monoclonals were used according to the supplier's specifications (Coulter Electronics, Hialeah, Florida). Mononuclear cells obtained by Ficoll separation, were washed once in phosphate buffered saline/ 2% foetal calf serum/0.2% sodium azide. Between 0.5 and 1.0×10^6 cells were incubated for a period of 20 minutes with respective monoclonal antibodies, at 4°C, in the dark. All Ficoll separated cell preparations were fixed with 2% paraformaldehyde and stored at 4°C until flow cytometric analysis. Alternatively, a whole blood procedure, *viz.* Coulter Qprep, (Coulter, Miami, Florida) was used for sample preparation (6 of 41 cALL cases).

Flow Cytometry.

Samples were analysed on an EPICS Profile-I ™Flow Cytometer. Daily calibration was performed prior to analysis with Immunocheck standardised beads (Coulter Electronics) and instrument alignment was set accordingly. Measurements were performed at the same excitation intensity for comparison of results (Bohmer *et al*, 1985). In addition, individual cases were analysed in one session, specifically to avoid artifacts of instrument variation.

CD10 Density evaluation.

Comparative analysis of CD10 expression was performed in 41 patients with cALL, *i.e.* 27 white, 12 black and 2 mixed race children. By using the relatively constant fluorescence intensity (FI) of normal background peripheral blood lymphocytes as an internal quantitative standard (Cadwell *et al*, 1987), it is possible to document alterations in the FI of unrelated antigens, *e.g.* CD10. A ratio was calculated for each patient based on the FI (measured as the mean channel number or MCN) of the CD10 positive fluorescent population and the mean FI (mean MCN) of the background normal T cell population, *i.e.* CD10 FI (MCN) divided by the mean FI (MCN) of CD2, CD3, CD4, CD5, CD7, and CD8, (or alternatively by the mean of CD2, CD3, CD5, and CD7 if CD4 and CD8 were not performed) of the remaining positive normal T lymphocytes present in each sample. The ratios thus determined were used to elucidate and document any differences of CD10 expression between patients. Although epitopes of CALLA are

described (Matsuzaki *et al*, 1987), to the best of my knowledge, no deficiencies of CD10 have been described in black or white children.

Cytogenetics.

Cytogenetic studies were performed in the cALL group examined for variation of CD10 density specifically to assess the presence of t(1;19) abnormalities. All cytogenetic studies were performed by the Division of Haematologic Cytogenetics of the Haematology Department at the University of the Witwatersrand Medical School/ South African Institute of Medical Research.

Epidemiological Evaluation.

Unfortunately actual incidence figures are unavailable owing to lack of accurate and detailed census information. *Population numbers as recorded in the official 1991 census are listed as follows:*

total population of Johannesburg : 1 647 259

total population of Whites : 326 691

total population of Blacks 1 156 252

(information as enumerated by the Central Statistical Service in the 1991 census, not adjusted for possible under- or over- count).

This following section was drawn from data supplied by Ms. F Adam. For the purpose of this study, the relative frequencies were expressed as ratios of cALL and T ALL in relation to race groups and age. Patients attending

various hospitals are no longer racially segregated, but Johannesburg Hospital serves mainly white patients and Baragwanath Hospital mainly black patients.

Statistics.

Comparisons inside race groups between sexes, and inside sexes between race groups were done using Student's t-test. These findings were confirmed by the Mann-Whitney test as sample numbers were small. The method of least square means, following a two-way analysis of variance was employed to test the overall effects of sex and race.

RESULTS

Epidemiology.

A total of 89 cases of French American British defined ALL (Bennett *et al*,1976) were available for the purposes of this study. cALL was the predominant type found in white children, with a peak frequency of 2 - 4 years of age (Figure 1) : the male to female ratio was 1.3:1. Fewer black children were seen with cALL: the peak of frequency under 4 years was less pronounced and possibly occurred at a slightly later age than in white children. There were relatively more black children (50%) with cALL in the age group of 5 - 9 years, when there was a suggestion of a second peak of frequency (Figure 1): the male to female ratio was 1.6:1. cALL was diagnosed in 5 female and 3 male white adults, and in only one male black adult.

There were 10 black children with T ALL, with a peak frequency at 5 - 9 years (Figure 2). There was a markedly skewed ratio of 9:1 boys versus girls amongst the black children in this latter group. Only two white children with T ALL were noted. Both were aged less than 4 years.

FIGURE 1 : Frequency of common acute lymphoblastic leukaemia versus age distribution in Johannesburg

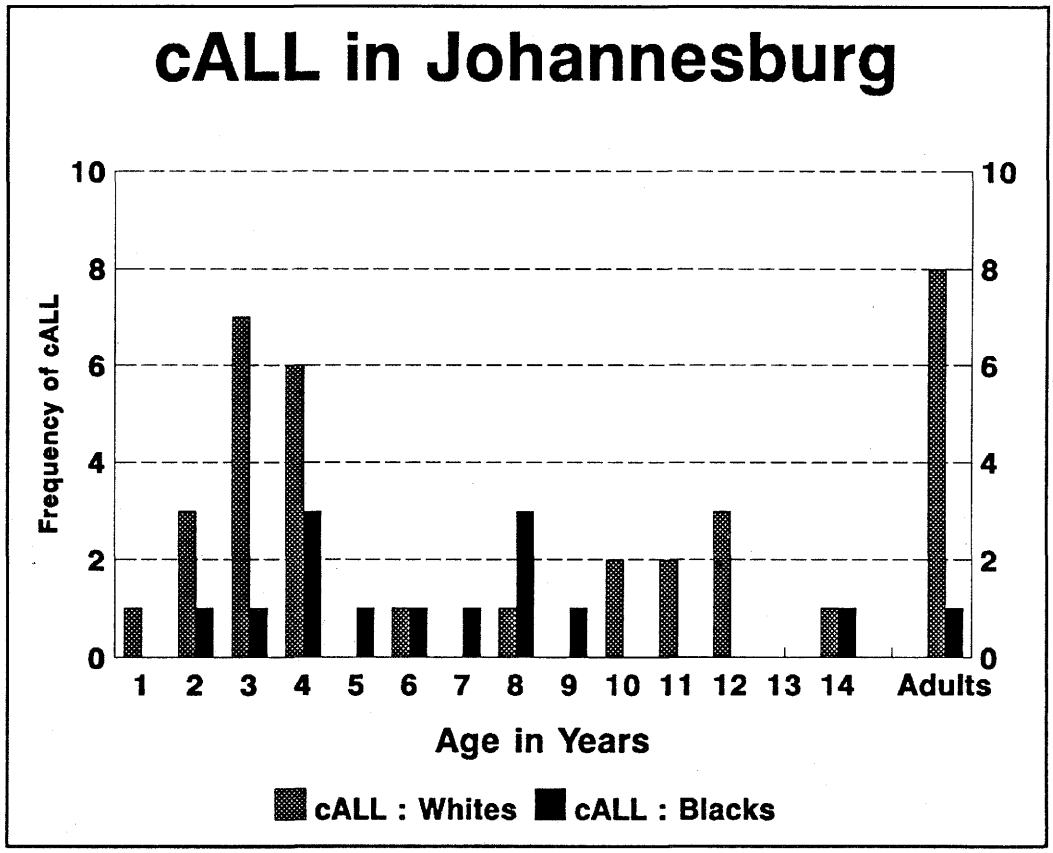
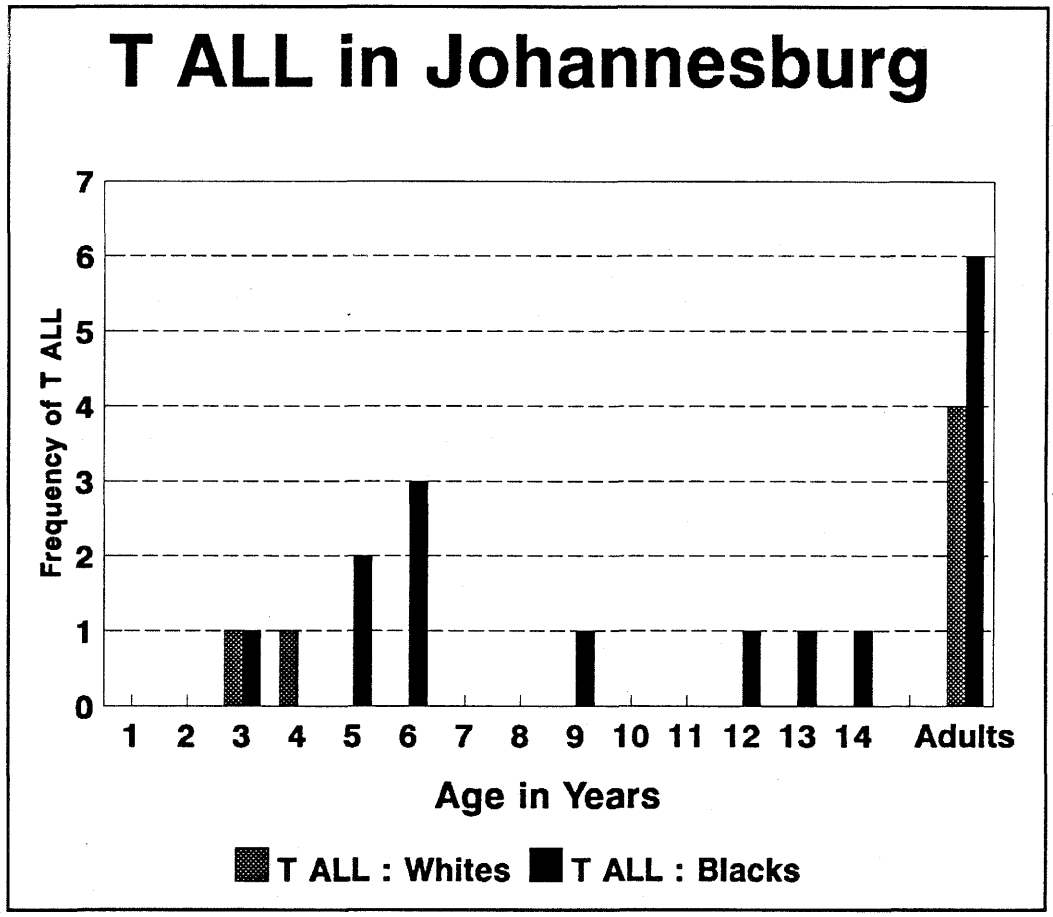


FIGURE 2 : Frequency of T acute lymphoblastic leukaemia versus age distribution in Johannesburg



When comparing relative frequencies of diagnosis (Table 1), in childhood, T ALL was 5 times more common in Blacks than in Whites, while cALL was twice as common in Whites as in Blacks. Amongst adults, T ALL was once again more frequent in Blacks than Whites but the ratio was only 1.5:1 : in contrast, cALL was seen in 8 Whites and only one Black. During childhood, cALL was seen nearly 14 times more frequently than T ALL in Whites; cALL was only slightly more common than T ALL in Blacks. In adults, cALL was seen twice as often as T ALL in whites, whereas there were 6 Blacks with T ALL and only one with cALL.

Six white adults were classified as undifferentiated acute leukaemia : two of these patients expressed both HLA-Dr and CD7. Only one Black adult male was classified as undifferentiated ; CD7 was not performed in this patient.

In the null ALL group, 3 of 4 white adults and the only black adult recorded were noted to be young males aged between 16 and 25 years; amongst the children, one white female aged 2 years, and one black male aged 13 were also shown to have null phenotypes. The 4 remaining patients included two mixed race female children with cALL, one Asian male child with T ALL and one white male child with a B ALL

TABLE 1 : Relative frequencies of diagnosis of cALL and T ALL, in Blacks and Whites, children and adults.

CHILDREN			Observations	Ratios
	cALL	Blacks vs Whites	13:27	0.5:1
	T ALL	Blacks vs Whites	10:2	5.0:1
	Blacks	cALL vs T ALL	13:10	1.3:1
	Whites	cALL VS T ALL	27:2	13.5:1
ADULTS				
	cALL	Blacks vs Whites	1:8	0.1:1
	T ALL	Blacks vs Whites	6:4	1.5:1
	Blacks	cALL vs T ALL	1:6	0.2:1
	Whites	cALL vs T ALL	8:4	2.0:1

CD10 Comparative Analysis.

A pattern of segregation of CD10 density was noted with regard to sex and race in the cALL group (Tables 2, 3, and 4). Black males had the lowest overall CD10 density (Mean = 0.60) and were significantly different from white males (Student's p = 0.0251, Mann Whitney p = 0.0054), as well as white females (Student's p = 0.0030, Mann Whitney p = 0.0013). There were significant differences between white males and white females (Student's p = 0.0300, Mann Whitney p = 0.0281), with the highest CD10 density pattern found in the white female group (Table 3). Differences between

black females and white females and between black males and black females were not statistically significant, but the numbers in the black female group were small. The 2 mixed race female children (not included in the CD10 non-parametric analysis due to insufficient numbers), showed similar CD10 density segregation patterns to the white female children (Table 2).

The method of least square means following a two-way analysis of variance (Table 4), revealed that sex had contributed significantly to the difference noted between groups ($p = 0.0038$), whereas the contribution of race was only marginal ($p = 0.0530$). Small numbers within the black cALL group may however have contributed to this latter finding.

CD10 density statistical evaluations were performed both including and excluding those patients samples prepared by Coulter QPrep (whole blood preparation). Levels of significance were unchanged in the Student's t- and the Mann-Whitney tests when the QPrep group (6 of 41 patients) was excluded from the analysis, suggesting that the QPrep procedure itself had not contributed to the variation in CD10 expression.

TABLE 2 : Children with common acute lymphoblastic leukaemia and their respective calculated CD10 ratios

No	PT	AGE	R	S	CD10 ratio	No	PT	AGE	R	S	CD10 ratio
1	TM	2	B	M	0.32	23	SM	9	B	F	0.32
2	PM	7	B	M	0.38	24	VP	14	B	F	0.79
3	EH	8	B	M	0.42	25	LA	6	B	F	1.21
4	N	5	B	M	0.47	26	EB	4	B	F	2.28
5	LM	8	B	M	0.61	27	LM	4	B	F	2.76
6	M	8	B	M	0.63	28	AG	3	W	F	0.94
7	TB	4	B	M	1.37	29	C	3	W	F	0.99
8	DJ	12	W	M	0.48	30	M	4	W	F	0.96
9	VV	2	W	M	0.6	31	LH	4	W	F	1.17
10	GK	3	W	M	0.65	32	SS	4	W	F	1.61
11	NB	4	W	M	0.7	33	CS	12	W	F	1.61
12	AD	2	W	M	0.72	34	M	4	W	F	1.64
13	D	8	W	M	0.89	35	SP	12	W	F	1.66
14	JM	3	W	M	0.93	36	CF	4	W	F	2.19
15	GR	10	W	M	0.95	37	GA	1	W	F	2.58
16	JL	3	W	M	1.09	38	LK	3	W	F	3.42
17	M	2	W	M	1.38	39	JBJ	3	W	F	3.64
18	QG	10	W	M	1.49	40	SB	12	M	F	1.74
19	JPJ	11	W	M	1.67	41	M	12	M	F	3.57
20	RC	11	W	M	1.87						
21	GT	4	W	M	2.05						
22	SB	6	W	M	2.43						

Abbreviations : R=race; S=sex; No=patient number; Pt=patient identified by initials

TABLE 3 : Mean CD10 ratios and Standard Deviation values for the cALL group stratified according to race and sex.

Race and sex	Mean	S.D.	Number
Black Males	0.60	0.36	7
White Males	1.19	0.59	15
Black Females	1.47	1.02	5
White Females	1.87	0.92	12

TABLE 4 : GENERAL LINEAR MODELS PROCEDURE

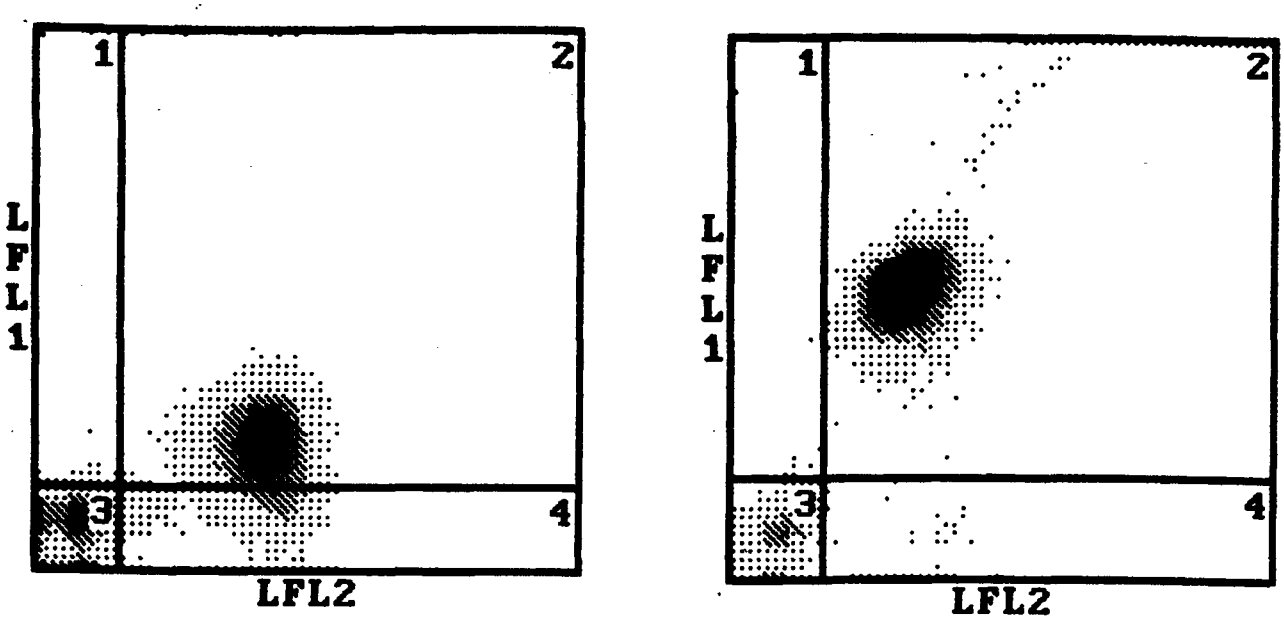
A two-way analysis of variance to show the effect of race and sex in the childhood cALL group analysed for variation of CD10.

	Overall Mean CD10 ratio	Weighted Mean CD10 ratio	p value
Total Females	1.75	1.67	
			0.0038
Total Males	1.00	0.90	
Total Whites	1.49	1.53	
			0.0530
Total Blacks	0.96	1.04	

FIGURE 3 : **CD10 DENSITY IN cALL.** Figure 3(a) patient SM, with a **low density CD10 pattern,** and 3(b) patient JB, with a **high density CD10 pattern.**

Both histograms (quadrant 2) show the dual expression of CD10 on the y-axis (LFL1) and CD19 on the x-axis (LFL2). Note the different positions of the CD10 expressing populations on the y-axis.

(a) (b)



Variation of CD19 FI in the cALL group was also noted. Similar ratios were calculated based on the FI (MCN) of the CD19 expressing population divided by the mean FI (MCN) of the background T cell population. No significant differences were found between race or sex with regard to CD19 FI. In addition, no relationship was found between CD10 to CD19 expression in the cALL group.

Cytogenetics.

Specimens for cytogenetic analysis were received in 33 of 42 children with cALL, but failed in 3. Abnormalities were detected in 17 of 30 patients. Only one mixed race child (SB, excluded from CD10 ratio analysis) showed a t(1;19) abnormality (Crist *et al*,1990), suggesting that differentiation toward a pre-B phenotype is not related to CD10 antigen density. Hyperploidy or polyploidy (Look *et al*,1985; Pui *et al*,1989) was seen in 6 children, none of whom had CD10 ratios below 0.90 (Table 2: Patients JM, JL, JP, SB, LM and SP respectively). A t(9;22) (Fletcher *et al*,1991) was noted in 3 patients (QG, GT and MR, the latter of whom was noted to have a lower CD10 antigen density. No t(4;11) abnormalities (Pui *et al*,1991c) were found.

DISCUSSION

Because accurate current census information is not available in South Africa, especially now during the major population movements from rural to urban areas that are occurring following relaxation of the apartheid related influx laws in 1986, we are unable to determine crude and age-specific incidence figures for ALL in the various population groups. It was possible, however, to evaluate relative frequencies of the various ALL subtypes as a function of age in black and white patients. It is evident from this study that white children displayed the characteristic age distribution of children in developed countries elsewhere, whether black or white (Bowman *et al*,1984; Reaman *et al*,1984; Kalwinsky *et al*,1985; MacDougall,1985).

Although the relative frequency of cALL was less in black than in white children, as shown by others (Bowman *et al*,1984) and recently in another Southern African country (Paul *et al*,1991), there were slightly more cALL than T ALL in the black children. Furthermore, when cALL occurs in South African blacks, it is a disease overwhelmingly of children, with a rather wider, or possibly bimodal, age distribution. The relative frequency ratios also suggest that T ALL is truly more common in Blacks than Whites, in children and in adults, and is not merely relatively common as a consequence of a deficit of cALL.

With the recent attention given to the population epidemiology of cALL in Britain (Alexander *et al*,1990; Kinlen *et al*,1990; Kinlen,1991; Fleming,1991), it is relevant to address the issue of whether the occurrence of cALL in black

South African children is a new phenomenon, consequent upon the relatively recent population fluxes that are now occurring in South Africa. The only previous study which attempted to define ethnicity of cALL in this area was that of MacDougall in 1985. In that study, no childhood peak of cALL was documented in Blacks, but this could have reflected a particularly high failure rate for immunophenotypic assignment in the majority of black children, as compared with a minority of such failures in white children. The immunophenotypic analysis, which was carried out in this laboratory, antedated the acquisition of the flow cytometric apparatus used in the present study. The main difficulty experienced at the time was in demonstrating presence or absence of CD10, as it frequently appeared to be very weak, bordering on negative or non-specific, as judged by fluorescence microscopy. The density data generated in the present study may explain these difficulties, and suggests that the occurrence of cALL in black South African children may not be a very recent phenomenon, and possibly was present in the early 1980's prior to the relaxation of influx control regulations in 1986. Black children with ALL, including those with cALL (Crist *et al*,1989), have long been known to fare worse than their white counterparts (Walters *et al*,1972; Reaman *et al*,1984; Bowman *et al*,1984; Greaves *et al*, 1985; Kalwinsky *et al*,1985; MacDougall,1985), and males with cALL worse than females (Crist *et al*,1989). Density of CD10 antigen on cALL cells has been documented to correlate directly with prognosis (Look *et al*,1984), independently of cytoplasmic immunoglobulin expression. In the present study, it is of interest that the black males, who are the worst prognostic

group, had lower CD10 density than the other groups, while white females, known to constitute the best prognostic group, had significantly higher CD10 density than other groups. Black females and white males occupied intermediate positions with respect to CD10 density of expression. Overall, females had higher expression than males, and whites higher than blacks. While these rankings could be coincidental and unrelated to prognosis, it is tempting to suggest they are not. If they are related, this study reinforces the notion that CD10/NEP, is directly concerned with prognosis (Letarte *et al*,1988; Lebien *et al*,1989), possibly through an influence on drug metabolism. In this regard it is of interest that the level of NEP activity has been shown to correlate with antigen density by flow cytometry (Tran-Patterson *et al*,1990). However, it is still too early to evaluate directly the impact of CD10 density on prognosis in our patient sample. The results suggest that ethnically determined polymorphisms and/or gender may influence CD10 expression density and that the adverse prognosis seen in black children reflects biological rather than sociological differences. Alternatively, low density cALL may constitute an entirely separate disease entity from high density cALL with differences of race, age and prognosis.

CHAPTER 3

Part II : DNA PLOIDY ANALYSIS IN ACUTE LEUKAEMIAS

COMPARISONS OF S PHASE.

SUMMARY

Forty six patients with acute leukaemia presenting to the Johannesburg, Baragwanath and Hillbrow Hospitals between February and June, 1991 were studied. This group included 2 patients in blastic transformation of Chronic Myeloid Leukaemia and 9 patients in acute leukaemic relapse. DNA ploidy analysis (S phase fraction estimation and DNA Index calculation) was performed on all samples.

Bone marrow aspirates were received in 33 of 46 patients, and studied w.r.t. S phase percentage. No significant differences were noted of the S phase percentages between presentation ALL and AML or between presentation (*denovo*) cALL and relapsed cALL. Further evaluation of 16 AML patients revealed an apparent, although not statistically significant, segregation of AML FAB subtype with S phase percentage.

Nine patients, with greater than 70% blasts in matched peripheral blood and bone marrow samples, were noted to have higher S phase percentages in the bone marrow aspirates when compared with corresponding peripheral blood samples ($p = 0.021$).

A tendency to underestimate peripheral blood blast percentages by morphological assessment alone was noted after consideration of the S phase percentage noted by flow cytometry. DNA ploidy/S phase analyses are therefore recommended as an adjunct to routine differential assessment of peripheral blood blast percentages.

Details of ABSTRACT from PART II.

An abstract entitled **'Peripheral blood blast percentages: WYSIWYG or is it?'** based on the data contained in PART II of this thesis, will be presented at the **32nd Congress of the Federation of the S.A. Societies of Pathology**, to be held at the Wild Coast Sun, during **June 1992.**

INTRODUCTION

THE S PHASE DEBATE

Comparison of leukaemic and normal bone marrow proliferation.

Over the last few decades, many investigators have attempted to define the proliferative nature of acute leukaemias (Mauer and Fisher, 1962; Clarkson *et al*, 1970a, 1970b; Cheung *et al*, 1972; Wagner *et al*, 1972; Hillen *et al*, 1975; Dosik *et al*, 1980a; Holdrinet *et al*, 1983; Suarez *et al*, 1985; Ffrench *et al*, 1987; Raza *et al*, 1987a; 1987b; 1990; 1991). Although initially encouraged by in vivo and in vitro tritiated thymidine techniques *i.e.* labelling index (LI), this field was further inspired by advancements in flow cytometry and development of fluorescent dyes for DNA staining as previously mentioned. Generally, the results of these latter studies varied considerably, but overall showed that leukaemic cell populations were distinctly different from their normal population counterparts. Leukaemic bone marrows were shown to have lower S phase distributions and longer cycle times *i.e.* slower doubling times than normal active bone marrow (Clarkson *et al*,1972a and 1972b; Raza *et al*, 1990, 1991). Leukaemic cells in bone marrow and peripheral blood compartments were also shown to have different characteristics; peripheral blood leukaemic blasts were documented as having lower S phase percentages than their bone marrow counterparts (Mauer and Fisher, 1962).

Drug studies.

The effects of drug induced changes on cell cycle distribution of acute leukaemias were evaluated during the 1970's. These studies were based mainly on techniques involving in vivo tritiated thymidine incorporation (later DNA histogram analysis by flow cytometry was used), and were focused on the recruitment of leukaemic cells into S phase by administration of cycle active drugs (Burke *et al* 1971; Vogler *et al*, 1974; Wantzin *et al*, 1976; Rentchler *et al*, 1978; Hiddeman *et al*, 1982).

Proliferation assessment and prognosis.

Correlative studies of prognosis and complete remission rates or response to chemotherapy, with S phase percentages in acute leukaemias, have been conflicting. The adverse prognostic significance of higher S-phase fractions in determining the length of the first remission was reported in childhood ALL (Scraffe *et al*, 1980; Dow *et al*, 1982), as well as in adult ALL (Holdrinet *et al*, 1983) and adult AML (Preisler *et al*, 1984, Raza *et al*, 1990). Later Hiddeman *et al* (1982), showed that S phase was not related to the likehood of attaining a complete remission, or predictive for therapeutic response. Dosik *et al* (1980a) had, in contrast, shown that higher pretreatment S phase percentages correlated well with the degree of cytoreduction but not however, with rate of complete remission.

Other studies reported opposing views. Early reports (Hart *et al*, 1977) which had shown that young AML patients with higher S phase fractions had the best remission rates, were confirmed by later by flow cytometric studies

(Hall *et al*, 1983; Riccardi *et al*, 1986). Andreeff *et al* (1980, 1986) had, in addition, shown that adult AML patients with higher RNA content were also more likely to respond to therapy than those patients with lower RNA values.

Look *et al* (1982) had shown similar findings in childhood ALL and further, demonstrated a correlation of low percentages of S-phase with lack of response to induction therapy in some children with ALL. Recently, Raza *et al*(1990 and 1991) has reported that although S phase percentage was a good indicator of response to therapy, it was not predictive of curability.

Explanation of variable reporting.

Factors contributing to the variable and conflicting S phase reporting are numerous, and include amongst others, the diverse techniques utilised (tritiated thymidine labelling index ,flow cytometry, different dye combinations etc), variable haemodilution of aspirate samples (Burke *et al*, 1971; Hiddeman *et al*, 1982; Dosik *et al*, 1980b; Riccardi *et al*, 1986; Raza *et al*, 1987a), differences in therapy protocols of the groups studied, and patient selection (Look *et al*, 1985).

Overview of S Phase percentages.

Overall, in spite of the apparently conflicting literature and terminology which appeared at this time, a common theme was nevertheless evident. Although patients with higher percentages of S phase cells or a higher RNA content were more likely to respond to therapy, these patients, once in remission, were also most likely to relapse *i.e.* their period of remission would be short,

and their overall outcome poorer. Patients with higher bone marrow blast proliferative activity (S phase percentage) responded more readily to chemotherapy, but had a shorter duration of first response compared with patients with lower proliferative activity (Andreeff *et al*, 1986; Riccardi *et al*,1986).

By way of contrast, those patients with low S phase fractions (or low RNA content) were unlikely to be cured, or respond actively to chemotherapy. However, if a response was seen in these patients, this would be the group most likely to have extended remissions. Patients with lower S phase estimates were reported to have longer remissions once attained, than patients with higher S phase estimates (Andreeff *et al*, 1986). Acute leukaemias with minimal or no S phase fractions have been reported and are described as being non responsive to chemotherapy (Look *et al*, 1985; Bejar-Lozano *et al*, 1989).

AML versus ALL.

Higher percentages of S phase cells were found in ALL than in AML in adults (Hart *et al*, 1977; Dosik *et al*, 1980a; Holdrinet *et al*, 1983a). Andreeff *et al* (1986), however, found no difference between the percentage S phase cells in adult ALL as opposed to AML. Higher S phase percentages have been noted in relapsed than presentation acute myeloblastic leukaemias (Murphy *et al*, 1977; Dosik *et al*, 1980a). This has however been refuted by others (Holdrinet *et al*, 1983), although the latter report did show higher percentages in relapsed than presentation ALL patients.

S phase differences in ALL subtypes.

The cells of B ALL FAB subtype L3 have been reported to have a significantly higher S phase percentage than the cell populations of other ALL FAB subtypes, L1 or L2 (Ffrench *et al*, 1987). Similar higher S phase percentages have been reported in the cell populations of ALL FAB subtype L2 when compared to FAB subtype L1 (Suarez *et al*, 1985). In addition, the leukaemic blast populations of childhood T ALL have been reported to have higher S phase fractions than the cells of other lymphoid leukaemic groups (Murphy *et al*, 1977; Dow *et al*, 1982). Other studies have shown that the cell populations of childhood B and T cell leukaemias, have higher S phase percentages than those cells found in non T-non B or undifferentiated leukaemias (Look *et al*, 1982). This phenomenon has, however, not been documented in adult ALL studies (Ffrench *et al*, 1987).

Aneuploidy and prognosis.

Correlation of flow cytometry aneuploidy to prognosis has been extensively reported in childhood ALL, and has been reviewed recently (Pui *et al*, 1990a). Briefly, Look *et al*, (1985) first reported that hyperdiploid childhood ALL was associated with a good prognosis. Later Pui *et al*, (1989) showed that within this latter group, those patients most likely to fail therapy had, in addition, structural chromosomal abnormalities. Pseudo-diploid subtypes were also shown to be associated with a poorer (intermediate) prognosis (Look *et al*, 1985), and may be attributed to the presence of translocations involving chromosomes 1,8,9,11,14,19,22, including the t(1;19), t(4;11), and

t(9;22) abnormalities (Secker-Walker *et al*, 1989; Williams *et al*, 1986). The association of a poorer prognosis in children with hypodiploid ALL was reported by Pui *et al*, (1987). Further characterisation of aneuploidy in childhood ALL has included the poor prognostic groups with near haploid/hypodiploid DNA content (with less than 45 chromosomes) (Pui *et al*,1990b), and the near-triploid or tetraploid group (Pui *et al*, 1990c). Recently Pui *et al*, (1991a) has reported hyperdiploidy as the best indicator of event-free survival the first 2,5 years of continuing therapy in childhood ALL.

In adult ALL and AML, a relative paucity of studies of DNA aneuploidy by flow cytometry exists, especially when compared to the childhood ALL group. Hiddeman *et al* (1986) reported that aneuploidy in acute leukaemia was seen as often in adults as in children. A higher aneuploidy rate was recorded in adult ALL than in adult AML, but identified as a favourable prognostic factor in adults with AML (Barlogie *et al*, 1977). Andreeff *et al* (1986), however, reported that the presence of an aneuploidy in adult ALL or AML held no significance with regard to response to induction therapy, complete remission, or survival. Clearly, more studies are required to assess the importance of DNA content in adult leukaemias.

The aim of this study was to confirm previous reports of lower S phase percentages in peripheral blood as opposed to bone marrow aspirate samples, and further, to evaluate if any correlation existed between the acute myeloid leukaemia FAB subtype and S phase percentage.

MATERIALS AND METHODS

Patients

Between January and July 1991 bone marrow aspirates and/or peripheral blood specimens from 46 acute leukaemic patients referred from the Johannesburg, Baragwanath and Hillbrow Hospitals for immunophenotypic workup, were studied (Tables 5 and 6). This group included two CML transformation (CMLt) patients. One of the latter patients, with documented evidence of lymphoid transformation (CD10 and CD19 expression), was included in the presentation ALL group. A second patient in the CMLt group was shown to be myeloperoxidase positive, and therefore included in the presentation AML group.

TABLE 5 : LIST OF PATIENTS WITH ACUTE LEUKAEMIA PRESENTING TO THE JOHANNESBURG HOSPITAL BETWEEN FEBRUARY AND JUNE, 1991.

Pt.	*Dx*	*WCC*	*Bl% Pb*	*Bl% Bm*	*%S Pb*	*C%S Pb*	*%S Bm*	*DI Pb*	*DI Bm*
BH	nALL	235.0	83	96	6.5	7.8	9.1	1.13	1.12
LM	cALL	43.9	77	90	0.9	1.2	3.8	1.05	1.04
BM	cALL	117.0	96	95	8.1	8.4	10.8	0.98	1.04
AG	cALL	222.0	95	97	4.3	4.5	10.0	1.06	1.05
LA	cALL	286.0	93	90	7.5	8.1	13.5	1.04	0.98
HR	AMLnoFAB	3.3	86	85	3.2	3.7	3.9	1. 5	1.07
WM	AML M2	36.6	80	84	4.1	5.1	9.3	1.08	1.06
FH	CMLt ALL	57.7	72	89	5.8	8.1	6.8	1.21	1.21
QG	cALLr	33.7	82	72	6.0	7.3	8.2	1.07	1.06
AM	AML M2	6.9	40	90	3.5	8.8	8.6	1.02	1.06
MM	AML M2	23.8	31	70	3.5	11.3	10.8	1.06	1.02
NM	AML M3	6.3	30	90	4.3	14.3	12.5	1.10	1.08
MO	AML M4	12.4	4	90	0.5	12.5	10.8	1.03	1.00
AM	AML M4	12.3	67	87	2.6	3.8	10.8	1.03	1.04
PK	AML M5	4.1	19	42	1.4	7.4	5.3	1.03	1.07
RM	AML M1	7.5	39	86	1.2	3.1	14.0	1.08	1.06
MK	cALL	25.2	69	91	4.2	6.1	19.7	1.10	1.07
MD	cALL	4.5	36	90	2.4	6.7	13.7	1.00	1.02
LL	cALLr	10.1	32	90	7.3	22.8	21.0	0.96	0.93
SB	cALLr	14.3	5	80	0.5	10.0	10.7	1.00	1.03
LK	cALL	7.6	0	96	2.1	-	6.7	1.03	1.30
JP	cALLr	0.5	0	84	O.0	-	8.5	0.90	1.11
AN	cALLr	6.3	0	85	0.3	-	11.7	1.01	1.05
WC	AML M3	1.3	0	90	3.2	-	3.4	1.02	0.99
LG	AMLeMDS	2.8	0	30	0.9	-	3.2	0.98	1.21
HH	AML M5	2.2	0	38	1.1	-	5.3	0.97	1.05
BH	nALLr	19.0	94	98	1.9	2.0	-	1.08	-
PL	T ALL	0.9	20	95	0.9	4.5	-	0.99	-
PD	nALLr	23.2	90	95	2.6	2.8	-	1.07	-
NN	eT ALL	6.1	0	75	-	-	5.8	-	1.14
MR	CMLt AML	8.6	6	95	-	-	16.4	-	1.14
DR	eT ALL	15.1	52	90	-	-	14.3	-	1.00
LR	nALL	4.7	0	94	-	-	3.7	-	1.16
vW	AML M3	0.3	32	95	-	-	7.6	-	1.08
AK	AML M2	-	-	90	-	-	7.6	-	1.04
RM	AML M3	0.4	-	90	-	-	4.6	-	0.91
GS	AML M3	1.0	1	96	7.8	780.0	-	0.95	-
FM	AML M3	40.8	30	90	8.5	28.3	-	0.97	-
JB	AML M5	43.0	81	97	5.5	6.8	-	1.09	-
HL	AML	106.0	78	59	1.4	1.8	-	1.10	-
MM	AML	39.7	52	35	11.7	22.5	-	0.99	-
JW	cALL	59.9	88	90	2.3	2.6	-	1.16	-
DW	cALL	37.9	74	90	6.5	8.8	-	1.04	-
AN	cALL	7.5	99	97	2.1	2.1	-	1.10	-
KD	cALLr	41.8	62	85	2.2	3.5	-	1.01	-
SP	cALLr	200.0	90	98	15.9	17.7	-	1.15	-

Table 5 abbreviations

%S = S phase percentage, C%S = S phase percentage corrected according to Pb blast percentage, DI = DNA index, Pb = peripheral blood, Bm = bone marrow, Bl = blasts. See text for further details.

TABLE 6: DETAILS OF PATIENT SAMPLES. Details of type of sample received in 46 patients with acute leukaemia

GROUP	Paired	Pb only	BMA only	TOTAL
AML	12	5	3	20
ALL	8	3	3	14
ALLr	5	5	-	10
CMLt	1	0	1	4
TOTAL	26	15	7	48

Pb = peripheral blood, Bm = Bone marrow, CMLt = Blastic Transformation of Chronic Myeloid Leukaemia, ALLr = Acute Lymphoblastic Leukaemia relapse

DNA staining.

Peripheral blood or bone marrow samples were prepared for flow cytometry as previously described (Fried *et al*,1978). For each estimation, 0.5 ml of Propidium Iodide (PI) solution(PI:50ug/ml, 0.1% Triton X100(pfs), and 0.1% Sodium Citrate; obtained from Sigma®) was added to approximately 10^6 cells. Sex matched controls were prepared concurrently for comparison of the patients DNA content, and assessment of background non-specific staining. Flow cytometric analysis followed incubation of the samples at 4°C, in the dark, for 15-20 minutes.

Flow Cytometry.

All specimens were analysed on an EPICS Profile I Flow Cytometer. Daily calibration and alignment of the instrument was performed using standardised beads (DNACheck, Coulter Electronics, Hialeah, Florida). PI stained nuclei were excited at a wavelength of 488nm, at an intensity of 20mW. The red fluorescence was monitored through a 550 LP dichroic filter, and 575 band pass filter. To ensure optimal specimen analysis, flow rate was adjusted to 200-300 events per second. For standardisation, the current (voltage) of the red (FL2) photomultiplier tube (PMT) was adjusted so that the mean channel number (MCN) for nuclei of the normal diploid lymphocytes of control specimens fell in channel 198-202 (of 1023). Vigorous vortexing was performed prior to analysis to disperse doublets, triplets etc, to prevent them from being analysed and contaminating the DNA histogram. If obvious doublets were noted to be present, in spite of vigorous vortexing procedures, preparation and analysis of the sample was repeated. The limits of the G0/G1, S phase and the G_2M populations was set visually by the author, at the points of maximum inflection on the DNA histogram, after collection of all the data (10^3 events).

DNA indices were calculated by dividing the MCN of the patient's G0/G1 population by the MCN of the sex matched control G0/G1 population. The DNA indices can be seen in Table 1, but have not been addressed further in this study.

Statistics and Data Analysis.

Bone marrow aspirates were studied w.r.t. S phase percentage in 33 patients with acute leukaemia. Mean S phase percentages were calculated overall, and within presentation AML (n=16) or ALL (n=12) subgroups. Comparisons between the latter groups, and between presentation(*denovo*) cALL (n=7) and relapsed cALL (n=5) patients were performed. Wilcoxon nonparametric testing was used. Insufficient patient numbers in the T, null ALL, and relapsed AML groups did not permit these groups to be analysed comparatively. In 16 patients with AML, the S phase percentage of the bone marrow (%S Bm) was compared to the FAB subtype (Bennett *et al*,1976) assigned at diagnosis. This latter group included one patient with myeloperoxidase positive transformation of CML. As no FAB classification was assigned at time of blastic transformation, this patient was allocated to the 'unclassified ' group. The numbers where unfortunately too small in each FAB subgroup for statistical analysis, and these data were therefore represented as a dot-plot distribution.

In 9 patients, where matching peripheral blood and bone marrow samples had greater than 70% blasts, the absolute and the corrected peripheral blood S phase percentages were compared with the corresponding bone marrow S phase percentages (%SBm). The S phase percentages of the peripheral blood samples (%SPb) were adjusted according to the blast count, as per routine morphological assessment. This correction was based on the assumption that in acute leukaemia only blasts will contribute to the S phase percentage noted in the blood. No adjustments were made on the bone marrow S phase

percentages; bone marrow samples were considered to be representative of the leukaemic population (Dosik *et al*,1980). The contribution of background normal myelopoiesis to the S phase percentage of acute leukaemias is regarded as being negligible (Clarkson *et al*, 1970a) and suggested to be due to leukaemic marrow inhibition of normal haemopoiesis (Clarkson *et al*,1967). Similar corrections for morphological peripheral blood percentages were made on all other peripheral blood samples studied w.r.t. S phase percentage. These values were not however used in the comparative analysis.

RESULTS

Bone marrow S phase percentages within Acute Leukaemia subgroups.

The mean overall bone marrow S phase percentage for new patients with acute leukaemia was noted at 9.2% (n=28). The mean S phase percentages of AML and ALL groups noted at 8.4 ± S.D. 4.0 (n=16) and 9.8 ± S.D. 4.8 (n=12) respectively. No significant differences of mean bone marrow S phase percentage was noted between these latter groups (p=0.4711), or between patients with presentation (*denovo*) cALL (mean=11.2% ± S.D. 5.2) and relapsed cALL (mean=12.0% ± S.D. 5.2) (p=0.9353). In spite of small numbers, an apparent trend of decreasing bone marrow S phase percentage means was observed, in order of B, T and null ALL immunophenotypes. All data shown is listed in Table 7.

TABLE 7 : Mean Bone Marrow S Phase percentages in AML and ALL.

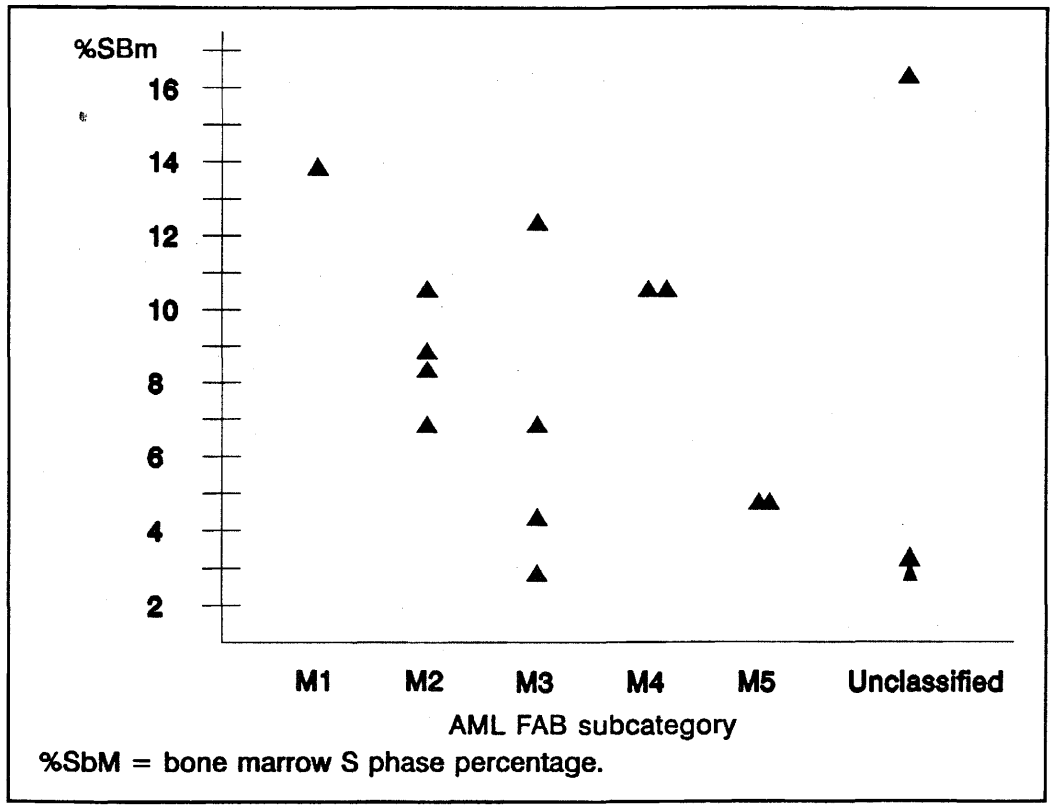
Diagnosis	N	mean %SBm
Presentation Acute Leukaemia	28	9.2
Presentation AML	16	8.4
Presentation ALL	12	9.8
i. cALL	7	11.2
ii. T ALL	2	10.1
iii. nALL	2	6.4
Relapsed cALL	5	12.0
TOTAL	33	10.1

Abbreviations: AML=acute myeloid leukaemia, ALL=acute lymphoid leukaemia, cALL= common ALL, T ALL= T cell ALL, nALL= null ALL, %SBm= S phase percentage of the bone marrow.

AML FAB subtype versus bone marrow S phase percentage.

Study of 16 patients with newly diagnosed AML revealed an interesting clustering of S phase percentages within AML FAB subgroups (Figure 4).

FIGURE 4 Bone marrow S phase percentage versus acute myeloid leukaemia FAB subtype



Comparison of peripheral blood and bone marrow S phase percentages.

Lower S phase percentages were found in peripheral blood than in the bone marrow samples, on comparison of both absolute ($p=0.004$) and corrected ($p=0.021$) peripheral blood values (Table 8). The levels of significance were not appreciably altered after the exclusion of one relapsed case ($p=0.023$). Patients with less than 70% peripheral blood blasts were not included in the comparative analysis of peripheral blood versus bone marrow but nevertheless had corrections performed as previously described. Amongst these latter patients, 6 of 11 patients with recorded blast values of between 1 and 70% blasts, had higher corrected S phase peripheral blood percentages than the S phase values obtained for the bone marrow. In addition, 5 of 6 patients had S phase percentages noted but peripheral blood morphological blast estimations of zero. One patient (GS), was found to have an absolute peripheral blood S phase percentage of 7.8%, and a morphologically assessed blast count of 1%.

TABLE 8: Comparison of peripheral blood and bone marrow S phase percentages in patients with acute leukaemia

Number of patients	Pb x + S.D.	p value	Bm x + S.D.
9 (all patients)	5.2 ± 2.4 **6.0 ± 2.5***	0.004 **0.021***	8.4 ± 3.2
8 (presentation patients)	5.1 ± 2.4 **5.9 ± 2.6***	0.008 **0.023***	8.4 ± 3.4

* **Bold figures** indicate values after correction for peripheral blood blast percentage.

DISCUSSION

The observation of peripheral blood S phase percentages in patients with few or no peripheral blood blasts was an unanticipated but nevertheless, important finding. This data suggests an overall tendency to underestimate peripheral blood blast percentages by morphological assessment alone. Morphology is subjective, even in the eyes of the most experienced reviewer, and is often perceived as being an actual and true account of the peripheral blood blast number by both haematologists (with laboratory insights) and clinicians. Objective assessment of a patient's peripheral blood blast percentage by measurement DNA ploidy (S phase fraction), can help to eliminate some of the problems associated with subjective morphological analysis. Although flow cytometry is beyond the reach of many small routine haematology laboratories, at least in academic centres where flow cytometry is available, this facility can be used to distinguish cycling/blast cells by measuring S phase percentages in routine samples. Regarded then as a complimentary aid to morphological assessment in those samples with a high index of suspicion, flow cytometry can be utilised to improve an otherwise subjective and biased service. Further extension of this facility may include improved detection of early relapse or minimal residual disease.

The study of the mean bone marrow S phase percentages was overall unremarkable. The mean values obtained for the acute leukaemia subgroups were similar to previous reports (Dosik *et al*, 1980a), with lower (Andreeff *et*

al, 1980; Hiddeman *et al*, 1982; Murphy *et al*, 1977) and higher values (Holdrinet *et al*, 1983; Riccardi *et al*, 1986) reported.

An interesting clustering of bone marrow S phase percentages with AML FAB subtypes was however noted. Andreeff *et al*, (1986) and Raza *et al*, (1991) have reported no significant differences of bone marrow S phase percentages between AML FAB subtypes. However, Raza *et al*, (1991) reported that AML M0 had significantly higher bone marrow S phase percentages than other FAB subtypes. This latter author also observed that AML M3 appeared to have a lower (but not statistically significant) mean bone marrow S phase fraction than other FAB subtypes. Interestingly, the only patient in this study assigned to the M1 category, had a much higher bone marrow S phase percentage (14.3%) than the mean for the AML group (7.6%). Clearly, further investigation is required to investigate this apparent clustering of S phase fractions within AML FAB subtypes, which, if real, would add an interesting biological dimension to the AML FAB classification. Significantly lower S phase percentages were noted in the peripheral blood than the corresponding bone marrow samples. Although these findings confirm previous reports (Mauer and Fisher,1962; Holdrinet *et al*,1983), this data has been refuted by others (Cheung *et al*, 1972). Several biological explanations for the discrepancy between peripheral blood and bone marrow S phase percentages of acute leukaemias were outlined by Mauer and Fisher (1962). These authors suggest that lower S phase percentages may be attributed to the circulation of preferential resting cycle compartments, with

loss of environmental growth factors in the peripheral blood compartment. Alternatively, circulation of relatively more mature cells may occur *i.e.* some leukaemic cells may lose their ability to divide after several generations, resulting in a population of nonproliferating blast cells which then leave the bone marrow and circulate in the blood. Differences in generation time of heterogeneous groups within the same acute leukaemic population, may also contribute to differences noted between S phase fractions in peripheral blood and bone marrow compartments (Clarkson *et al*, 1972a; Raza *et al*, 1990). The relationship of the latter to loss of homing receptors in leukaemic cell populations is unknown. Selective homing of haemopoietic progenitor cells to bone marrow is reported as being mediated by receptors (C-type lectins) with galactosyl/ mannosyl specificities capable of binding and interacting with specific configurations of membrane glycoconjugates on haemopoietic stromal cells (Aizawa and Tavassoli, 1987). Loss, or at least alteration of homing receptor molecules on some leukaemic blast cells, may result in inefficient binding to bone marrow stromal cells (Hardy *et al*, 1990) and allow dissemination of these blasts into the peripheral blood, comparable to metastatic phenomena noted in solid tumours. Activation of homing receptors may initiate events of differentiation and maturation in normal bone marrow development (Hardy *et al*, 1990), with gradual loss of homing receptors as blood cells mature (Matsuoka *et al*, 1989). Therefore, if these observations are applicable to blast populations, the blasts with greater homing receptor losses may have less proliferative capacities. The latter would also most likely be the cells to leave the bone marrow compartment;

hence possibly contributing toward the lower S phase percentages noted in the peripheral blood blast compartment. Further study is therefore required in this regard.

In summary, this study has confirmed that lower S phase percentages are noted in peripheral blood blast than bone marrow compartments in patients with acute leukaemia. Differences of S phase fractions in acute myeloid leukaemia FAB subtypes, however requires further investigation. A tendency to underestimate peripheral blood blast percentages by morphological assessment alone was noted. DNA ploidy analysis by flow cytometry (specifically S phase percentage) is therefore recommended as an adjunct to routine differential assessment of blast percentages.

In FINAL CONCLUSION, although the value of immunophenotyping is well established, flow cytometry has contributed considerably toward the latter by enabling measurement of different aspects of antigen expression. Not only is flow cytometry easily practised and more objective, it has added interesting new dimensions and given refreshing alternative direction to diagnostic leukaemia immunophenotyping. Parameters of a diagnostic or prognostic nature, previously not recognised, or difficult to assess adequately with manual fluorescence microscopy, have became evident with the use of flow cytometry. Low density antigen expression, specifically CD10 outlined in this study, is one such example. Although CD10 expression has long been recognised as an important prognostic and diagnostic parameter, flow cytometry has enabled the detection of a subpopulation within the group designated as cALL. In addition to the latter, other subgroups of acute leukaemia are better recognised by flow cytometry. Acute leukaemias in which there is aberrant expression of antigens, not specific to the lineage of the leukaemia in question, are better recognised. Multiparameter flow cytometric analysis allows direct evidence of co-expression of antigens, through utilisation of the facility of dual or three colour fluorescence. Although these entities were previously recognised, long and tedious sample preparation with two or three colour fluorescence techniques negated the positive impact of monitoring aberrant antigen expression in leukaemias. Positive identification of biphenotypic acute leukaemia subtypes furthers confirms the value of flow cytometric immunophenotyping.

DNA ploidy analysis has however, played a lesser, but nonetheless, important role in the immediate identification of leukaemia subtypes. The detection of aneuploidy by flow cytometry, especially in the context of childhood ALL, has been well reported. Hyperdiploidy in cALL has been firmly established as an important prognostic factor (Look *et al*, 1985). Recently, Pui *et al*, (1991a) reported hyperdiploidy in cALL as the best indicator of eventfree survival in the first 2,5 years of continuing therapy. The value of S phase evaluation has, however, been less clear. Overall, studies have been conflicting and unreliable. However, the combination of S phase measurement with related parameters *e.g.* bromodeoxyuridine uptake, proliferating nuclear cell antigen measurement (PCNA® Coulter Electronics), or Ki67 (Dako®) etc, would further help to define and improve the enumeration of the S phase measurement.

Flow cytometry continues to evolve, and plays an increasingly important role in understanding the cellular biology of leukaemias. The versatility of flow cytometry to simultaneously detect multiple parameters in a single cell *i.e.* proteins, enzymes, metabolites, oncogene products, DNA or RNA content, etc, ensures an important role in research applications. Compact, user friendly instruments, which allow rapid and accurate analysis, have allowed flow cytometry to enter the routine clinical arena, with wide and varied application.

In summary, Flow Cytometry is a valuable and accessible tool for all modern pathologists.

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