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ENZYMIC CHARACTERIZATIONS OF LEUKOCYTES IN ACUTE LYMPHOCYTIC LEUKEMIA

by

Sandra A. Russo

A dissertation submitted to the Graduate Faculty in Bicmedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1988

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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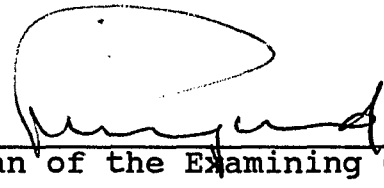

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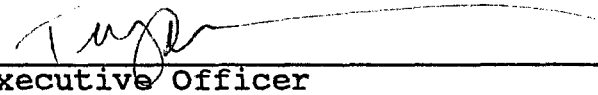

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The City University of New York
1988

Abstract

ENZYMIC CHARACTERIZATIONS OF LEUKOCYTES
IN ACUTE LYMPHOCYTIC LEUKEMIA

by

Sandra A. Russo

Advisor: Professor Olga Greengard

Thymidine kinase (TK), the proportion of its isozymes (TK1, TK2), and γ-glutamyltranspeptidase (GGT) were studied in peripheral white blood cells and bone marrow from children with acute lymphocytic leukemia (ALL). Of the 47 cases, 43 were classified as early pre-B cell ALL. The blood lymphoid fraction of ALL (as compared to normal) subjects showed significant elevations in the activity of TK, and in the ratio of its isozymes 1 and 2. The GGT activity of the same homogenates was significantly below normal, and this was also revealed in whole cells assayed with a fluorimetric method we adapted for use in flow cytometric analysis and sorting of leukocytes on the basis of GGT content.

During chemotherapy induced remission, the enzyme concentrations in ALL blood lymphocytes (as well as bone marrow) became normalized, and changed again toward the untreated values during relapse. The proportion of TK isozymes followed a similar pattern, but TK (and GGT) activities were more sensitive indicators of disease state. Over the untreated and relapsed population, the number of lymphocytes as well as of blasts per ml blood correlated

significantly with the lymphocyte fraction's TK and (in the opposite sense) GGT activity per mg protein. Elevated TK and subnormal GGT was also exhibited, however, by the blast free preparation from several relapsed or untreated patients, indicated the presence (to varying extents) of a subpopulation of maldifferentiated or functional defective (but morphologically normal) lymphocytes. Incubation of ALL lymphocytes with mitogens resulted in blast formation, and in variable (often striking) increases of TK as well as thymidine incorporation, but (unlike in lymphocytes from normal subjects) PWM failed to stimulate GGT activity. This enzyme was also diminished in the granulocytes (and bone marrow) of ALL subjects; in one third of the cases it was 10-20% of normal.

The results of this investigation indicate that quantification of GGT and TK in blood leukocytes 1) detects functional abnormalities in lymphocytes that are not evident by microscopic examination, 2) reveals disease heterogeneity in subjects with the same ALL (as judged by antigen typing) and similar hematological characteristics, and 3) contributes objective criteria to monitoring the treatment efficacy and predicting relapse.

ACKNOWLEDGEMENTS

Many individuals helped make my graduate training a fruitful and pleasurable experience. Among these, my deepest appreciation is reserved for my advisor, Dr. Olga Greengard, who provided scientific advice, a comfortable space in which to work, and most importantly a genuine concern for my development as a person and a professional scientist. For her helpful criticism, thoughtful advice and sincere friendship, I shall always be grateful.

I also wish to thank Dr. Michael B. Harris for his collaboration and stimulating advice during the course of this thesis. Special thanks go to Dr. Oya Tugal for continuation of the project, Hanna Gungor for cytological determinations, Daniel Petrovich, Umran Gungor and the pediatric oncology nursing staff for their willing cooperation in providing patient data and samples.

Dr. Jonathan F. Head's friendship coupled with his patience and accuracy in teaching me laboratory techniques and procedures, made my work not only more productive, but enjoyable as well. For his and my fellow graduate students', Ruth Mc Chesney and Steven C. Bailey, helpful conversation, moral support and sympathetic ears, I am especially appreciative. In addition, I would like to thank Fan Yeen Liu, for her tolerance and pleasant disposition, which made it easy for me to burden her with the monotonous task of putting my data into the computer during her summer stay.

To the Biochemistry Department and the members of my defense committee, I offer my appreciation for their scientific training, constructive criticisms, comments and guidance.

Finally, to my parents and sister, I express my love and special thanks for their encouragement, help and everlasting vote of confidence. Their support was taken for granted, but not forgotten.

This work was supported by Research USPHS Grant CA25005 from the National Cancer Institute.

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ABBREVIATIONS

acP.....acid phosphatase
ADA.....adenosine deaminase
ALL.....acute lymphocytic leukemia
AML.....acute myelogenous leukemia
ATP.....adenosine triphosphate
cALLA.....common ALL antigen
CLL.....chronic lymphocytic leukemia
CML.....chronic myelogenous leukemia
CNS.....central nervous system
Con A.....concanavalin A
CTP.....cytidine triphosphate
dCTP.....deoxycytidine triphosphate
DMF.....dimethylformamide
ER.....E-rosettes
Est.....esterase
FAB.....French-American-British
FACS.....fluorescence-activated cell sorter
GGT..... γ-glutamyltranspeptidase
Hex.....hexosaminidase
hr.....hour
HuTLA.....human T cell antigen
Ia.....Ia-like antigen
Ig.....immunoglobulin
LDH.....lactate dehydrogenase
LSM.....lymphocyte separation medium
M.....molar
MC.....mammary carcinoma
min.....minutes
ml.....milliliter
mM.....millimolar
MNA.....4-methoxy-2-naphthylamide
5'-N.....5'-nucleotidase
NHL.....non-Hodgkin's lymphoma
nmole.....nanomole
NSA.....5-nitrosalicylaldehyde
PAS.....periodic acid-schiff
PHA.....phytohemagglutinin
pmole.....picomole
PWM.....pokeweed
RBC.....red blood cell
SAS.....statistical analysis system
TCA.....trichloroacetic acid
TdT.....terminal deoxynucleotidyl transferase
Tinc.....tritiated thymidine incorporation
TK.....thymidine kinase
UBL.....undifferentiated B-cell lymphoma
ug.....microgram
WBC.....white blood cell

CHAPTER I

INTRODUCTION

In 1845, Rudolf Virchow described a disease which he first called leukemia. Virchow suggested a disturbed regulation of maturation as the underlying pathophysiologic mechanism of the disease. By 1889, acute and chronic leukemia were recognized as two separate entities (Ebstein, 1889). In acute leukemia, the cells are less differentiated than in its chronic counterpart and, if untreated, it follows a rapidly fatal course. In 1900, Naegeli described the myeloblast cell and separated acute leukemia into two distinct categories: myelocytic and lymphocytic.

Acute lymphocytic leukemia (ALL) is a hematopoietic malignancy involving progressive infiltration and replacement of normal bone marrow and lymphatic tissue by an abnormal lymphoid precursor (Williams, 1983). Immunologic, enzymatic, and molecular investigations of human leukemias support the notion of unicellular (monoclonal) origin and an apparent maturation arrest or uncoupling of proliferation and differentiation (Korsmeyer et al., 1983; Greaves and Janossy, 1978). It is thought that leukemic cells are genetically more unstable than normal hematopoietic cells and that new subpopulations of leukemic cells result from clonal evolution. These subpopulations may have different biological characteristics than the original clone (Murphy, 1985).

ALL has a peak incidence between 2 and 10 years of age. It is the most common malignant disorder in childhood and the principal cause of death due to disease in the

pediatric age group (Cutler and Young, 1975). A peak frequency of ALL at about age 4, emerged in Great Britian in the 1920s, in the United States in the 1940s, in Japan in the 1960s, but has not been reported in the People's Republic of China (Miller, 1978). It has been suggested that the difference in the time of appearance of these peaks are due to environmental influences, perhaps related to differences in the rate of industrialization of these societies and thus to variation in the time of exposure to enviromental leukemogens (Miller, 1979).

The possibility of true leukemic clusters (i.e., the appearance within a given locale and time period of a greater number of leukemia cases than would be anticipated from the overall incidence of disease) is of obvious epidemiological interest, since this would suggest a common etiological factor and perhaps horizontal transmission of an infectious agent, such as a virus (Evatt et al., 1973). However, despite intense interest, most studies have failed to demonstrate statistically significant clustering in childhood leukemia (Smith, 1978). An obvious exception was the high incidence of leukemia observed in Hiroshima and Nagasaki as a consequence of radiation exposure (Miller, 1979).

ALL is more common in males than in females; the sex ratio (male:female) rises gradually after the first year and progressively increases through puberty (Gunz and Henderson, 1983). A higher incidence of ALL has been

observed in whites than in nonwhites (Court Brown, 1961).

The onset of ALL is nearly always acute. Symptoms have not been present ordinarily for more than a few weeks before diagnosis (Williams, 1983). The most common complaints include malaise, fatigue, weight loss, pallor, easy bruising or bleeding, moderate lymphadenopathy, splenomegaly and hepatomegaly, bone pain and joint swelling. Blood leukocyte count is usually increased in ALL, but in about one-third of patients the total count is normal or low. The absolute neutrophil and platelet count in the blood is almost always decreased and anemia is usually present at the time of diagnosis (Williams, 1983).

Progress in the therapy of ALL has been rapid and gratifying during the past three decades. The first description of an effective chemotherapeutic agent was by Farber et al., in 1948. The subsequent decade can be characterized as one in which several other effective agents were introduced and the important concept of combining agents to increase the proportion of patients responding was developed (Frei, 1965). Perhaps the single most important event of the 1960s was the recognition that the central nervous system (CNS) acted as a chemotherapeutic sanctuary for leukemic lymphoblasts (Aur et al., 1971). With the addition of a phase of treatment by means of cranial irradiation and intrathecal methotrexate in the middle 1960s, the proportion of patients alive and without evidence of disease 5 years

after diagnosis approached the 50% mark.

Thus, as discussed by George et al., 1979, it became increasingly important to design treatment strategies for curable ALL that would reduce the risks of the consequences of both the disease itself and therapy. Among the important problems are risk of central nervous system damage by CNS treatment as described by Price and Jamieson (1975) and the finding of intellectual impairment in some long-term survivors of treatment (Eiser and Lansdown, 1977).

Classification of ALL

The detrimental impact that certain clinical features at diagnosis, such as high white blood count, mediastinal mass, early central nervous system (CNS) leukemia, and age under two or over ten, have relationship to the expected clinical course of the disease was recognized in the 1970s (Simone, 1975). An important laboratory observation was added to these clinical risk features by Sen and Borella (1975). They found that leukemic lymphoblasts in a group of clinically high-risk ALL patients formed rosettes with sheep erythrocytes in a manner characteristic of normal thymic lymphocytes. This observation was the beginning of the biologically oriented classification for ALL.

The French-American-British (FAB) scheme for the subclassification of ALL, proposed by Bennet, et al., in 1976, was based upon the cellular morphology of lymphoblasts that they termed L1, L2, and L3. The L1

morphology makes up about 85 percent of ALL cases. The cells of these patients are fairly uniform in size and relatively small, the diameter no more than twice that of a normal lymphocyte. The appearance of nuclear chromatin ranges among patients from being finely dispersed to irregularly clumped but is homogeneous in a given patient. Nuclear shape is regular, but indentation, clefting or folding may be present. The nucleolus is inconspicuous, and cytoplasm is scanty and less basophilic than in L2 or L3.

L2 refers to patients in whom most lymphoblasts are more than twice the size of a normal small lymphocyte. Heterogeneous cell size in a given patient is the rule as are gross irregularities of nuclear shape, with frequent indentations, clefting and folding. Nucleoli are variable in number, as is the degree of basophilia. Abundant and deeply basophilic cytoplasm is common. L2 morphology make up 15 percent of ALL cases.

The rare Burkitt cell ALL, a B-cell variant, presents with larger lymphoblasts, usually with deeply basophilic, heavily vacuolated cytoplasm, and represent the L3 type of morphology.

The phenotypic subtypes of ALL can be predictive of clinical response, for example, remission duration. They also provide a rational explantation of much of the clinical and hematological heterogeneity of this disease; variation in growth rates, tumor burden, compromise of

marrow function and age-associated prognosis have been correlated with phenotypic subtypes (Sen and Borella, 1975; Chan et al., 1985). The routine multiparameter examination of leukemic cell phenotype, therefore, provides a practical contribution to accurate diagnosis.

Data from a number of sources indicate that there is significant heterogeneity in cellular phenotype when ALL cells are studied using immunologic markers. This heterogeneity is in part a reflection of the fact that the malignancy involves lymphoid cells that appear to be arrested at various stages of maturation along the differentiative pathways of T or B lymphocytes (Kersey et al., 1979). Immunologic studies (Netzel et al., 1979; Thiel et al., 1980) using antisera against common ALL antigen (cALLA), Ia-like antigen (Ia), human T cell antigen (HuTLA), the ability to form E-rosettes (E), and the fluoresce membrane Ig (cytoplasmic immunoglobulin, CyIgM and surface bound immunoglobulin, SmIg) are used to classify ALL into common ALL (cALLA+, Ia+), common T cell ALL (cALLA+, HuTLA+), pre-T-ALL (cALLA-, HuTLA+, E-), T-ALL (HuTLA+, E+), pre-B ALL (cALLA+, Ia+, CyIgM+) and B cell ALL (smIg+,Ia+). The main leukemic phenotype and the corresponding normal precursor cell types are shown in Figure I-1 - see section on Enzyme Markers in ALL for discussion of TdT (Hoffbrand et al., 1981).

Definite survival advantage following therapy is seen in the cALL group compared to T-ALL which shows a shorter

FIGURE I-1

THE MAIN LEUKEMIC PHENOTYPES AND THE CORRESPONDING NORMAL PRECUSOR CELL TYPES

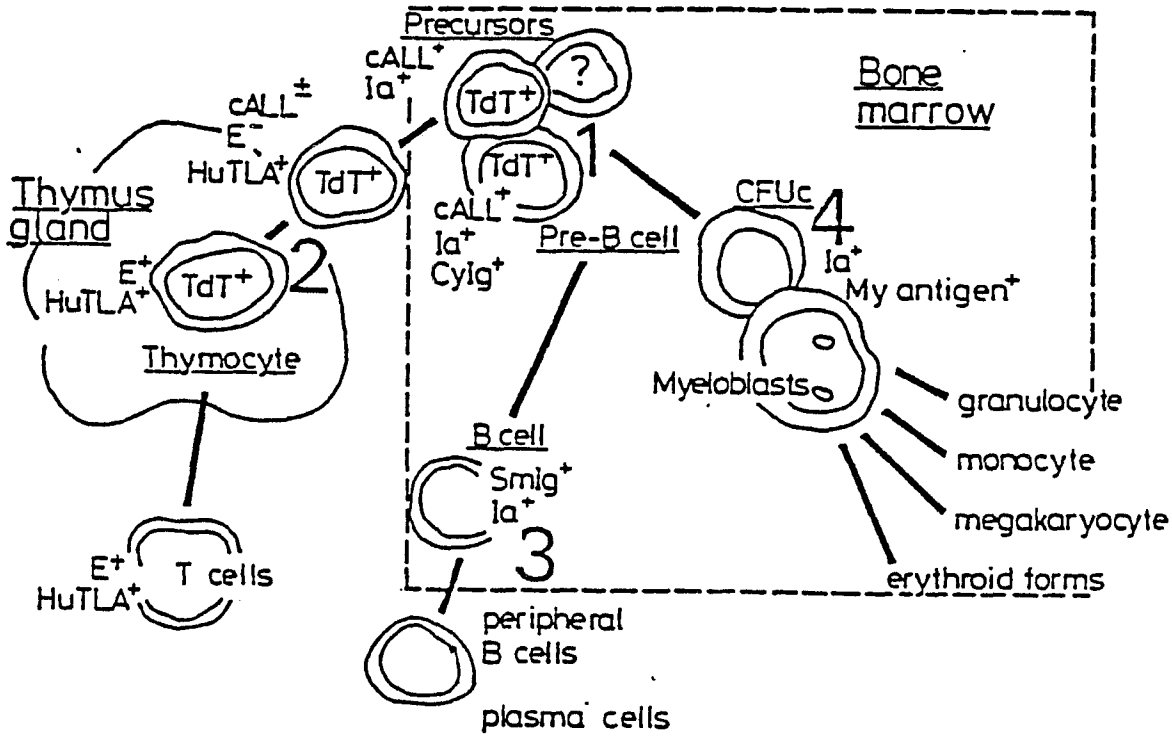
1 Common ALL and pre-B ALL; both are cALLA+, Ia+, TdT+, SmIg-, HuTLA-. In addition, pre-B ALL blast cells have cytoplasmic IgM. Note that normal TdT+ pre-B cells (CyIgM+) are extremely rare while approximately 30% of common ALL cases are pre-B (TdT+, CyIgM+).

2 Two forms of thymic ALL; both are HuTLA+, TdT+, Ia-, SmIg-. The rare "early" forms are E- and weakly cALLA+; the typical Thy-ALL is E+, cALLA-.

3 B-ALL: SmIg+, Ia+, TdT-, E-, HuTLA-, cALLA-.

4 AML: Ia+, TdT-, E-, HuTLA-, SmIg-, cALLA-.

? The phenotypic characteristics and leukemic equivalents of the human pre-lymphoid, pre-myeloid, pluripotential stem cells are unknown.



remission time than cALL and pre-B ALL group (the rare B-ALL has the worst prognosis) (Thiel et al., 1980); T-ALL also presents with a number of clinical and hematological features (e.g., male sex bias, high white blood count) that are known to be indicators of poor prognosis (Pinkel, 1979). There appears to be a clear correlation between immunologic subtype and clinical response, however at present time these immunologic subtypes of ALL add no further insight into the therapy of ALL (Foon et al., 1980).

There still exists an overlap between cases of cALL positive ALL and the subgroup of pre-B and T-cell ALL. Greaves, et al., 1981, reported that approximately 25 percent of the cALL positive group have more than 10 percent lymphoblasts which are identifiable as pre-B cells. Koresmeyer, et al., 1981, demonstrated than even though most leukemic non-T, non-B, cALL positive cells have not yet acquired the capacity to synthesize detectable intracytoplasmic immunoglobulin, they have undergone immunoglobulin gene rearrangements that may already commit them to B cell development.

The antigenic components of the leukemic cell phenotype used as discriminatory markers, while having a selective association with particular hematopoietic lineages or maturation compartments, are not necessarily cell type-specific. No convincing reports of leukemia-specific antigens in ALL or other lymphoid neoplasms in

humans have been shown; those that appeared to be possibly leukemia/lymphoma specific, such as the Burkitt lymphoma glycolipid antigen (Wiels, 1982), have usually lost their candidature once appropriate normal tissues were analyzed (Greaves, 1982). The common ALL associated antigen, though extensively applied as a valuable diagnostic marker (Greaves et al., 1983), is present on kidney tubules and some other nonlymphoid cells (Metzgar et al., 1981), and several other antigens expressed in leukemic subsets are shared with neural tissues or kidney epithelial cells (Kemshead et al., 1982; Platt et al., 1983). Leukemia-associated antigens initially appeared to be maturation stage linked but are now regarded as activation or proliferation associated.

In the human lymphoid lineages there is a reasonably clear, though still incomplete, picture of sequential changes of membrane antigenic phenotype (Reinherz and Schlossman, 1981; Lobach et al., 1985) and of rearrangement and expression of immunoglobulin and T-cell receptor genes (Korsmeyer, 1985) that accompany normal B and T lymphocyte maturation. The dominant antigenic, enzymatic, and molecular profile of T-cells corresponds to cortical thymocytes or to the proliferating blast cell populations in the subcapsular thymic cortex. The latter correspond to no more than 2% of the total cell population of a pediatric thymus. Similarly, in normal bone marrow 1 to 3% of the population of lymphoid cells have nuclear terminal

deoxytransferase (TdT) and the same monoclonal antibody cell surface characteristics as the common (B-cell precursor) form of ALL. These latter, rare cell populations are present at higher relative frequencies in fetal tissue and regenerating marrow (Bonati et al., 1984; Greaves et al., 1983). The selective expression of antigenic determinants (enzymes and other markers) in ALL and other lymphoid malignancies is therefore primarily determined by lineage of origin, maturation status and proliferative activity.

The definitions of ALL subclasses have gone through an evolutionary process depending on the available markers (Table I-1) (Kamps and Humphrey, 1985). Conventional markers, such as sheep erythrocyte rosetting (E) and surface membrane immunoglobulin (SmIg) expression used for the classification of the disease into T-ALL, B-ALL, and non-T, non-B-ALL, were supplemented in the 1970s with heteroantisera. These are being rapidly supplanted by monoclonal antibodies produced with the hybridoma technic (Greaves et al., 1980). Due to the exquisite specificity of monoclonal antibodies, new subsets of normal and abnormal T- and B-cells were found that aided in more precise definition of the stages of their differentiation. Examples of such monoclonal antibodies are OKT3, OKT4 and OKT5, reactive with all normal human T cells, a subset of helper T cells, and a subset of suppressor T cells, respectively; and OKI1, an anti-Ia-like antibody reactive

TABLE I-1

THE EVOLUTION OF SUBGROUPS OF ACUTE LYMPHOCYTIC LEUKEMIA

Generation (Approx Time)	1st (early 1970s)	2nd (mid 1970s)	3rd (early 1980s)	4th (from 1982)
Methods	sIg, E_{EAC}	Heteroantisera	Monoclonal Antibodies	Gene Rearrangement
Subgroups of ALL	B	B	B	—
	Non-T Non-B	Null	HLA-DR$^+$	Non Pre-B, T, B
		Pre-B	Pre-B	Precursor B
		Common	Various	—
			Subgroups	Precursor B
	T	Pre-T	Prothymocytic	
			Early-thymocytic	—
		T	Mid-thymocytic	
			Late-thymocytic	—

with all B cells, but not with resting T-cells (Reinherz and Schlossman, 1980).

Chromosomal Abnormalities in ALL

Confirmation of the role of genetic factors in the etiology of ALL is based on four major observations (Sandberg, 1980); the occurrence of familial leukemia, the high incidence of leukemia in identical twins, the increased susceptibility to leukemia of patients with certain chromosomal syndromes, and the presence of karyotypic abnormalities in patients with ALL.

Individuals with trisomy 21 (Down's syndrome) have approximately 15 times the risk of developing ALL than persons in the general population (Miller, 1970). The occurrence of ALL in patients with Down's syndrome may reflect the fact that their unstable genome is more susceptible to the effects of extrinsic leukemogenic agents. An increased incidence of ALL has also been documented in Bloom's syndrome (Miller, 1968) and Fanconi's anemia (Sasaki and Tonomura, 1973), two rare, recessively transmitted chromosomal fragility disorders.

About half the patients with ALL have lymphoblasts with aneoploid or pseudodiploid chromosomal abnormalities at diagnosis. A number of specific chromosomal subgroups of ALL have been recognized including Philadelphia chromosome, Ph1+ (22q-) ALL, translocation (4;11) ALL, deletions of the long arm of chromosome 6 (6q-) ALL and 8 (8q-) ALL, and 14q+ ALL (Sandberg, 1980). Some of these

karyotypic subgroups appear to have distinct clinical features and response to treatment. In a recent large study of 136 patients, chromosome number was shown in multivariate analysis to be the strongest single predictor of outcome and to be the only variable that added significant prognostic information to leukocyte count (Williams et al., 1982). Children in the hyperdiploid (more than 50 chromosomes) category had the best response to treatment, and those in the pseudodiploid category had the poorest. Abnormalities of the DNA content of leukemic cells can be assessed by flow cytometry. The degree of DNA content abnormality detected by flow cytometry is correlated with the number of whole chromosome gains (hyperdiploidy) or losses (hypodiploidy) (Look et al., 1982).

Several studies have recently correlated karyotype with immunologic phenotype (Bloomfield et al., 1981; Williams et al., 1982). All cases of B-ALL so far reported had abnormalities (8q- or 14q+), compared with 68% of non-T, non-B-ALL and only 45% of T-ALL. The most common chromosomal abnormality in non-T, non-B-ALL is a Philadelphia chromosome (Ph1), most commonly t(9;22)(q34;q11). The next most commonly occurring abnormality is the translocation t(4;11)(q21;q23). ALL with a modal number >50 is usually restricted to non-T, non-B-ALL, accounting for as many as 20% of cases of this condition, and appears to carry an usually good prognosis

(Williams et al., 1982).

A frequent, yet poorly characterized chromosomal abnormality in ALL (Oshimura and Sandberg, 1976) is represented by an apparently heterogeneous group of deletions of part of the long arm of chromosome 6 (6q-). The c-myb proto-oncogene has also been localized on chromosome 6 (Dalla-Favera et al., 1982). c-myb is involved in amplification which, as shown for c-myc (Barletta et al., 1987) and c-abl (Collins and Groudine, 1983) occasionally accompany nearby chromosomal recombinations. In view of the apparent hematopoiesis-specific expression of this gene, the possible relationship between the 6q- abnormality found in hematopoietic neoplasms and the c-myb gene is particularly intriguing; c-myb messenger RNA (mRNA) has primarily been found in hematopoietic cells, where relatively high levels are detectable in immature myeloid and lymphoid precursors (Gonda et al.,1982). Expression of c-myb is induced in proliferating immature hematopoietic cells (Westin et al., 1982) and suppressed in terminally differentiated cells (Craig and Bloch, 1984). This suggested that the myb gene may be involved in the control of proliferation and/or differentiation of hematopoietic cells. The high constitutive expression of this gene in ALL may be involved in malignant transformation by promoting the continuous proliferation of these cells and preventing further progression along the differentiation pathway.

These alterations and other genetic events not involving chromosomal changes visible by karyotyping, for example, mutations in ras genes (Bos et al., 1985) seem likely to be involved at various stages in the multistep evolution of lymphoid malignancy.

Enzyme Markers in ALL

Besides immunologic subclassification, enzyme marker analysis has become an important tool in biological leukemia research. Diagnostic accuracy can be increased when reagents to membrane antigens are used in combination with each other or with antisera detecting enzymes. Most enzyme studies of leukemic cells are based either on histochemical demonstration or on quantitative biochemical analysis of total enzyme activity. In addition to the analysis of the levels of several enzymes in leukemic cells, the qualitative analysis of isozyme patterns has reflected subtle differences between cell types and between stages of cell maturation. The identification of these normal gene products that are consistently and selectively expressed on subtypes of leukemia has led to their utilization as direct targets in new therapeutic strategies.

The most extensively investigated enzymes are terminal deoxynucleotidyl transferase (TdT), 5'-nucleotidase (5'-N), adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), lactate dehydrogenase (LDH), hexosaminidase (Hex), acid phosphatase (acP) and esterase (Est).

Various cytochemical stains have also been studied in ALL. The degree of periodic acid-Schiff (PAS) has been suggested to correlate with survival. Patients with higher counts of lymphoblasts containing PAS-positive, coarse granules in blocks (aggregates) had longer remissions (Palmer et al., 1980). The PAS stain is positive in various immunologic ALL subtypes, but most common in cALL (Huhn et al., 1981). Acid phosphatase (AcP) is the cytochemical stain most frequently suggested to discriminate immunologic classes of ALL; it has been reported to be a marker for T-ALL and pre-T-ALL (Huhn et al., 1981). However, positives in the alternate classes prevent a substitution of immunologic markers by cytochemical enzyme stains.

Terminal Deoxynucleotidyl Transferase (TdT)

This enzyme, which has received the greatest recent interest as a marker for leukemia, was first identified in calf thymus (Bollum, 1962). TdT is a polymerizing enzyme that, unlike other DNA polymerases, catalyzes the addition of deoxyribonucleotide triphosphates to the 3'-OH end of poly or deoxyribonucleotide primers without requiring a template (Bollum, 1974). TdT activity can be measured in a population of cells with a quantitative enzyme assay and in individual cells with specific immunofluorescent antibody (Bollum, 1975). The immunofluorescence assay, particularly when used in combination with antisera to surface membrane antigens, has proved a sensitive technique for detecting

lymphoblasts which might otherwise be difficult to recognize.

The exact biological role of this enzyme is presently unknown. TdT activity has been found to be associated with immature and proliferating cells; increased TdT activity has been reported in normal thymocytes (Mc Caffrey et al., 1975) and in most blast cells of cALL (Coleman et al., 1974; Mc Caffrey et al., 1973). Since TdT is found primarily in fetal and infantile thymus, as well as the T-cell region of lymphnodes, it has been suggested that a relationship between TdT activity and the development of immunocompetence of lymphatic cells may exist (Bollum, 1979).

Hoffbrand and co-workers (1977) demonstrated a striking positivity of TdT in cALL and T-ALL blastic cells. (See also Figure I-1, Hoffbrand et al., 1981). In contrast, TdT is absent or present in low levels in B-ALL (Brouet et al., 1979). Thus, high TdT activity in peripheral blood and bone marrow leukocytes is present at initial diagnosis and relapse in almost all cases of T-ALL and cALL, whereas low-to-undetectable activity is found in normal leukocytes and B-ALL. In normal or regenerating bone marrow, no TdT positive cells can be detected, whereas in the marrow of a patient treated for T-ALL even a single TdT positive cell indicates residual leukemia (Hoffbrand and Janossy, 1981). These findings demonstrate that TdT is a useful enzyme marker for differentiating immature blastic

lymphoid cells as well as distinguishing T-ALL and cALL from B-ALL. However, repeated measurements of TdT activity in cells from peripheral blood or bone marrow have not yet proven of value in the management of childhood ALL (Hutton et al., 1982).

Recently, a new test for the detection of TdT, a quantitative solid phase enzyme immunoassay (EIA) has been shown to be 100 times more sensitive than previous tests for enzyme activity, and to have a definite advantage over the immunofluorescence test (Coleman et al., 1985).

5'-Nucleotidase (5'-N)

This enzyme is localized in the external surface of the plasma membrane of many cells, including lymphocytes, and catalyzes the dephosphorylation of 5'-nucleotides to produce the corresponding nucleosides (De Pierre and Karnovsky, 1974). Since 5'-N is surface membrane bound with its active center facing the extracellular space, it is classified as an ectoenzyme. Its precise function *in vivo* is not known, but in cells such as lymphocytes that do not generally synthesize purines *de novo* (Scott, 1962), it is postulated to provide metabolites that can be actively transported into the cell (De Pierre and Karnovsky, 1974). The importance of quantitative differences in 5'-N activity is also unclear.

In recent studies, the activity of this enzyme was found to be greater in peripheral B cells than in peripheral T cells (Thompson et al., 1979), and it was

suggested that the levels of 5'-N might reflect the lineage of specific cell population. Moreover, among T cells, 5'-N has been found to correlate with degree of cell maturity (Blatt et al., 1980). On the basis of measurement of 5'-N activity in normal fibroblasts and their virally transformed malignant counterparts, it has been postulated that decreases in 5'-N activity are related to the transformation process (Raz et al., 1978).

Low 5'-N activity has been consistently found in lymphocytes from patients with chronic lymphocytic leukemia (CLL) (Lopes et al., 1973), B-immunoproliferative disorders (Kramers et al., 1976), in the malignant T cells (Sezary cells) of some patients with T-cell lymphoma and in T-cell ALL (Kramers et al., 1976) and may reflect their immature state. In contrast, suprisingly high 5'-N activities were recorded on ALL blasts with common ALL antigen and in lymphoid blast crisis of chronic myelogenous leukemia (CML).

Several other cell membrane enzymes which serve function in amino acid and sugar transport were examined. Leucine aminopeptidase, maltase, and trehalase were found to be low in T- or non-T,non-B-ALL, AML, and B-CLL compared to normal peripheral blood lymphocytes, whereas they were normal or above normal in Sezary syndrome and T-CLL (Kramers and Catvosky, 1978).

Adenosine Deaminase (ADA)

In addition to 5'-nucleotidase, the partial or

complete absence of two other purine-pathway enzymes, adenosine deaminase and purine nucleoside phosphorylase have been linked with specific immunodeficiencies (Giblett et al., 1972; Giblett et al., 1975). The importance of ADA in lymphoid development has been recognized since the observation that cogenital ADA deficiency was a cause of T-cell deficiency (Giblett et al., 1972). ADA is involved in the degradation and recycling of purines, converting deoxyadenosine and adenosine to deoxyinosine and inosine, respectively (Conway and Cooke, 1939). Although the enzyme is present in most of the body, the highest concentration occurs in thymic cortical cells (Barton et al., 1979) and the concentration is higher in mature T than B cells (Tung et al., 1976). Evaluation of normal lymphoid tissue has shown that intracellular ADA activity reflects both the T-cell or B-cell origin of a cell population as well as its degree of maturation; the ADA activity of leukemic blast cells also appears to correlate with the cells from which they are derived (Blatt et al., 1980).

Initial studies showed raised levels in AML and ALL, while lower levels in CLL than in normal peripheral blood cells were reported (Smyth and Harrap, 1975). More recently, the incidence of raised levels has been shown to be higher in ALL than in AML, the highest level occurring in T-ALL blasts (Coleman et al., 1978). A radioassay in cells or serum, with antibody to ADA has confirmed the high levels in T-ALL blasts. However, the diagnostic value of

the enzyme assay is limited. Although T-ALL blasts tend to show higher ADA and lower TdT concentrations than cALL blast (Coleman et al., 1978), the overlap is too great for reliable distinction. Similarly in the chronic lymphoproliferative disorders, ADA is higher in the T-cell (Sezary's syndrome, T-CLL or T-prolymphocytic leukemia) than in B-cell disorders, but the overlap is again substantial (Prentice et al., 1980).

The observation that deoxycoformycin, an ADA inhibitor, inhibits T-cell proliferation (Smyth et al., 1978), and has striking therapeutic effects in relapsed patients with T-ALL refractory to conventional chemotherapy (Russel et al., 1981), is of considerable importance. The drug action is extremely selective; cALL blasts proving resistant to ADA inhibition.

Purine Nucleoside Phosphorylase (PNP)

PNP acts sequentially with ADA in reversibly converting purine nucleosides, including inosine, guanosine, and deoxy analogues to their corresponding purine bases (Parks and Agarwal, 1972). Partial or complete deficiencies of PNP leads to congenital immunodeficiency with severly defective T-cell functions but normal B-cell function (Giblett et al., 1975). PNP activity has also been reported to be lower in immature T-cells than in normal peripheral-blood T-cells, however, these decreases are not as profound as those noted for 5'-N (Blatt et al., 1980).

Compared to non-T, non-B-ALL and to normal peripheral blood leukocytes, significantly low PNP values were found in T-ALL (Blatt et al., 1980). In contrast, the malignant T lymphocytes of CLL were shown to have PNP content similar to that of normal mature T cells (Borgers et al., 1978).

The relative activities of purine-pathway enzymes and TdT in normal and malignant lymphoid tissue are shown in Table I-2 (Blatt et al., 1980).

Lactate Dehydrogenase (LDH)

LDH is a tetrameric enzyme that, with the cofactor nicotinamide adenine dinucleotide, converts lactate to pyruvate (Dawson et al., 1964). LDH is composed of two subunits, the varied combination of which results in five isozymes, LDH1 through LDH5 which have a spectrum of anaerobic metabolic activities (Dawson et al., 1964). The isozyme patterns of LDH have been found to be characteristic of different tissues (Papadopoulos and Kintzios, 1967) and of certain solid tumors (Wieme et al., 1968).

In normal lymphoid tissue, the intracellular LDH-isozyme pattern has been used to distinguish B cells, which have a high percentage of LDH3 and LDH5, from mature T cells, which have relatively more LDH1. In contrast to mature T cells, thymocytes exhibit a high percentage of LDH3 activity characteristic of immature cells and fetal tissue, thus, LDH isozyme patterns may also reflect their degree of T-cell differentiation (Blatt et al., 1980).

TABLE I-2

RELATIVE ACTIVITIES OF PURINE-PATHWAY ENZYMES AND TdT IN NORMAL AND MALIGNANT LYMPHOID TISSUES

5'N denotes 5'-nucleotidase, ADA adenosine deaminase, PNP purine nucleoside phosphorylase, TdT terminal deoxynucleotidyl transferase, plus signs relative amounts of intracellular enzyme, 0 absent, and ND not done.

CELL TYPE OR SOURCE	5'N	ADA	PNP	TdT
Normal lymphoid cells				
T lymphocytes	++	++ to +++	++	0
B lymphocytes	++ to +++	++	+ to ++	0
Peripheral-blood mononuclear cells	++	++	++	0
Thymocytes	+	++++	+	+
Acute lymphocytic leukemia				
T-cell	+	++++	+	+
Non-T, non-B cell	++	++ to +++	++	+
B-cell	ND	+	ND	0 to +
Chronic leukemia				
Chronic myelogenous leukemia in blast crisis	ND	++ to +++	ND	0 to +
Chronic lymphocytic leukemia	0 to +++	+ to +++	+ to ++	0
Sézary syndrome	+ to ++	++ to +++	+	0
Lymphoma				
Lymphoblastic	ND	ND	ND	+
Burkitt's	++	+	++	0
Diffuse histiocytic	ND	ND	ND	0 to +
Thymoma	ND	ND	ND	+

Hexosaminidase (Hex), Acid Phosphatase (acP) and Esterase (Est)

The first publication on the isozyme pattern of hexosaminidase in leukemic cells was by Ellis et al., 1978, in which they demonstrated by automated ion-exchange chromatography the abnormal expression of one band of intermediated mobility, the so-called hexosaminidase I (Intermediate) in cALL blasts. This isozyme was not seen in T-ALL or B-ALL. This distinctive pattern in cALL blasts has been found to recur at relapse and to disappear with remission, although the absolute amount of hexosaminidase I does not seem to correlate with the number of lymphoblasts in the bone marrow that are positive for cALLA (Ellis et al., 1978). Similar abnormalities of isozymal patterns in cALL have been found in studies of several other lysosomal hydrolases (Besley et al., 1978).

Characteristic acid phosphatase isozyme patterns were also found for T- and cALL (Gaedicke et al., 1979). Although isozyme 3b, which is especially found in embryonic lymphocytes (Li et al., 1970), was the predominant acid phosphatase of ALL lymphoblasts, cALL showed very heterogeneous acid phosphatase isozyme pattern.

Li et al., 1973 also reported the isozyme pattern of nonspecific esterase. As described for the acid phosphatase isozymes, the cAll cases showed polymorphism in their Est isozyme profiles (Gaedicke and Drexler, 1982). Although cALL appeared for a long time to be a homogeneous entity, this leukemic subtype can be divided into subsets

by enzyme marker analysis. In demonstrating different isozyme patterns in cALL cells, these studies support the assumption that various differentiation stages of lymphopoietic development are involved in cALL. Whether these differences in the differentiation stages of ALL cells are of clinical importance remains to be seen.

Thymidine Kinase (TK)

Thymidine kinase (TK), an enzyme of the "salvage" pathway of DNA synthesis, catalyzes the phosphorylation of thymidine to thymidine 5'-monophosphate (Okazaki and Kornberg, 1964) an essential precursor of DNA-thymidine. This activity is very low in normal tissues but increases in many proliferating animal tissues such as fetal liver (Kemper and Haynes, 1968), fetal lung (Greengard et al., 1980) tumor tissues (Bulkobsky and Roth, 1965; Greengard and Herzfeld, 1977; Greengard et al., 1982), and regenerating liver (Nakai et al., 1966).

Changes in activities of enzymes in pyrimidine metabolism have received little attention. CLL with its slow proliferative rate is characterized by relatively low TK activity (Nakai et al., 1966), while elevated TK levels are found in ALL (Hartje and Wilman 1980; Vertongen et al., 1984).

Previous reports have shown the existence of several forms of TK which posses different physical and biochemical properties (Taylor et al., 1972; Adler and McAuslan, 1974; Ellims et al., 1981). In human cells, the enzyme occurs as

two isozymes designated TK1 and TK2. TK1 migrates more slowly on polyacrylamide electrophoresis than TK2.

The ratio of the enzyme activity with CTP over ATP as the phosphate donor can also be used to discriminate between predominance of TK1 or TK2 isozyme activity (Taylor et al., 1972). The TK1 isoenzyme has a specificity for ATP, while TK2 also uses CTP, with activity reaching 80-90% of that obtained with ATP. A CTP/ATP activity ratio of less than 0.4 is considered to indicate predominance of TK1 activity (Ellims et al., 1981).

Inhibition of TK2 by deoxycytidine triphosphate (dCTP), in contrast to the lack of inhibition of TK1, may be used as a means to distinguish different forms of the enzyme (Taylor et al., 1972). Deoxy-CTP causes only 15-20% inhibition of TK1 activity, but 70-80% inhibition of TK2. Studies (Bresnick and Karjala, 1964) on the nature of d-CTP inhibition of TK have confirmed the "simple" competitive type; the binding of the inhibitor and substrate on the same site of TK. The ability of these catalytic properties to distinguish between TK1 and TK2 isozyme has been confirmed by thymidine-Sepharose affinity chromatography (Ellims et al., 1981).

The specific role of the TK1 isozyme is still unclear. It has been suggested (Taylor et al., 1972) that it could reflect a demand by the cell for maximum nucleotide biosynthesis during a particular stage of cell development. Although TK1 predominates in tumor tissue (Ellims et al.,

1981), Adler and McAuslan (1974) have proposed that the expression of different forms of TK is more a characteristic of the stage of growth or non-growth than a function of transformation. TK1 activity closely parallels changes in cell DNA synthesis, with a marked increase occurring upon transition of the cell from the dormant to the dividing state. The TK1 isozyme, therefore, is thought to play an important regulatory role in cell proliferation, to be rate-limiting in the incorporation of thymidine into DNA, and to exhibit maximum activity during the S phase of the cell cycle (Kit and Leug, 1974). In contrast, the TK2 isozyme is the predominant activity in mature non-proliferating cells and remains relatively constant during the cell cycle.

Ellims, et. al.,(1981) have recently demonstrated for non-Hodgkin's lymphoma (NHL) a correlation between TK isozyme type and cell morphology, in that cell immaturity is associated with the appearance of TK1 isozyme and a stepwise increase in this activity occurs with progressive degrees of cell differentiation. In CLL a change from a clinically indolent to aggressive disease is a well-documented occurrence, and has been associated with the appearance of immature lymphocytes in the peripheral blood (Enno et al., 1979) as well as with enhanced thymidine incorporation by <u>in vitro</u> leukocytes (Moayeri and Sokal, 1979).

γ-Glutamyltranspeptidase (GGT)

GGT catalyses the transfer of the γ-glutamyl compounds to a variety of amino acids and peptides (Meister, 1973; Tate and Meister, 1974). In this context, it functions as part of the glutathione synthesis and degradation cycle, i.e., it catalyzes the transfer of γ-glutamate from glutathione to mono and dipeptide acceptors such as glycylglycine.

GGT is localized on the external surface of the plasma membrane of epithelial cells, such as those of the intestinal mucosa, proximal renal tubules, bile ducts and seminal vesicles that are intimately involved in transport processes and the secretion of specialized body fluids (Meister and Tate, 1976). Novogradsky, et al., 1976, have shown GGT is also present on the external surface of lymphoid cells, which are of mesenchymal origin, indicating that this enzyme enjoys a broader distribution in mammalian cells than previously recognized. The ability of lymphoid cells to interact with γ-glutamyl donor substrates such as S-acetophenone glutathione and L-γ-glutamyl-p-nitroanilide that do not enter the cell further indicates that the active site of the enzyme is on the cell surface. Thus, GGT is classified as an ectoenzyme.

In tissues that are to perform secretory or absorptive functions, the concentration of GGT is low prenatally and rises precipitously during the critical postnatal period of functional maturation. Previous studies (Phares and

Vanderlaan, 1979; Farber, 1984) have shown that GGT activity in the rat liver is elevated in such hepatoproliferative tissues as developing, premalignant and malignant liver. Thus, in fetal or neonatal liver and also hepatocellular carcinomas which exhibit much higher GGT content than the cognate mature organ (Fujiwara et al., 1982), GGT is sometimes referred to as a "marker of undifferentiation." In contrast, its titers in normal fetal, and also neoplastic variant of kidney or pancreas is much lower than in the mature ones (Fujiwara et al., 1982). Thus, in these systems, GGT is looked upon as a "marker of differentiation."

The surface localization of GGT and the fact that it may be readily determined, should make this enzyme useful as a surface marker for normal and neoplastic hematopoietic cells in various stages of differentiation and maturation. In this context, the chronic B-cell disorders, in which the specific activity of GGT is lower than that found in normal lymphocytes (Kramer and Catovsky, 1978), may be examples of immunologically and presumably enzymatically immature cell types (Preud'Homme et al., 1974). In contrast, myeloma cells are highly differentiated B-cells which secrete immunoglobulin (Litwin et al., 1974) and have largely lost their surface membrane immunoglobulin. The greater maturity of the myeloma cells seems to correlate with their elevated levels of cell membrane enzymes as compared with circulating lymphocytes.

In acute leukemia, as in the chronic B-cell disorders, the specific activity of GGT was low as compared with those measured in normal blood lymphocytes (Kramer and Catovsky, 1978). Most of the other studies on acute leukemia have centered on variations in GGT activity to distinguish between lymphoid and nonlymphoid leukemia. In a series of 37 cases of AML, it was shown that only four cases had the low GGT activity seen in ALL (Heumann et al., 1985). This result was confirmed in 29 out of 32 AML and acute mylomonocytic cases (Morell et al., 1986).

Flow Cytometry

Dolbeare, et al., (1977) developed a fluorescent method for the cytochemical localization of cells with GGT activity in which the liberated fluorescent product is coupled with a trapping agent to form an insoluble Schiff-base product. Schiff-base formation does not quench the fluorescence of naphthylamines, but rather shifts the fluorescence to a longer wavelength. The principal advantage that this method offers is the ability to sort cells on the basis of fluorescence, using a fluorescence-activated cell sorter (FACS), and to obtain a purified population of GGT positive cells from an initially heterogeneous cell suspension. However, the method has not been previously applied to normal or leukemic white blood cells.

Fluorescence-activated cell sorters are being used increasingly for both clinical and phenotyping of leukemia

samples (Herzenberg, 1978). Physical and biochemical characteristics on single cells can be analyzed at rates of 10^5 cells per minute with the use of high speed cell sorting systems. Such a technique permits rapid and simultaneous analysis of light scatter (which measures approximate cell size and detects properties of cytoplasmic and nuclear components), protein and other biochemical properties on the same cell with the added option of electronically sorting from a give population particular cells fulfilling a set of preselected criteria. Thus, from a large heterogeneous population a relatively small homogeneous fraction of inconspicuous cells can be numerically enriched and sorted for further biochemical and morphological identification.

In Vitro Studies in Lymphocytes

Intensive studies of the characteristics of cell growth in human acute leukemia began in 1959 with the development of the nuclear label, tritiated thymidine (Hughes, 1959). Two characteristic phases of leukemic blast cell status were proposed for acute childhood leukemia; one actively proliferative and the other nonproliferative.(Maurer and Fisher, 1966; Gavosto et al.,1967) By means of in vivo labeling with tritiated thymidine, the cells in the actively proliferative phase were identified as being large with fine nuclear chromatin, while the cells which could not be demonstrated by these studies to be involved in proliferative activity were

smaller cells with dense coarse nuclear chromatin. The proliferating blast cells divided one or more times in the bone marrow and then became smaller as they apparently stopped further division and joined the nonproliferating blast cell population.

Gavosto et al., (1967) demonstrated that during steady-state conditions the large blast cell population is incapable of maintaining itself because loss of cells to the small blast population is greater than the number of cells produced by cell division. New cells must therefore continually enter the large blast cell compartment to prevent its depletion. The large proliferating cells are thus replaced by the reentry of cells from the small blast cell population (Saunders and Mauer, 1969).

With the aid of flow cytometry, the percentage of cells in the S-phase of the cell cycle can also be assessed. The median percentage of cells in S-phase or the labeling indices were significantly higher for B cell and T cell ALL compared with non-T, non-B ALL in several studies of children (Tsukimoto et al., 1976; Look et al., 1982; Dow et al., 1982).

Dow et al., 1981, found that the greater the initial proliferative activity of the blood and marrow leukemic cell population at diagnosis the greater the likelihood of early relapse. In these patients with greater initial leukemic cell proliferative activity, relapse was not only more likely, but occurred earlier than in patients who did

relapse but whose initial proliferative activity was less.

The induction of cell proliferation in resting tissues by application of appropriate stimuli, provides a helpful model for the study of metabolic events leading to the initiation of DNA synthesis and mitosis and may provide insight into the control of these processes in the cells.

Human peripheral blood lymphocytes are thought to be well differentiated cells at the end stage in lymphoid maturation. They seldom grow or divide *in vivo*. This quiescent behavior is usually maintained when these cells are cultured. However, the addition of phytohemagglutinin, PHA, an extract of the red kidney bean (Phaseolous vulgaris) (Rigas and Osgood, 1955), to lymphocyte cultures caused a striking transformation; as many as 90% of the cells enlarged, synthesized DNA, divided, and assumed the less differentiated appearance of lymphoblasts (Nowell, 1960).

The PHA-induced blastoid cells (Litwin et al., 1974) (enlarged cells with deeply basophilic, nongranular cytoplasm and a large nucleus which contains homogeneously stained chromatin and prominent nucleoli) have some resemblance to normal primitive cells and cells sometimes present in diseases such as acute leukemia (Tanaka et al., 1963). However, such a morphological resemblance does not necessarily mean that these cells have the same origin, function, or potentialities as the blastoid cells formed *in vitro*.

In CLL, the response to PHA is decreased and delayed (Smith et al., 1972), the peripheral WBC shows a negative correlation with the response to PHA (Sharman et al., 1966), and effective chemotherapy restores the normal response pattern (Bouroncle et al., 1969). The reason why patients with poor response to chemotherapy had low in vitro lymphocyte function tests (Green, et al., 1974) may not have been "immunodeficiency," but rather, the fact that the poor-responders tend to have a greater percentage of blasts in the peripheral blood. The question of whether the abnormal mitogenic stimulation in CLL is due to the dilution of a normal lymphocyte population by non-PHA-responsive malignant cells or to an intrinsic lymphocyte abnormality is not resolved at present. It is possible that these patients are truly immunologically abnormal and that their morphologically normal lymphocytes do not respond to PHA (Zusman and Nesbit, 1979).

Whereas lymphocytes from ALL patients in remission responded well to all stimuli tested, lymphoid cells from patients in leukemic stages with at least 80% lymphatic cells in the differential leukocyte count, uniformly displayed strongly reduced or even absent responses (Melief et al., 1973). The PHA response was not only reduced but also delayed. A definite respone was absent in ALL if the peripheral WBC count was very high (Melief et al., 1973; Zusman and Nesbit, 1979). This is in agreement with the findings in CLL. These data indicate that the response of

cells from leukemia stages are due to the presence of a small proportion of residual normal lymphocytes whereas the leukemic cells themselves are unable to respond.

Since the response to PHA in diseases appears to be directly related to the total leukemic cell mass, observations by Zusman and Nesbit (1979) suggest that an elevation of the peripheral WBC in CLL and in ALL does not always mean a greater proliferation of malignant cells. Since it is not possible to differentiate malignant from nonmalignant cells on a morphologic basis in many leukemic cases, the PHA test could be utilized in conjuction with the patient's WBC and the relative percentages of T and B cells to quantitate the leukemic cell mass in this disease.

The induction of replication in nondividing cells requires the synthesis or activation of a number of enzymes functioning in the DNA-synthetic pathway. TK activity has been shown to increase in human lymphocytes during transformation by PHA. Thus, Loeb, et al., (1970) found a 2-10 fold increase in the activity, expressed in relation to the cell number present in culture than before stimulation and the increase coincided with labelled thymidine incorporation into DNA. Pegoraro and Bernengo (1971) reported a 40-fold increase in the activity, expressed in relation to the protein content, but the increase was delayed 12 hours with respect to thymidine incorporation. Peterson and Tyrsted (1977) reported TK activity, expressed in relation to the DNA content,

increased approximately 170-fold in the cells and closely correlated to the increase in the incorporation of (Me-^{3}H)-thymidine into DNA. Their results also demonstrated that the TK of stimulated lymphocytes could be separated into two fractions by DEAE-Sephadex column chromatography, whereas only one enzyme fraction was obtained in normal lymphocytes. It was suggested that the pronounced increase in TK during transformation of the lymphocytes might reflect increased synthesis of the enzyme present or induction of a new enzyme in the resting cells by PHA.

Treatment of human peripheral lymphocytes and rat lymph node lymphocytes with mitogenic agents led to a marked increase in GGT (Novogradsky et al., 1976). Studies of the kinetics of induction of GGT in mitogen-stimulated rat lymphocytes showed that the increase in activity preceded the stimulation of DNA synthesis by 21 hours, a finding in accord with earlier data on protein synthesis in mitogen-stimulated cells (Ling and Kay, 1975), and continued to increase with increased DNA synthesis. It was also noted that because periodate-treatment, which induces blastogenesis (Novogrodsky and Katchaski, 1972), rapidly inactivates lymphocyte GGT, the observed increase in enzyme activity of periodate-treated rat lymphoblasts most probably can be ascribed to <u>de novo</u> synthesis of enzyme.

The quantitation of enzymic response to mitogens may be useful as a prognostic factor and as a nonmorphologic

indicator of the degree of leukemic cell proliferation in the peripheral blood of these patients. Prospective studies on patients with ALL may be worthwhile in order to conclusively establish whether the response to mitogens is useful in predicting a poorer clinical course for some children with this disease.

Recent in vitro experiments using a variety of maturation inducers (phorbol diester TPA, retinoids, butyric acid, DMSO) indicate that the maturation arrest can also be bypassed in some human leukemias (cell lines and fresh cells), entailing more differentiated cell phenotypes. Thus, some T-ALL cell lines can be induced by TPA to irreversibly modulate their composite phenotype from that of an immature or thymic variety to that of a mature T cell subset (Nagasawa and Mark, 1980). Tumor cells of patients with common ALL lacking cytoplasmic IgM, expressed this pre-B marker after in vitro treatment with TPA (Nadler et al., 1982). Cytoplasmic immunoglobulin (CyIgM) synthesis was demonstrated in ALL cells where a genetic B commitment (pre-B cell phenotype) was identified by gene probes before culture (Cossman et al., 1982); the antigenic changes were accompanied by a decrease or slowing in cell cycling in all experiments in which this was investigated. This provides further support for the view that maturation arrest in leukemia may be a reversible regulatory defect which uncouples maturation and proliferation (Sachs, 1981).

CHAPTER II

METHODS

Human Subjects

Peripheral blood and bone marrow from children with ALL and control subjects was collected in heparinized tubes. All patients were children between 3 and 13 years old. Diagnosis was based on clinical, hematologic, cytochemical and immunologic criteria. Patients were subjects of early pre-B, pre-B, B-cell or T-cell ALL.

A total of 47 ALL patients were investigated. The 27 untreated patients refer to children in the first leukemic phase before any therapy had been started. Treated patients refer to children in remission with chemotherapy. Treatment consisted of combination chemotherapy of vincistrine, L-asparaginase, 6-mercaptopurine, methotrexate and prednisone. Treated patients with 25% or more blast cells in their bone marrow were considered relapsed. In the 10 relapsed patients examined, blood was collected before intensive chemotherapy was resumed. Remission blood samples were drawn immediately before administration of drugs. Control samples were obtained from healthy volunteers, and from off-treatment ALL children who, in remission for at least six months, were considered cured.

Examination of bone marrow is a mandatory procedure for establishing a definite diagnosis and is routinely used to confirm relapse, since the circulation is often devoid of recognizable blasts or of abnormal WBC count. Bone marrow samples (before treatment and at the time of routine examination during remission or relapse) were collected

simultaneously with the blood samples. Under most circumstances, the amount of bone marrow aspirates obtained are just enough for diagnosis, however, in several cases the amount was sufficient to use a portion of it for enzyme analysis.

Normal and Tumor-Bearing Rats

Mammary carcinoma 5A (MC) was carried by sequential subcutaneously transplantation in adult male rats of the Fischer strain. Blood from normal and tumor-bearing rats was collected in heparinized tubes. Bone marrow was obtained by squeezing through each well-cleaned femur 10 ml of 0.9% NaCl solution using a 22-gauge needle.

Separation of Leukocytes

Blood lymphocytes and granulocytes were obtained by a method similar to that of Boyum (1968). Whole blood was mixed with 2 volumes of 1.5% Dextran T-500 (Pharmacia Fine Chemical AB, Uppsala, Sweden) in 0.9% NaCl solution (w/v) and allowed to stand for 30 minutes at room temperature in a centrifuge tube. RBCs settled to the bottom of the tube, while the leukocytes remained in the upper Dextran layer. In studies where only a leukocyte fraction was desired, this upper layer was carefully collected, diluted with the same volume of 0.9% NaCl solution, and centrifuged. Remaining RBCs were lysed with 20 ml of 0.2% NaCl solution and gently vortexed; twenty ml of 1.8% NaCl was added to restore osmolality and the suspension centrifuged.

Sedimented WBCs were resuspended in 0.9% NaCl solution. In other studies, in which a lymphocyte and granulocyte fraction was needed, the upper Dextran layer was placed carefully on top of 15 ml of Lymphocyte Separation Medium, LSM, (Bionetics Laboratory Products) and centrifuged at 2,000 rpms for 30 minutes at room temperature in a swinging bucket rotor of an International centrifuge. Pasteur pipets were then used to collect the upper layer consisting mostly of lymphocytes, which were washed with 0.9% NaCl and resuspended. In the lower layer, enriched with granulocytes, contaminating RBCs were lysed as indicated above and the cells were centrifuged down and resuspended in 0.9% NaCl solution.

The purity of the lymphocyte and granulocyte fraction was examined in cytospin preparations fixed in 95% ethanol and 5% glacial acetic acid and stained with Diff-Quik staining solutions (American Scientific Products). In the lymphocyte fractions, 70-90% of the cells were found to be lymphocytes, while the granulocyte fractions were 90-100% pure.

In some cases, further separation of the lymphocyte fraction using a discontinuous percoll gradient similar to that described by Ulmer and Flad (1979) resulted in a blast and lymphocyte fraction with only 5-10% cross contamination.

Lymphocyte and granulocyte enriched fractions from bone marrow were obtained by the method described above for

blood. Rat bone marrow cells suspended in 0.9% NaCl (10 ml/femur) were centrifuged for 10 minutes, washed twice with 0.9% NaCl and the sedimented cells were resuspended in 0.9% NaCl.

Thymidine Kinase (TK) Assay

The assay of TK activity in cell free preparations, was based on that of Machovich and Greengard (1972). With the optimal substrate and cofactor concentrations used, (0.01mM thymidine, 5mM ATP, 2.5mM $MgCl_2$) the rate of product formation was constant and proportional to the amount of enzyme added. The results (after subtracting enzyme free, incubated blanks) are expressed in units (pmoles of thymidine phosphorylated per min.) per mg protein. Protein was determined by the method of Lowry et al., 1951) using bovine serum albumin as the standard.

The proportion of TK isozymes 1 and 2 was examined by two routinely used methods (Ellims et al., 1981; Taylor et al., 1972). One involved measurement of activity with CTP as well as ATP as the phosphate donor and taking the ratio of the two measurements. The other method consisted of determining the percent inhibition of activity by dCTP (in amount equimolor to ATP).

γ-Glutamyltranspeptidase (GGT) Assay

The methods of Seymour and Peters (1977) with minor modifications (Koss and Greengard, 1982) were used for the quantification of GGT in cell homogenated in the presence

of Triton X-100. The assay mixture was buffered with 71mM diethanolamine (pH 8.5) and contained 1mM concentration of the substrate, γ-glutamyl-2-naphthylamide. The fluorescence of the liberated 2-naphthylamine (from the glutamylnaphthylamide using glycylglycine as the acceptor) was measured in a Perkin-Elmer spectrofluorimeter with an excitation wavelength of 340 nm and an emission wavelength of 410 nm. The results are expressed as units (nmole per min.) per mg protein or per million cells.

Statistical Analysis

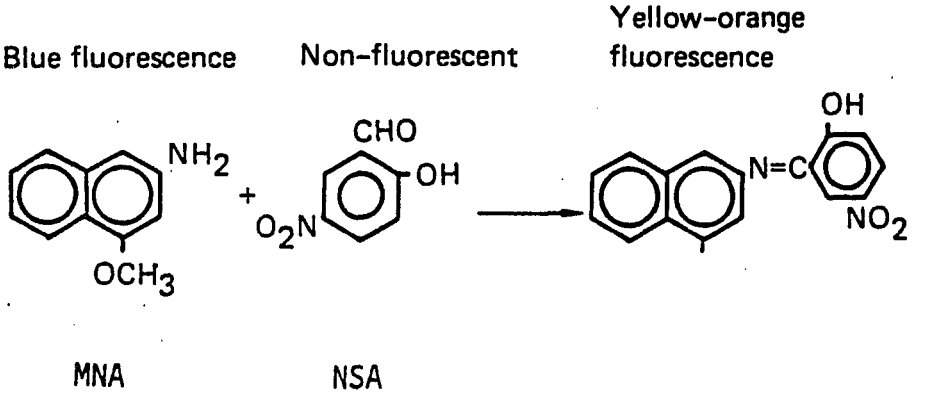
Statistical analyses (t-test, Pearson parametric and Spearman rank correlations, and linear regressions) of the data were done using a Stastical Analysis System (SAS) package.

Fluorescent Detection of γ-Glutamyltranspeptidase (GGT) in Intact Cells

In intact cells, GGT fluorescence was accomplished under similar conditions as Vanderlaan, Cutter, and Dolbeare (1979). The enzyme substrate, γ-glutamyl-4-methoxy-2-naphthylamide (γ-glu-MNA) (Sigma Chemical Co.), was dissolved in a small volume of 1:1 dimethylformamide (DMF) and 1N HCl. The MNA released by GGT was coupled to 5-nitrosalicylaldehyde (NSA) (Eastman Kodak Co., Rochester, NY) to form a Schiff-base product (Figure II-1, Dolbeare and Smith, 1977); NSA was dissolved in DMF. In all cases the final concentration of DMF in the assay medium was kept below 3%. The standard assay mixture contained 1mM γ-glu-

FIGURE II-1

REACTION OF MNA AND NSA TO FORM A SCHIFF-BASE COMPLEX



MNA, 1mM NSA, 0.1M Tris (hydroxymethyl)amino methane, pH=8.0, 80mM glycylglycine, 60mM NaCl, and approximately a million cells per ml; it was incubated for 0-60 minutes at 37° C.

A Zeiss Universal fluorescence microscope with a mercury arc lamp was equipped with an additonal emission filter (590 nm long pass filter) which eliminates the non-specific green fluorescence of the NSA trapping agent without significantly reducing the yellow orange fluorescence of the MNA-NSA product. Figure II-2, shows the excitation (Curve A) and emisson (Curve C) spectra of hepatoma (HTC) cells incubated with either NSA alone or γ-glu-MNA and NSA (Vanderlaan et al., 1979). Curve B, the emission spectra of NSA, indicates that cells incubated with NSA alone should show a nonspecific green fluorescence, while Curve C, the emission spectra of MNA-NSA product shows that cells with GGT activity should fluoresce yellow-orange. Kodak Tri-X pan, ASA 400 film and Ektachrome 400 slides were used for photography.

Flow cytometric analysis and sorting was carried out with a fluorescence-activated cell sorter (FACS 440) (Becton-Dickinson Co., Mountain View, CA) equipped with a 4 watt argon ion laser (Model 164-05, Spectra-Physics Co., Mountain View, CA). A similar multisensor cell separator system (Steinkamp et al., 1974) is diagrammed in Figure II-3. Processed signals were stored in a Becton-Dickinson Consort 40 Computer. These signals can be used

FIGURE II-2

EXCITATION AND EMISSION SPECTRA OF CELLS INCUBATED WITH EITHER NSA ALONE OF γ-GLU-MNA AND NSA

The excitation spectra of cells incubated with NSA alone or with substrate and NSA were identical (Curve A). Curve B is the emission spectrum of cells incubated with NSA alone, while Curve C is the emission spectrum obtained from a single cell filled with MNA-NSA crystals after incubation with γ-glu-MNA and NSA.

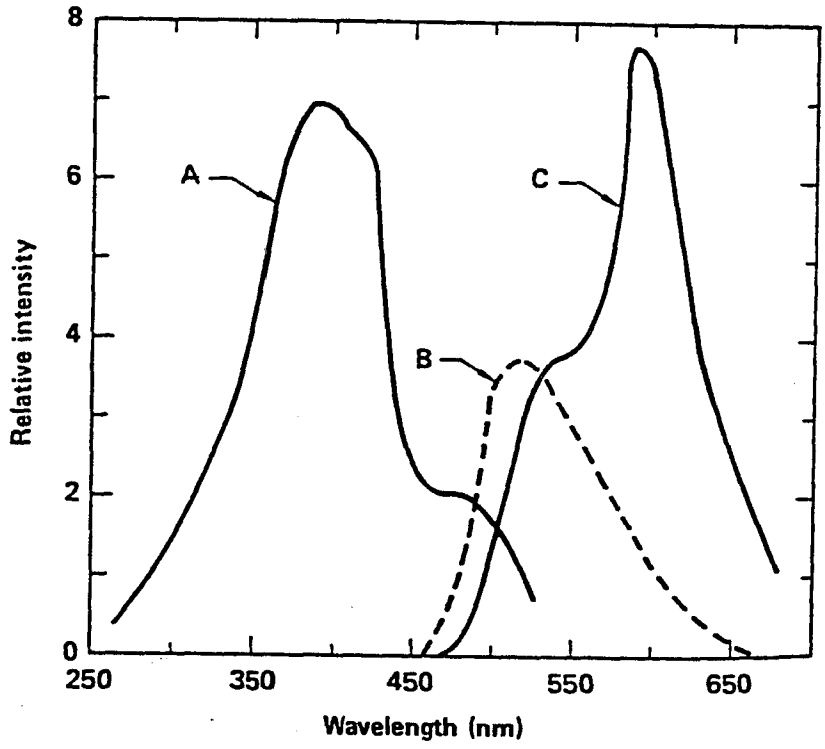
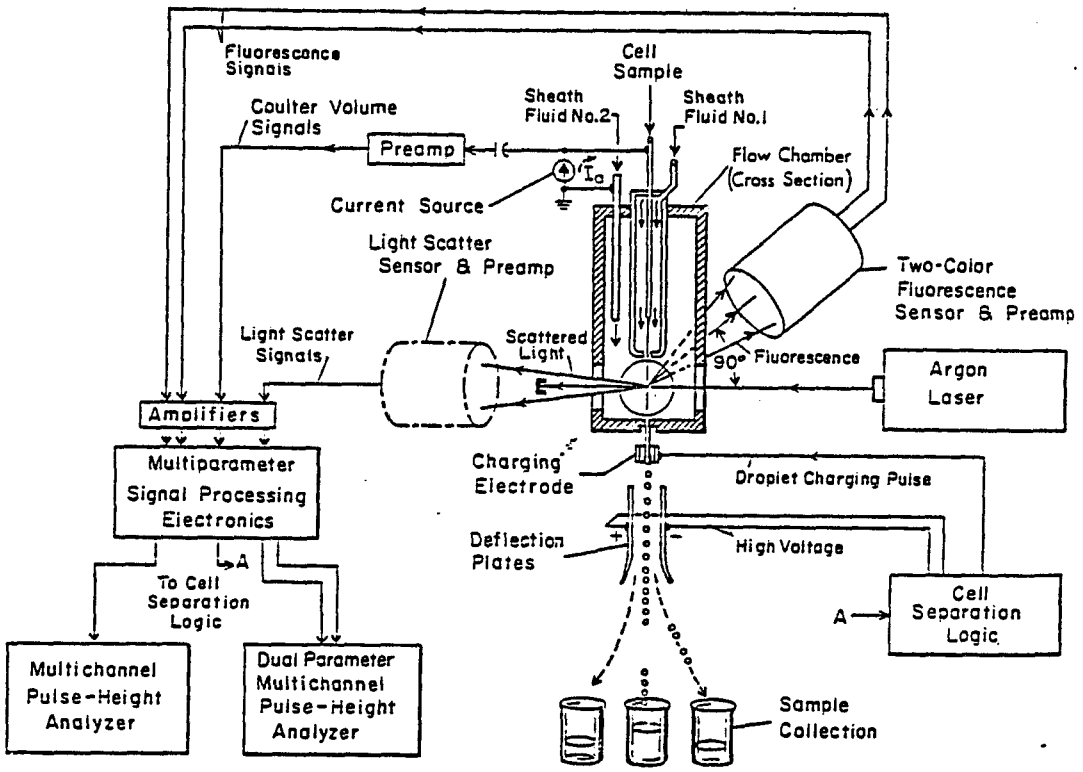


FIGURE II-3

CELL SEPARATOR SYSTEM

Multisensor cell separator system illustrating flow chamber, laser illumination, cell sensors, signal processing and cell separation electronics, and droplet charging and deflection scheme.



to activate cell sorting according to selected criteria by electrically charging droplets containing cells and electrostatically deflecting them into collection vessels. The GGT fluorochrome was excited at 457 nm and emission was filter through a 575/26 (575±26) nm band pass filter. Low angle and 90 degree light scatter were also measured.

Mitogen Studies

Human peripheral blood lymphocytes were separated using sterile LSM and subsequently cultured in the absence and presence of mitogen as descibed previously (Petersen et al., 1973). Lymphocyte suspensions from ALL patients were used only when the differential white blood count contained 50% or more non-blastic lymphocytes. Pokeweed mitogen (PWM) and Concanavalin A mitogen (Con A) reactivity were determined by culturing the cells in the presence of 10 ug/ml of each; the concentration of mitogen selected yielded maximal stimulation as determined in each case by a dose-response curve.

Normal and ALL lymphocytes suspensions were adjusted to a concentration of $1x10^6$ cells per ml. Cells were cultured in RPMI 1640 medium (Gibco Laboratories) containing glutamine (2 umoles/liter), 5% fetal calf serum (heat inactivated) and supplemented with penicilin (100 units/ml) and streptomycin (100 ug/ml). Cultures were incubated at 37^0 in an atmosphere of 95% air and 5% CO_2 for varying periods up to 6 days. Cells were then washed two times with 0.9% NaCl and counted using a hemocytometer.

Aliquots were then taken for enzyme assays, and also for cytospin slide preparation (stained with Diff-Quik solutions) in order to determine the % blastic cells.

In the thymidine incorporation studies, 1uCi/ml of (^{3}H)thymidine was present for the last 6 hours of incubation in the presence (or absence) of mitogen. The washed cells (Stiles et al., 1972) were then treated with 5 and 10% trichloroacetic acid (TCA). The precipitate, after washing with cold methanol, was dissolved in 1 ml of 0.1M NaOH and scintillation fluid, and counted in a Isocap/300 liquid scintillation counter. The results are expressed as radioactivity (cpm) incorporated into TCA-insoluble material per million cells.

CHAPTER III

LYMPHOCYTE THYMIDINE KINASE AND TREATMENT RESPONSE IN ACUTE LYMPHOCYTIC LEUKEMIA

INTRODUCTION

Evidence that quantification of TK in cancer tissues can provide information about features of the disease that may not be revealed by microscopic pathology came initially from studies on solid neoplasms of non-hematopoietic origin. In rat tumors with common histogenesis (e.g. among hepatic, mammary, or renal carcinomas) TK concentration was found to vary in parallel with growth rate in vivo and with loss of functional differentiation (Knox, 1976; Weber, 1977; Machovich and Greengard, 1972). It also paralleled these criteria of clinical malignancy in human colonic carcinomas (Weber et al., 1981; Herzfeld and Greengard, 1980), and in human lung neoplasms the radiologically determined volume doubling time (which is an established, but not routinely available, predictor of disease outcome (Joseph et al., 1971; Straus et al., 1978)) bore a highly significant quantitative (inverse) correlation to their biopsy samples' TK concentration (Greengard et al., 1985).

According to a 1981 study (Ellims et al., 1981) in lymphoma subjects, the biopsy samples' TK activity provides an objective indicator of the severity of the disease. The hope that in leukemia assays of peripheral leukocytes may suffice, came from the finding of higher TK activity in acute than in chronic lymphocytic leukemia (Eng Gan et al., 1982). This result on 9 ALL cases was confirmed on another 6 cases (Vertongen et al., 1984) . Both studies also showed

that (as in most solid tumors) isozyme 1 predominates in ALL, but whether quantitative variations in this isozyme may be indicative of disease outcome remained unknown, because neither the TK1 nor the TK titers have been compared with hematological or clinical data on the same individuals. Such a comparison was the main objective of the present investigation. We show that lymphocyte TK activity (per mg protein) correlates significantly with the number (per ml blood) of WBCs and lymphocytes as well as with the number of blasts. The proportion of isozyme 1, though it followed the same pattern, appears to be a less reliable discriminator between the individuals' disease state than the total TK activity. The latter rose consistently when treatment was no longer effective and, since this was also true for cases whose blood was devoid of morphologically recognizable blasts, the level of this enzyme in lymphocytes may constitute a sensitive indicator of relapse. The finding that lymphocyte TK activity remains uniformly low during successful ALL therapy is also of interest, since information about such an effect of chemotherapy has hitherto been restricted to a study of chronic myelocytic leukemia in which higher TK activity was found in the lmphocytes of 7 untreated cases than in 8 treated ones (Nakai et al., 1966).

RESULTS

Thymidine kinase (TK) activity (per mg protein) in the lymphocyte fraction of untreated ALL children (Table III-1, column 1) was generally high but extended into the normal range (see end of Table III-1) and the difference between the two means (31.5±8.9 and 7.0±0.7, respectively) failed to reach statistical significance ($p<.058$). The variability among the untreated cases (the reason for which will be discussed later) contrasts strikingly with the uniformly low TK level of lymphocyte samples from 17 patients obtained during chemotherapy. The latter values were within the normal range, and the mean, 5.3, was significantly different ($p<.02$) from the untreated mean, 31.5. This decrease in TK activity is also apparent from comparing pre- and post-treatment results on the same individuals (see first 6 cases in Table III-1). In 4 of these 6 subjects chemotherapy reduced the TK activity from 212.5 - 10.8 to below 8; however, in cases C.P. and S.N., where the pre-treatment values were low (even lower than in most control subjects) chemotherapy caused no further decrease.

The mean value for the 10 relapsed subjects (24.8) was almost 5 times higher ($p<.004$) than the mean of the 17 in the "Treated" column (5.3) (and was also significantly higher ($p<.02$) than the control mean). More importantly, observations on 8 of these cases (see results with the same initials in the "Treated" and "Relapsed" columns) show that

TABLE III-1

THYMIDINE KINASE (TK) ACTIVITY IN THE BLOOD LYMPHOCYTE FRACTION OF ALL SUBJECTS

Untreated				Treated		Relapsed				
Case	TK	WBC	Blast	Lymph	Case	TK	Case	TK	WBC	Blast	Lymph
SL	212.5	253.0	177.1	55.7	SL	5.0					
JK*	24.9	789.0	789.0	0	JK*	4.4	JK*	55.9	178.0	153.0	10.7
RM	13.3	27.5	4.1	1.4	RM	7.5					
KS	10.8	10.5	6.0	2.9	KS	6.2					
CP	2.9	3.5	1.8	1.6	CP	4.5	CP	7.0	1.8	0	0.6
SN	2.3	3.6	2.5	0.8	SN	9.5					
NL	17.6	9.7	1.4	1.4			NL	28.5	10.4	0.2	2.6
DV	101.0	84.4	67.5	15.2	HA	5.1	HA	81.5	36.2	17.0	9.1
MD	74.6	27.2	14.7	11.7	VF	3.6	VF	30.6	11.6	6.8	3.8
JO	55.7	68.7	42.6	18.6	WZ	6.0	WZ	9.6	3.9	0	3.0
VV	53.4	61.4	46.7	8.6	HS	3.7	HS	8.4	3.4	0	1.8
DH	31.9	48.0	16.3	30.2	SB+	6.3	SB+	8.0	2.6	0	2.1
RB	31.1	4.8	0.5	3.7	MK	5.4	MK	6.7	2.2	0	1.2
MC	30.7	2.9	0	1.9			CR	11.6	3.4	0.7	0.5
JF	19.2	32.6	26.4	3.9	AP	5.3					
CB	17.6	3.9	0	3.7	JA	6.5					
FB	15.6	22.4	0.5	7.8	LS	3.6					
MX	13.3	1.9	0	1.7	SC	3.1					
GG	13.1	21.6	16.2	3.2	VB	4.9					
KP	12.3	9.5	4.9	1.6							
RL	11.5	8.2	3.9	3.6							
JG	8.5	10.7	6.4	4.3							
AW	5.7	2.0	1.3	0.5							
CN	3.9	4.6	0.5	2.4							
AK	3.8	4.3	0	3.2							

	TK, Mean±SE
Untreated	31.5±8.9 (25)
Control	7.0±0.7 (13)
Treated	5.3±0.4 (17)
Relapsed	24.8±8.1 (10)

Patients (for definition of "Treated" and "Relapsed" see Methods) were subjects of early pre-B or pre-B-cell ALL, except where otherwise indicated (*, T-cell; +, B-cell). Columns "WBC", "Blast", and "Lymph", represent the number of WBCs, lymphoblasts, and non-blastic lymphocytes per ml blood $x10^{-6}$ determined by standard hematological methods on one portion of each blood sample. From the remainder of each blood sample, the lymphocyte fraction (containing lymphocytes plus blasts, if any) was separated and TK was assayed; the results are expressed in units per mg protein.

even though the TK activities varied among the different relapsed cases, the value in a given individual was always higher than found in the same individual before relapse. This was true even though no detectable blast cells appeared in the peripheral blood of the majority of relapsed cases, and bone marrow sampling was needed to confirm relapse.

In three cases (J.K., C.P., and N.L.) in which blood samples on the same individual were received at diagnosis and during subsequent relapse, TK activity in the lymphocyte fraction was substantially higher during relapse than at initial diagnosis. In one of these cases (C.P.) particularly low TK activity was observed at the time of diagnosis and it more than doubled at relapse.

It should be noted that TK activity was determined at optimal substrate and ATP concentrations and that assays of admixtures of preparations from different individuals (e.g. one with low and one with high activity) gave additive results; thus, the higher values obtained in untreated and relapsed subjects than in treated ones are not attributable to (endogenous or drug-derived) dissociating activators or inhibitors, but denote differences in the amount, structure, or covalent ligands of the enzyme molecules themselves.

The TK results so far described were obtained with whole lymphocyte fractions (containing blasts and lymphocytes), so that the TK values reported in Table III-1

represent an "average " for the different cells present. There were large individual variations in the number of blasts and lymphocytes (Table III-1). This heterogeneity is a possible explanation for the large Standard Error of the mean TK activity in the untreated ALL groups.

The methods of physically separating blasts from leukemic lymphocytes are notoriously inadequate. However, in some cases it was possible to obtain a blast and a lymphocyte fraction with only 5-10% cross contamination using a discontinuous percoll gradient similar to that described by Ulmer and Flad (1979). It may be seen from Table III-2 that TK activity(per mg protein) was higher in the blast fraction. However, in two out of the three cases the lymphocyte fraction also exhibited elevated TK activity (cf. values 16.1 or 18.9 with the control mean of 7.0 in Table III-1). This was consistent with the similarly high TK activity of the lymphocyte fraction of several patients with no or minimal blast count (Table III-1).

To further examine this question, quantitative comparisons were made between TK activity in the lymphocyte fraction and hematological parameters. The results, based on data under the "Untreated" and "Relapsed" columns in Table III-1, are summarized in the left side of Table III-3. It may be seen that TK activity per mg protein (determined in the fraction containing lymphocytes plus blast) correlated quantitatively with the number of blasts per ml blood ($p < .0009$) but that even higher correlation

TABLE III-2

THYMIDINE KINASE ACTIVITY IN SEPARATED LYMPHOBLAST AND LYMPHOCYTE PREPARATIONS

	Thymidine Kinase Units/mg protein	
Case	Lymphoblasts	Non-Blastic Lymphocytes
1	19.2	6.0
2	31.9	16.1
3	59.4	18.9

The preparations from the blood of 3 untreated ALL cases were obtained as described in the text.

TABLE III-3

CORRELATIONS BETWEEN ENZYMIC AND HEMATOLOGIC PARAMETERS

Number/ml blood	TK Units/mg protein		CTP/ATP Ratio		% Inhibition by dCTP
	(n=35)		(n=28)		(n=14)
	r	p<	r	p<	
Log WBC	0.63	.0001	-0.48	.01	NS
Log Blasts+Lymph	0.63	.0001	-0.51	.005	NS
Log Blasts	0.54	.0009	-0.47	.01	NS
Log Lymphocytes	0.63	.0001	-0.27	NS	NS
Lymphocytes	0.87	.0001	-0.13	NS	NS
	(n=28)				
TK CTP/ATP Ratio	-0.51	.006			

The number of the indicated cells in the whole blood of 35 ALL subjects was compared with the TK activity (per mg protein) of the lymphocyte fraction (containing lymphocytes and blasts, if any) from the same blood samples. In 28 of these cases, TK was also assayed with CTP as the phosphate donor and divided by the activity obtained with ATP ("CTP/ATP Ratio"), and the percent inhibition of TK by dCTP (in amount equimolar to ATP) was determined in 14 cases. The results, referring to correlations between hematologic and enzymic parameters (first 5 lines) or between "CTP/ATP Ratio" and TK (last line), are presented as Pearson parametric correlation coefficients (r) with statistical significance (see p< columns; NS, not significant). The Spearman rank correlations were also significant (p<.0001).

coefficients and lower p values were obtained when comparing this activity with the number of WBCs or lymphocytes, or lymphocytes plus blasts (see also Figure III-1) per ml blood. These Pearson parametric correlations are not attributable to clusters of very high and low values, since the Spearman rank order correlations (not shown) were also significant, $p<.0001$.

The experiments in Table III-4 examine two previously used criteria for evaluating the proportions of isozyme 1 and 2 of TK. Addition of dCTP to the assay system (containing saturating concentrations of ATP) caused a significantly smaller inhibition in lymphocyte fractions of untreated ALL than in control subjects. Since isozyme 1 is insensitive to dCTP (Taylor et al.,1972), this observation is in accord with the previously concluded predominance of isozyme 1 in leukemic cells (Eng Gan et al,1982; Vertongen et al., 1984; Ellims et al., 1981). The percent inhibition by dCTP was significantly greater ($p<.02$) in treated cases than in untreated cases. These results suggest that the treatment has a selective effect and that the decrease it causes in total TK activity shown in Table III-1 is mainly attributable to a decrease in isozyme 1.

Similar conclusions may be drawn from experiments using the other criterion of isozyme distribution (see left side of Table III-4). The ratio of activity with CTP and ATP as the phosphate donor (TK CTP/ATP) in untreated ALL, 0.24, was significantly lower than in control subjects

FIGURE III-1

RELATIONSHIP OF THE NUMBER OF LYMPHOID CELLS PER ML BLOOD TO THE SPECIFIC ACTIVITY OF THYMIDINE KINASE

Blasts plus lymphocytes per ml (abscissa) counted in whole blood of ALL subjects were plotted against TK per mg protein (ordinate) measured in their isolated lymphoid fraction; Pearson correlation coefficient, $r=0.70$, $p<.0001$. Each circle refers to an individual untreated or relapsed ALL patient in Table III-1; square refers to the mean (bar=1SE) for 13 control subjects.

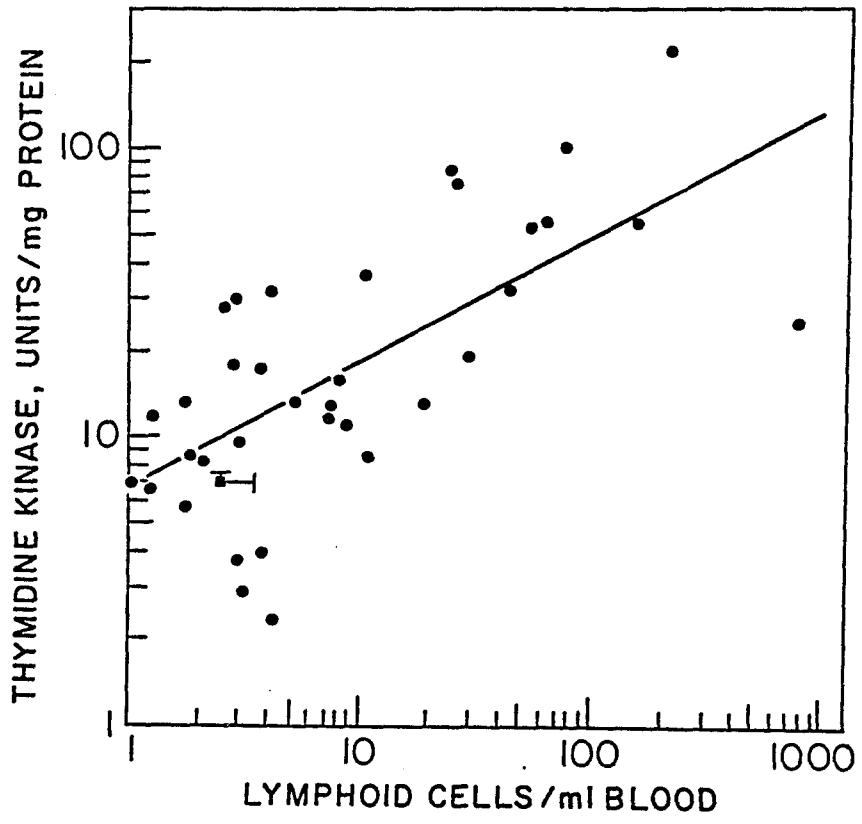


TABLE III-4

THYMIDINE KINASE ISOZYMES IN LYMPHOID CELLS OF ALL SUBJECTS

	TK Activity	
	CTP/ATP Ratio	% Inhibition by dCTP
Control	0.67±0.05 (10)	46.00±8.43 (6)
Untreated ALL	0.24±0.03 (21)	25.77±4.88 (14)
Treated ALL	0.63±0.02 (10)	44.06±2.66 (7)
Relapsed ALL	0.34±0.10 (7)	

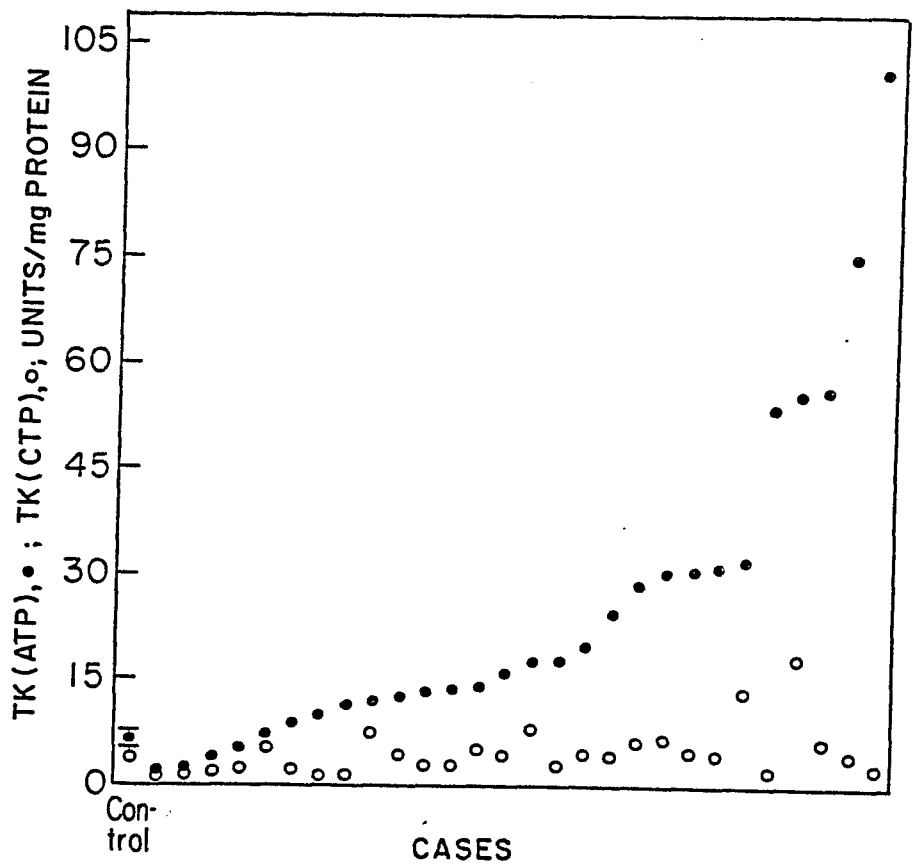
The results, referring to the separated blood lymphocyte fraction (including blasts, if any), are means ±SE with the number of individiuals studied in parentheses. Low TK activity with CTP as compared to ATP as the phosphate donor (see Ratios), and low percent inhibition of the latter activity by dCTP, indicate relatively high TK isozyme 1 proportion.

(0.67) and below the value of 0.4 which is considered to denote predominance of isozyme 1 (9). Again, treatment abolished this predominance, raising the ratio to 0.63 (i.e., towards the control value of 0.67). During relapse this ratio reverted to the low value of 0.34. Table III-3 shows (last line) that this ratio correlated with subject to subject variation in TK (i.e., activity with ATP), however, the isozyme ratio (last 2 columns) showed less close or no correlation with hematological parameters in the same subjects than did TK. It is also evident from the comparison of individual ALL subjects in Figure III-2 that activity with CTP encompasses a relatively narrow range and that (as explained under Discussion) the concentration of TK is a more sensitive indicator of hematological differences between individual ALL subjects than is its isozyme pattern.

FIGURE III-2

TK ACTIVITIES WITH ATP OR CTP AS THE PHOSPHATE DONOR

The results refer to the same lymphocyte fractions of 28 (untreated and relapsed) ALL patients and 10 control subjects as Table 4. For each patient, listed in the order of increasing TK activity with ATP (●) , activity with CTP as the phosphate donor (O) is shown at the same abscissa point. Points with SE bar denote the mean of these two activities for control subjects.



DISCUSSION

The number of ALL subjects in this study made it possible to examine for the first time whether there is a consistent parallelism between lymphocyte TK activity and any hematological parameter known to be indicative of the severity of disease. The single most important risk factor in ALL, the number of WBC per ml blood (Mauer et al.,1982), was found to bear a highly significant correlation to TK activity per mg protein in the lymphocyte fraction. Blast count was a somewhat less close (though also statistically significant) correlate than WBC or lymphocyte count. Individual variations in TK activity could only in part be attributed to differences in the number of blasts cells present in the fraction assayed. One indication of this was the much higher than normal activity in several subjects with no or minimal blast count (see Table III-1). Also, while the results on blood samples from three individuals permitting clean separation were in accord with the well known enrichment of blasts with TK, the (blast-free) lymphocyte fraction of two of these cases (Table III-2) showed higher than normal TK activity (Table III-1). Therefore, it seems that measurement of this enzyme also may reveal the existence of maldifferentiated lymphocytes or of small blasts which are not recognizable as such by microscopic examination. Temporarily non-dividing small blasts (which replenish the leukemic bone marrows proliferative compartment (Gavosto et al.,1964; Saunders

and Mauer, 1969) are likely candidates; they are present in the circulation of some ALLs (Mauer and Murphy, 1979) and, being derived from large blasts, they may still be relatively rich in TK.

Chemotherapy reduced the lymphocyte TK activity to uniformly low levels; the mean for 17 cases, 5.3±0.4, was similar to the normal, control mean of 7.0±0.7. However, when treatment was no longer effective, the TK units rose to a mean of 24.8. Bone marrow samples are routinely used to confirm relapse since the circulation is often devoid of recognizable blasts or of abnormal WBC count. This was indeed so in several of the present patients. Nevertheless, each of these patients (as well as those with blasts in their blood) showed higher lymphocyte TK activity than they did during remission (see results with the same initial in the "Treated" and "Relapsed" columns in Table III-1). Thus, while the bone marrow probably exhibits even greater elevation in TK, its assay in the peripheral cells (if compared with values during remission of the same individuals) appears to be sufficient to confirm relapse. This sensitivity suggests that measurement of TK may predict relapse before suspected on clinical or hematological grounds.

It has been a consistent observation in ALL that the growth potential of the bone marrow cells at relapse is greater than at the time of diagnosis (Mauer et al., 1982). The present results indicate that this is reflected by the

peripheral lymphocytes' TK activity : it was higher at relapse than at first diagnosis in each of the subjects studied at both times (see initials J.K., C.P., and N.L. in Table III-1).

Previous investigations have indicated that the predominance of TK isozyme 1 may provide a clearer distinction of leukemic from non-leukemic subjects. The present results are in harmony with this, and also demonstrate that the decrease in TK during chemotherapy is attributable to the loss of isozyme 1. Thus, even though the two parameters paralelled one another in untreated CLL (Ellims et al., 1981) as well as in the present ALL cases, the isozyme proportion may conceivably have a somewhat better ability to reveal salient differences between individual subjects of the same type of leukemia. However, in ALL at least, this is not so. As seen from Table III-3, none of the hematological criteria correlated significantly with percent inhibition by dCTP, lymphocyte count failed to show significant parallelism to the ratio of activity with CTP over ATP, and the number of WBC or blasts per ml blood showed a less close correlation to this ratio than to TK activity (per mg protein). The nature of the isozyme determination methods is one reason for this: both methods are based on a ratio of two measurements which magnifies the usual random error associated with any one measurement. Even without such error, the narrow range (compared to TK) of the isozyme values over the different individual ALLs

implies a relative lack of sensitivity. It may also be noted that activity with CTP (see small range in Figure III-2) contributes no resolving power. We believe, therefore, that for purposes of assessing disease state as it varies among ALL subjects or during treatment, determination of the TK isozyme pattern (at least with the routinely applicable methods) is of far less practical value than is simple quantification of TK (i.e., with only ATP as the phosphate donor).

If the correlations of known hematological criteria of disease state to TK were perfect (rather than merely of high statistical significance) then its measurement would, of course, serve no practical purpose. Some of the deviations from the expected relationships, such as the high TK activity during relapse even in the absence of blasts from the circulation, are the observations which suggest that this activity may be a potentially useful prognostic indicator. It appears that the TK assay can possibly detect heterogeneity in a cell population with apparently homogeneous, normal morphology, and thus may reveal differences in disease condition that are not evident upon microscopic examination of the leukocytes.

CHAPTER IV

DIMINISHED LYMPHOCYTE AND GRANULOCYTE GGT ACTIVITY IN ALL AND RESPONSE TO CHEMOTHERAPY

INTRODUCTION

The GGT concentration of cancer tissues does not conform to a general pattern, in that liver tumors (Cheng et al., 1978) and adenocarcinomas of the human lung (Greengard et al.,1986) show high levels compared to the cognate tissue, whereas neoplastic transformation of kidney or pancreas (Fujiwara et al., 1982), for example, is associated wth a loss of GGT. ALL's appear to be analogous to the latter category, for lymphoid cells from subjects of this disease were reported to exhibit subnormal GGT activity (Kramer and Catovsky, 1978; Heumann et al., 1985; Morell et al., 1986). These previous studies, concerned primarily with discrimating between different types of chronic and acute leukemias, did not extend to comparison in ALL of the GGT activity of lymphoblasts with that of other leukocytes in the blood or bone marrow. Nor have there been investigations on the possible relationship of quantitative variations in this enzyme among individual ALL subjects to hematological or clinical parameters. To obtain such information was the purpose of the present study on ALL children, most of which were diagnosed as "early pre-B" or "pre-B". It shows that the low GGT activity in the lymphoid fraction of their blood and bone marrow was normalized during successful chemotherapy, and that low activity prior to treatment or at relapse was characteristic not only for blastic cells but also for the apparently normal blood lymphocytes of several subjects.

Moreover, the granulocytes also exhibited GGT deficiency which was quite severe in some cases, and indicated that these cells as well as lymphocytes (or subpopulations thereof) had undergone incomplete functional differentiation. Because of the relatively large number of cases available for study, it was possible to establish some significant correlations between enzymic and hematological parameters. The results suggest that treatment efficacy may be reflected by blood leukocyte GGT titers; the possible prognostic significance of variations in these titers among individual ALLs prior to treatment, however, requires more extensive study.

RESULTS

GGT activities (per mg protein) in blood lymphocytes from each ALL case studied are presented in Table IV-1 under the columns headed by "L". The mean for the 27 untreated ALL children, 1.05 ± 0.15, was lower ($p < .0002$) than the control values and rose significantly ($p < .0001$) during chemotherapy-induced remission to 2.47 ± 0.26 (Table IV-1). This rise is also apparent from comparing pre- and post-treatment GGT results on the same individuals (see first 6 cases in Table IV-1). Similar results on granulocytes ("G" column in Table IV-1) indicate that the abnormality in ALL, and its reversal by treatment, extends to the granulocytic population. In fact, the granulocyte GGT might reflect the abnormality more strikingly: in several untreated subjects it was 1-5% of the control mean, while the lymphocyte GGT was never less than 10% of the control mean. In some cases (see C.N. and C.B.) the GGT activity of the granulocytes was clearly subnormal even though the lymphocyte values were in the normal range. Chemotherapy also normalized the granulocyte number per ml blood; among the 27 untreated ALLs, 18 had very low granulocyte count (0.34 ± 0.08), whereas the significantly ($p < .0001$) higher mean value for the 29 subjects in the "Treated" column of Table IV-1 (2.08 ± 0.20) was in the normal range.

Comparison of the same individuals at and before relapse (see results with the same initials in the

TABLE IV-1

GGT ACTIVITY IN THE BLOOD LYMPHOCYTE FRACTION (L) AND GRANULOCYTE FRACTION (G) OF ALL SUBJECTS

Untreated			Treated			Relapsed		
Case	L	G	Case	L	G	Case	L	G
JK*	0.56	-	JK*	3.45	1.83	JK*	0.40	0.50
RM	2.22	1.45	RM	3.47	2.11	RM	2.53	-
CP	0.63	0.02	CP	1.70	-			
SN	0.22	0.02	SN	1.37	0.91			
SL	0.23	0.11	SL	2.51	1.75			
JG	0.53	-	JG	1.12	2.05			
			HA	1.71	0.93	HA	0.40	1.41
DV	0.38	-	VF**	1.02	0.67	VF**	0.67	0.02
MD	0.56	0.09	WZ	3.63	0.45	WZ	0.78	0.01
VV	0.16	0.15	HS	1.56	0.93	HS	2.05	0.01
RB	1.69	2.10	SB+	6.61	0.84	SB+	6.07	0.05
MC	1.83	2.37	MK	1.93	1.34	MK	1.18	0.39
JF	0.29	1.37				CR	0.25	0.28
NL	1.49	1.21	BZ	2.38	0.39			
FB	1.44	2.02	JA	2.33	0.58			
GG	2.54	2.85	LS	1.66	1.16			
KP	0.63	0.57	KD	0.79	0.12			
RL	0.79	0.63	AB	2.90	1.76			
AW	0.31	0.06	AP	3.38	1.66			
CN	2.07	0.25	ES	4.31	3.02			
AK	0.64	0.59	HR	2.97	1.31			
KS	0.98	0.98	JT	1.60	0.50			
MX	2.32	1.91	KH	2.54	0.94			
DH	0.24	0.06	MS	2.31	1.23			
CB	2.43	0.87	OH	5.93	3.41			
JO**	0.96	0.53	RJ	1.89	0.71			
IF	1.09	-	SC	1.89	0.43			
AC	1.19	-	SI	0.65	4.37			
			VB	1.15	0.57			
			VR	3.71	2.06			

GGT, Mean±SE

	(L)	(G)
Untreated	1.05±0.15 (27)	0.92±0.19 (22)
Control	2.25±0.30 (16)	1.59±0.23 (16)
Treated	2.47±0.26 (29)	1.34±0.19 (28)
Relapsed	1.59±0.62 (9)	0.33±0.17 (8)

Patients (for definition of "Treated" and "Relapsed" see Methods) were subjects of early pre-B ALL, except where otherwise indicated (*, T-cell; **, pre-B; +, B-cell). In each blood sample, the lymphocyte fraction (containing lymphocytes plus blasts, if any) and the granulocyte fraction were separated and GGT was assayed. The results are expressed in units per mg protein. Means±SE refer to the number of individuals in parentheses.

"Treated" and "Relapsed" columns, Table IV-1) showed that in 7 out of the 8 cases relapse was associated with a return of the lymphocyte GGT activity to the low values seen before chemotherapy. The decrease of GGT activity at relapse in the granulocyte fraction in most individuals was much more marked than in the lymphocyte fraction, and the mean granulocyte GGT activity for relapsed cases (0.33) was significantly lower ($p < .0009$) than the mean for treated cases (1.34). Neutropenia tended to return during relapse: out of the 9 cases, 6 had very low granulocyte count (0.45 ± 0.16 per ml blood). These counts showed no statistically significant quantitative correlation to granulocyte GGT activity per mg protein, and this activity was also low in a few relapsed (or untreated) patients with normal numbers of granulocyte per ml blood.

The results in Table IV-2 indicate that in the untreated ALLs not only the blood leukocytes but also the bone marrow is deficient in GGT. This was true in both the lymphocyte enriched and the granulocyte enriched fraction of bone marrow. Chemotherapy which normalized the GGT activity in the peripheral blood lymphocytes and granulocytes raised the GGT of the subject's bone marrow cells as well, and the decrease during relapse was also evident in both compartments.

In Tables IV-1 and IV-2 the blood lymphocyte values for untreated and relapsed subjects represent "average" GGT activities for the varying number of blast and lymphocytes

TABLE IV-2

GGT ACTIVITY IN THE LYMPHOCYTE FRACTION (L) AND GRANULOCYTE FRACTION (G) OF BONE MARROW AND BLOOD FROM THE SAME INDIVIDUAL

	Bone Marrow		Blood	
	L	G	L	G
Untreated	0.03	0.01	0.31	0.06
Untreated	0.11	0.02	0.63	0.02
Untreated	0.10	-	0.55	-
Treated	3.33	1.35	3.80	2.35
Treated	1.82	3.58	3.00	3.68
Treated(Mean)	3.4±1.6(5)	1.5±0.5(5)		
Relapsed	0.44	0.03	2.05	0.01
Relapsed	0.12	-	2.21	-
Control(Mean)	2.0±1.2(4)	1.2±0.3(3)		

Each horizontal line refers to GGT activity (units per mg protein) in the indicated bone marrow and blood preparations from the same individual, or to means (±SE) of bone marrow results on the number of individuals in parentheses. Control bone marrow samples were obtained from ALL children who had been off therapy for at least six months. For means of peripheral blood cell results see lower portion of Table IV-1.

TABLE IV-3

ENZYME ACTIVITIES IN SEPARATED LYMPHOBLASTS AND LYMPHOCYTE PREPARATIONS

Case	Lymphocytes	Non-Blastic Lymphocytes
	γ-Glutamyltranspeptidase	
1	0.01	2.90
2	0.11	0.16
3	0.27	0.42
4	0.66	2.95
	Thymidine Kinase	
1	19.2	6.0
2	31.9	16.1
3	59.4	18.9

The lymphoblast and lymphocyte preparations from the blood of 4 ALL cases were obtained as described under Methods. In cases 1 through 3, both GGT and TK were measured in the same preparation. The results are expressed in units per mg protein.

in this fraction. Table IV-3 refers to 4 untreated subjects where it was possible to separate a blast and a lymphocyte fraction from the blood with only 5-10% cross contamination. The results show that the former fraction had minimal GGT activity. However, the apparently normal lymphocytes may have also contributed to some of the subnormal GGT activities listed in Table IV-1, column 1, since in cases 2 and 3 (though not 1 and 4, see Table IV-3) the separated lymphocyte (as well as the blast) fraction exhibited activities (0.16 and 0.42) which were far below the control mean (2.25, see Table IV-1). Measurement of TK in the same fractions of cases 1-3, shown for comparison in the last part of Table IV-3, confirmed the well known enrichment of blasts with TK (control value = 7.0 ± 0.7 and the fact that in some subjects (see also cases 2 and 3 in Table IV-3) this enzyme was also elevated in the non-blastic lymphocytes.

The relationship in untreated and relapsed subjects in Table IV-1 of hematological parameters to the GGT activities in the whole blood lymphocyte fraction was examined. GGT (per mg protein) showed an inverse quantitative correlation to the number (per ml blood) of lymphoid cells (Figure IV-1), to the number of WBC, and an even closer one to the number of blasts (Table IV-4). These cell counts bore an opposite relationship to TK (see Figure IV-1 and Table IV-4), which is consistent with the finding that the specific activities of TK and GGT (last

TABLE VI-4

CORRELATIONS BETWEEN ENZYMIC AND HEMATOLOGICAL PARAMETERS

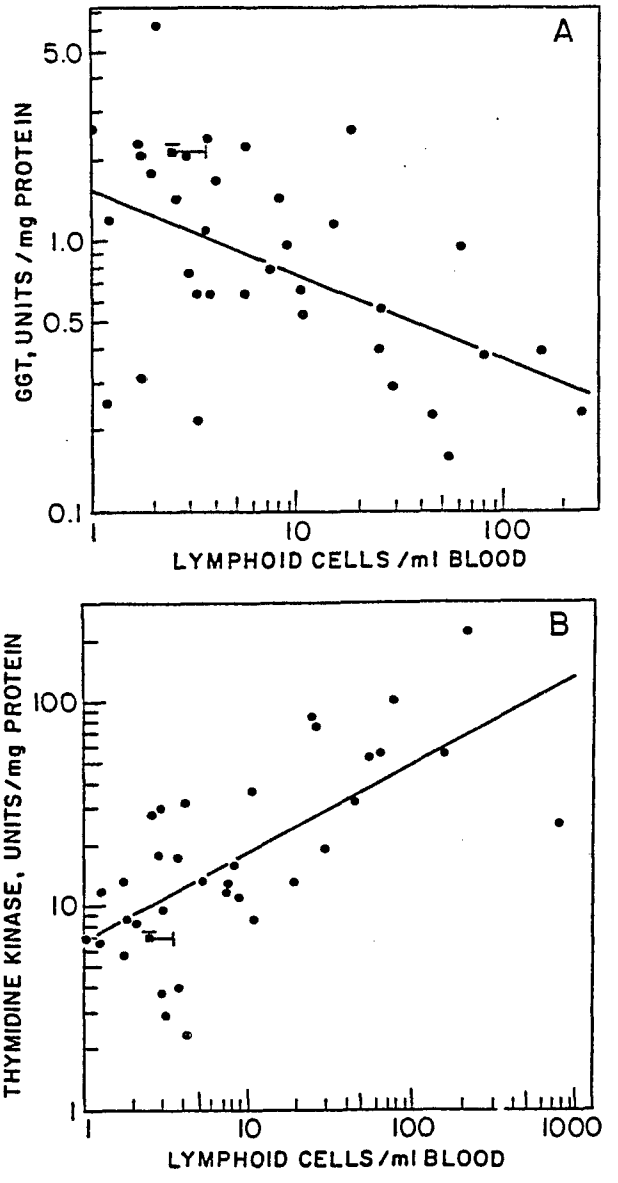
	Log GGT (n=35)		Log TK (n=35)	
Log Number/ml Blood	r	p<	r	p<
WBC	-0.43	.01	0.71	.0001
Lymphocytes + Blasts	-0.52	.001	0.69	.0001
Blasts	-0.61	.0001	0.57	.0003
Lymphocytes	-0.34	.05	0.61	.0001
			(n=63)	
Log GGT			-0.47	.0001

In the first 4 lines, the number of the indicated cells in the whole blood (per ml) of untreated and relapsed ALL subjects was compared with GGT and TK activity (per mg protein) of the lymphocyte fraction (i.e. lymphocytes plus blasts, if any) from the same blood samples. In the last line, the correlation of TK to GGT was calculated for all ALL cases in which both enzymes were measured. The results presented are Pearson parametric correlation coefficients (r) with statistical significance (p). The Spearman rank order correlations were also statistically significant in all cases.

FIGURE IV-1

RELATIONSHIP OF THE NUMBER OF LYMPHOID CELLS PER ML BLOOD TO THE SPECIFIC ACTIVITY OF GGT (A) AND TK (B)

Blasts plus lymphocytes x 106 per ml (abscissa) counted in whole blood of ALL subjects were plotted against GGT (A) and TK (B) per mg protein (ordinate) measured in their isolated blood lymphoid fraction. Each circle refers to an individual untreated or relapsed ALL patient. Squares refer to means (bar=SE) of results on control subjects.



line, Table IV-4) in the different subject's blood lymphocyte fraction bear an inverse correlation to one another.

DISCUSSION

As seen in Chapter III, the high TK content in the blood lymphocyte fraction of ALL subjects became normalized by successful chemotherapy and rose again during relapse. Results described in this Chapter identify a second enzyme which may reflect treatment response, but which (being deficient rather than elevated in untreated subjects) does so in the opposite sense. Lymphoblasts had minimal GGT activity, and the specific activity of the lymphocyte fraction showed a direct correlation to the number of blasts per ml blood. However, biochemical abnormalities in a subpopulation of apparently normal lymphocytes was also reflected by GGT. For subnormal activity was found in isolated lymphocytes from the blood of 2 (out of 4) ALL cases as well as in the lymphocyte fraction of 8 untreated or relapsed subjects with no or minimal blast count. This, like the TK elevations in such subjects, indicates that enzyme assay may reveal subpopulations of small blastic or maldifferentiated lymphocytes which, showing no microscopically detectable abnormality, are thought to be present in ALL patients (Gavosto et al., 1964; Gavosto et al., 1967; Mauer and Murphy, 1979).

Relapse is usually confirmed by inspection of bone marrow samples since the blood may still be devoid of blast cells. However, the apparently normal lymphocyte fraction of several such subjects had higher TK activity than they did during remission. The present observations on

GGT further suggest that assays of peripheral cells may detect or predict relapse before suspected on hematological or clinical grounds. Conclusions based on these two enzymes should be much safer than on any one alone; for the GGT, in contrast to the TK, activity of the lymphoid cells tends to decrease with diminishing treatment efficacy, and simultaneous occurrence of two such opposite changes is unlikely to be accidental. Also, the very different catalytic properties, subcellular localizations, and physiological functions of TK and GGT assure that extraneous factors in vivo or mistakes in assay in vitro which might confound the results on one enzyme would not affect the other.

The occurrence of neutropenia in ALL is well documented (Fernbach, 1984), however, previous investigations have not questioned the normalcy of the granulocytes that are present in the blood. The GGT deficiency we now found indicates that these cells - though they were devoid of microscopically detectable signs of abnormality or immaturity - have undergone incomplete differentiation. Whether the expression of several other genes associated with this process has been attenuated is an open question. However, in view of some unique attributes of GGT, deficiency in this enzyme alone may mean that the circulatory granulocytes of ALL subjects cannot effectively perform some of their mature physiological functions. GGT, with its predominantly plasma

membrane localization, can act on extracellular substrates (Novogrodsky et al., 1976; Inoue et al., 1977), is thought to play an important role in amino acid transport into cells (Griffith et al., 1979; Meister, 1983), and it is the only enzyme capable of initiating the hydrolysis of glutathione (Curthoys and Hughey, 1979). Also, GGT catalyses the production of leukotriene D_4 (Hammarstrom, 1983) which has been postulated (Miller and Weiner, 1986) to be essential for the response of normal bone marrow to granulocyte-macrophage colony formation stimulating factors.

The granulocyte GGT deficiency in ALL (seen in the bone marrow's granulocyte fraction as well) may also be pertinent to clinical diagnoses. In several subjects where chemotherapy ceased to be effective there was a more pronounced decrease in the GGT activity of granulocytes than in that of lymphocytes. Moreover, prior to treatment, very low granulocyte GGT activity was seen in some subjects without neutropenia. In view of the heterogeneous symptomatology and disease outcome of subjects with the same type of ALL (as judged by antigen typing), it seems worth considering the possible prognostic value of subclassifying these individuals on the basis of whether the specific GGT activity of their blood granulocytes is or is not severely depressed.

CHAPTER V

GGT ACTIVITY IN INTACT LEUKOCYTES: FLOW CYTOMETRIC ANALYSIS AND SORTING

INTRODUCTION

The induction of hepatocellular cancers by chemical carcinogen treatment is long known to be preceded by the appearance of hyperplastic nodules containing liver cells with high GGT activity (Richards et al., 1982). This phenomenon has provided the prime motivation for the development of sensitive GGT assays that would permit not only the early detection of preneoplastic lesions but also the flow cytometric detection of intact, GGT-containing cells. Dolbeare and Smith, 1977, succeeded in designing a method where GGT in intact cells is revealed by the formation of an insoluble fluorescent product, thus permitting flow cytometric analysis of these cells. The use of this method, or of an alternative one by Chen and Hakilll (1984) has hitherto been restricted to identifying GGT containing cells in the liver (Vanderlaan et al., 1979) and cervical tumors (Haskill et al., 1983).

Flow cytometry has been applied to leukemic leukocytes for purposes of revealing heterogeneity in terms of size, subcellular morphology, DNA content and surface antigen expression. GGT has not been used as a probe in studies of hematologic cancers perhaps because thay tend to be associated with a loss (Kramer and Catovsky, 1978; Heumann et al., 1985; Morell et al., 1986) rather than an increase in cellular GGT content. Our recent observation of the heterogeneity within the lymphocyte fraction of ALL subjects (Chapter IV) suggested that GGT may be a useful

parameter for flow cytometric identification of distinct leukocyte subpopulations. In this first attempt at the "GGT-labelling" of hematopoietic cells, however, we used an experimental system more readily available and reproducible than human leukemic cells, namely, cells from rats with (and without) a subcutaneous implant of mammary carcinoma 5A. The present study shows that the method of Dolbeare and Smith, 1977, with minor modifications, is sensitive enough to detect the GGT activities of normal peripheral leukocytes and bone marrow cells. Multiparameter flow cytometric analysis distinguished cell types that do, from those that do not exhibit a progressive increase in GGT activity in response to MC transplantation, and revealed an unexpected heterogeneity among peripheral granulocytes.

RESULTS

Initial qualitative studies using a fluorescent microscope showed that with the 0.7 mM concentration of the substrate (MNA) and 6 mM trapping agent (NSA) (which was suitable for detecting GGT-rich cells in hyperplastic liver (Vanderlaan et al., 1979), leukocytes from normal rats developed very little fluorescence. A somewhat higher concentration of MNA (1mM) and a lower concentration of NSA (1mM) were, however, suitable. Fluorescence intensity increased during the first 30 minutes of incubation, and showed very little further change during the next 30 minutes. Addition to the complete reaction mixture of excess amounts of the alternative substrate, glutathione, prevented formation of the fluorescent product which verified the specificity of the reaction for GGT. The results of this assay, therefore, will be referred to as "GGT fluorescence" or "fluorescence", so as to distinguish them from the results of the usual quantitative assay in cell homogenates which will be referred to as "GGT activity".

Rat Leukocytes

In the normal rat's blood leukocyte fraction, the GGT fluorescence intensities of individual cells were indistinguishable from one another. This was in harmony with the similar GGT activity of granulocytes and lymphocytes (Table V-1, lines 1 and 2). After subcutaneous

TABLE V-1

THE EFFECT OF TUMOR-BEARING ON THE GGT ACTIVITY AND NUMBER OF GRANULOCYTES

	Cell No. x 10^6/ml blood	GGT nmol/min/10^6 cell
Normal Rats		
lymphocyte	4.5±0.8	0.3±0.15
granulocyte	0.8±0.3	0.3±0.12
Tumor-Bearing Rats		
lymphocyte	5.8±1.0	0.6±0.19
granulocyte	4.6±0.5	4.9±1.1

Blood samples were taken from rats without tumors or 14 days after the transplantation of mammary carcinoma 5A. The values are means± SD of results on 3-4 animals.

implantation of mammary carcinoma 5A (MC), however, there was a striking rise in activity (per cell) in the granulocyte but not the lymphocyte fraction (Koss and Greengard, 1982). In the present animals this difference in GGT between granulocytes and lymphocytes was 8-fold (Table V-1, lines 3 and 4). In qualitative accord with this, the intact cell assay applied to the whole leukocyte fraction revealed major differences in the cell's GGT fluorescence (Figure V-1).

Quantitative information was then sought by subjecting these cells to flow cytometric analysis. Again, the whole leukocyte fraction was used, since the purpose was to determine whether this method can achieve a distinction between intact cells with relatively high and low GGT activity. Figure V-2A depicts the percent of cells (ordinate) with the indicated GGT fluorescence intensity (abscissa). It may be seen that in contrast to the smooth distribution for leukocytes from the control animal (solid line), the distribution of leukocytes harvested 14 days after MC implantation (broken line) showed 2 peaks (Figure V-2A). One of these coincided with the peak for control cells, while the other peak revealed the appearance of a population of cells with higher GGT activity. At 28 days after transplantation the first peak became negligible (Figure V-2B), indicating that the percent of cells with low GGT is greatly decreased (from 46 to 5 percent). The height of the other peak, and its coincidence with the 2nd

FIGURE V-1

FLUORESCENT MICROSCOPE VISUALIZATION OF GGT IN LEUKOCYTES FROM MAMMARY CARCINOMA BEARING RATS

Animals bearing a mammary carcinoma were bled 14 days after transplantation. Unfixed fresh leukocytes were incubated for 30 minutes with the GGT reaction mixture. Lightly stained cells are lymphocytes while those with intense, unevenly distributed fluorescence are granulocytes. Magnification is 500x.

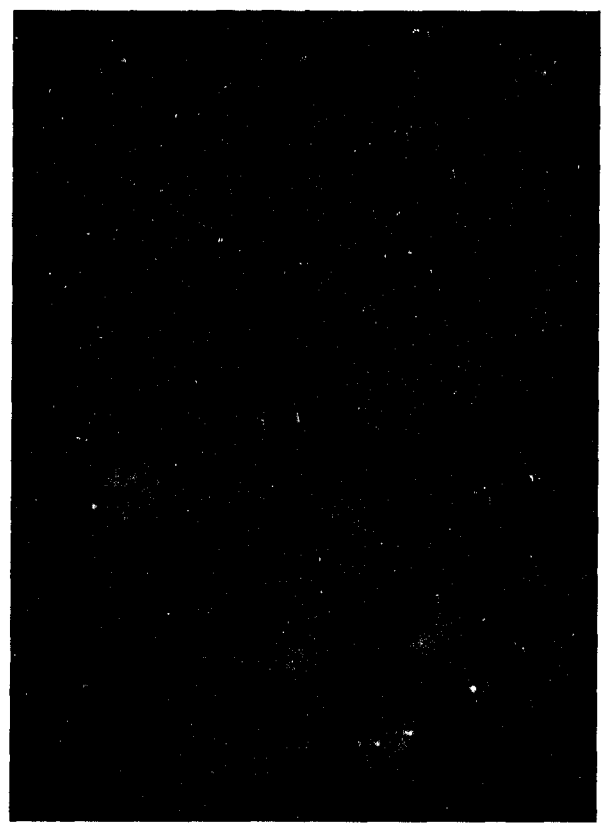
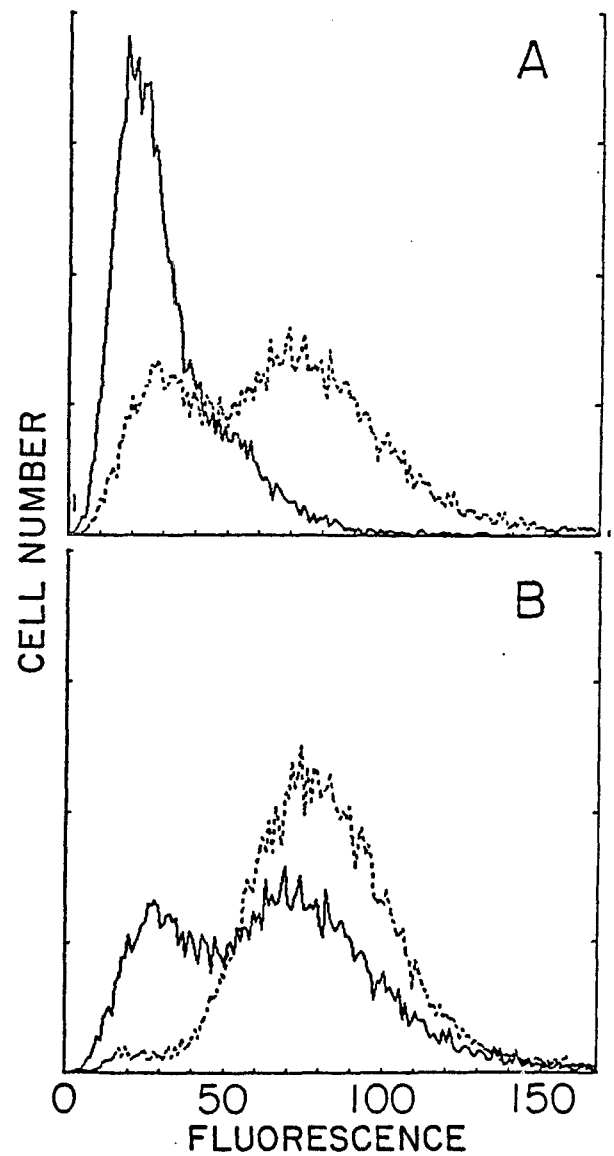


FIGURE V-2

FLOW CYTOMETRIC GGT ANALYSIS OF LEUKOCYTES FROM NORMAL AND MC (MAMMARY CARCINOMA) BEARING RATS

Cells were incubated for 30-45 minutes with substrate and trapping agent. Ordinate indicates the relative number of cells; approximately 20,000 cells were analyzed. Solid line in A refers to leukocytes from normal animals. MC bearing animals were bled 14 days (broken line in A and solid line in B) or 28 days (broken line in B) after transplantation.



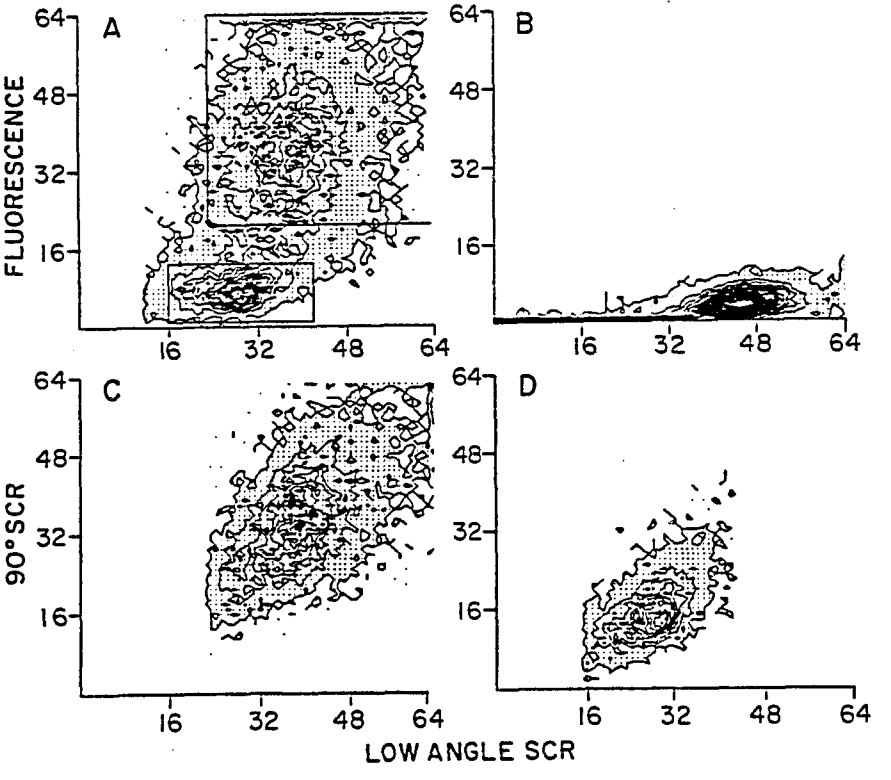
peak seen at 14 days, indicates that between the 14th and 28th day of transplantation there was an increase, from 54 to 95 percent, of cells (presumably granulocytes) with the high GGT fluorescence. Doubling the substrate concentration from 1mM to 2mM resulted in no further increase in GGT fluorescence. This fluorescence was again specific for GGT since it was absent from cells which have been incubated with the complete reaction mixture plus excess glutathione (see Figure V-3B).

Figure V-3A, referring to leukocytes obtained 14 days after MC transplantation, displays two parameters recorded simultaneously in each cell, low angle scatter and GGT fluorescence intensity. The contour lines show that there are two regions of particularly high cell density, one with relatively high and one with low GGT fluorescence. With respect to low angle scatter (which depends on cell size) the two regions overlap to some extent. The two regions were, therefore, "gated" (see boxes in Figure V-3A) according to GGT-fluorescence, and diplayed seperately against the two light scatter parameters (Figure V-3C and 3D). It may be seen that 90 degree scatter provides a better distinction between cells with high and low GGT fluorescence than does low angle scatter. Previous studies (Nicola et al., 1980) have shown that in the leukocyte population, for which both scatter profiles (i.e., low angle and 90 degree) are very low (but above that for damaged or dead cells), 90% of the cells are lymphocytes.

FIGURE V-3

CONTOUR DISPLAYS OF GGT FLUORESCENCE AND LIGHT SCATTER FOR LEUKOCYTES FROM A RAT 14 DAYS AFTER MAMMARY CARCINOMA TRANSPLANTATION

The 256 channels for each parameter are compressed to make a 64X64 array for each 2 parameter display. Approximately 20,000 cells were analyzed after incubation with the GGT reaction mixture (but in B, excess glutathione which competes with the fluorogenic substrate, was also added). GGT fluorescence versus low angle scatter is displayed in A; "gated" analysis of highly fluorescent cells (see upper box in A) is displayed in C as low angle versus 90 degree scatter."Gated" analysis of less fluorescent cells (lower box in A) is displayed in D. Shaded areas and sequential contours represent large numbers of cells, contour lines are shown for 5 or more cells.



Due to their larger size, the low angle scatter of neutrophils is higher and their 90 degree scatter, which depends on the number of internal reflective surfaces, is also higher. Mature granulocytes, with their segmented or lobulated nuclear morphology show particularly striking 90 degree scatter (Nicola et al., 1980). Thus, the gated cells with low (Figure V-3D) and high (Figure V-3C) GGT fluorescence were probably mainly lymphocytes and granulocytes, respectively.

The flow cytometer was, therefore, programmed for sorting the two gated populations. Examination of their cytospin preparations under the microscope confirmed that the high GGT fluorescent, gated population consisted of granulocytes while the less fluorescent gated cells were lymphocytes. The microscopic appearence of these cells is shown in Figure V-4A and 4B, respectively.

The fact that the number of granulocytes, rather than that of lymphocytes increases progressively with tumor growth (Koss and Greengard, 1982, see also Table V-2), is also apparent from the flow cytometric analysis of leukocytes that were not reacted with the GGT substrates. It may be seen from Figure V-5A, 5B, and 5C that the increase above normal in the percent of granulocytes (channels 40 through 60, LOW ANGLE SCR) is much more striking at 28 days (Figure V-5C) than at 14 days (Figure 5B) after transplantation; consequently, the percent of lymphocytes (channels 20 to 40, LOW ANGLE SCR), which are

FIGURE V-4

LIGHT MICROSCOPE APPEARANCE OF LEUKOCYTES SORTED ON THE BASIS OF GGT FLUORESCENCE

Blood leukocytes from rats 14 days after MC transplantation sorted (see boxes in Figure V-3A) on the basis of high and low GGT fluorescence are shown in A and B respectively. The shape and nuclear segmentation of the granulocytes (A) is slightly obscured by the accumulation of the crystalline GGT reaction product; lymphocytes (B), containing relatively little of this product, suffered less distortion. Magnification, 1250x.

(A)

(B)

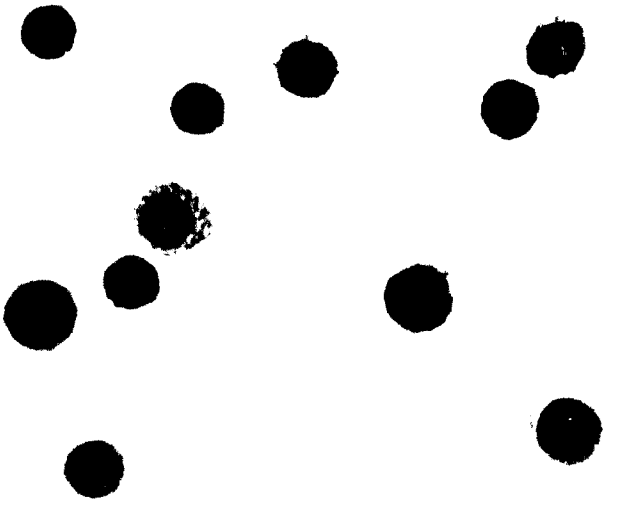


TABLE V-2

CHANGES IN GRANULOCYTE GGT ACTIVITY IN THE COURSE OF MAMMARY CARCINOMA GROWTH

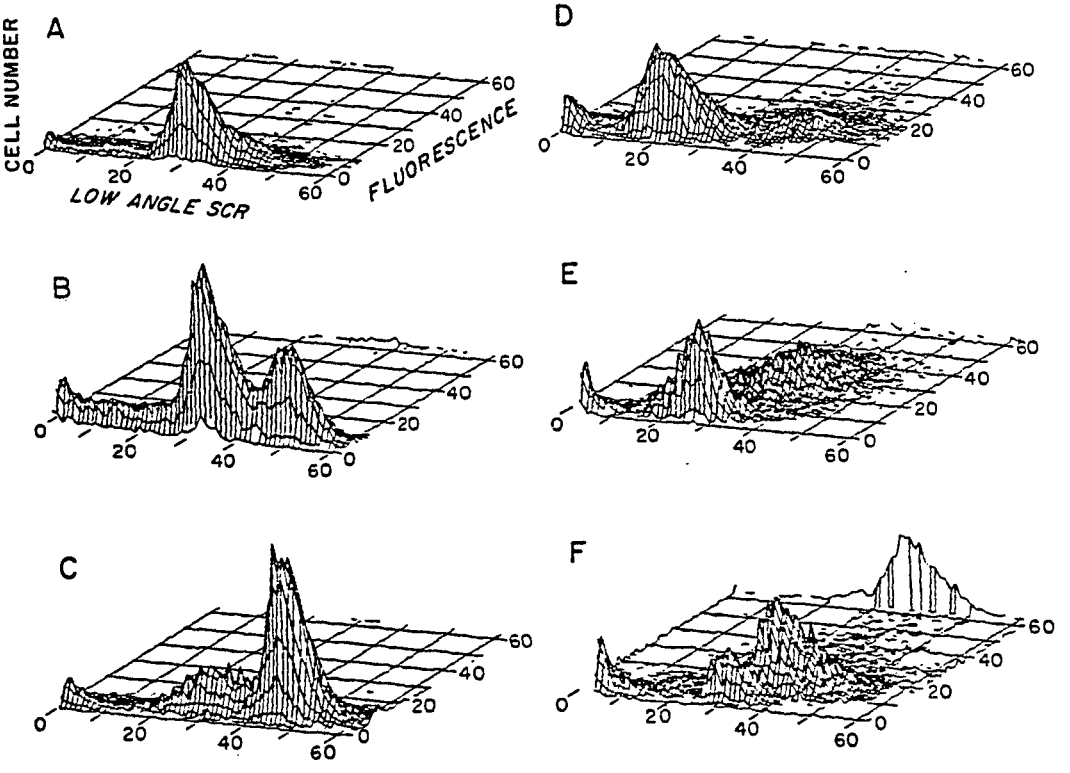
Tumor Wt (g)	Time After Transplantation (days)	Granulocyte GGT (nmol/min/10^6 cells)	Granulocyte No. (per ml bloodx10^6)
-	-	0.3±0.1 (4)	0.9±0.3 (6)
0.7	7	0.2	1.3
6.9	15	4.1	3.6
35.9	22	11.7	11.6

Animals were sacrificed, blood was collected, and tumors were weighed on the indicated days after subcutaneous transplantation. GGT was quantified in the blood granulocyte fraction. Granulocyte count (Column 4) was determined in whole, unfractionated blood. Each horizontal line refers to a different experiment (values without S.D.), or to the mean±S.D. of results on 4-6 individual, untreated rats.

FIGURE V-5

TWO PARAMETER PULSE HEIGHT DISTRIBUTION OF LEUKOCYTES FROM NORMAL AND MAMMARY CARCINOMA BEARING RATS

Peripheral leukocytes were from a normal rat (A and D), and from rats 14 days (B and E) or 28 days (C and F) after MC transplantation. A-C refer to leukocytes unreacted, and D-F to leukocytes reacted with the GGT reaction mixture.



the predominant cells normally (Figure V-5A), became smaller until on day 28 the leukocyte population consisted almost 100% of granulocytes (Figure V-5C). Figures V-5D, 5E, and 5F refer to leukocytes from the same three animals but after they have been reacted with the substrate for GGT. These again reveal a progressively increasing percent of granulocytes with significant GGT fluorescence (channels 10 through 40). More importantly, they illustrate the ability of the flow cytometric approach to provide evidence for heterogeneity that could not be obtained by conventional cell separations and assays. It may be seen from Figure V-5F that on the 28th day after transplantation (but not yet on the 14th day, see Figure V-5E) the granulocyte population is heterogeneous in that a significant portion of the cells, 30 percent, showed much higher (see channel 60) fluorescence although their low angle scatter (and thus size) was the same as for the other portion.

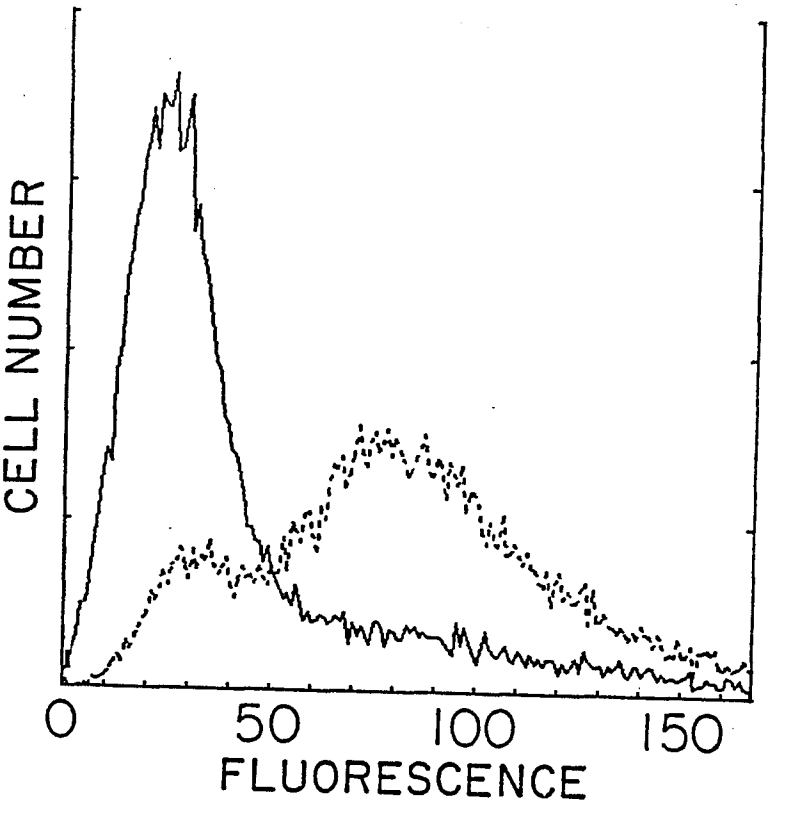
Bone marrow cells also undergo a progressive increase in GGT activity with increasing tumor size (Koss and Greengard, 1982; Greengard et al., 1984). This again is apparent from flow cytometric analysis. As seen from Figure V-6, bone marrow cells from animals 14 days after MC transplantation showed much lower GGT fluorescence intensity than 28 days after transplantation (cf. solid and broken line).

In gated analysis of these bone marrow cells, the

FIGURE V-6

FLOW CYTOMETRIC GGT ANALYSIS OF BONE MARROW CELLS FROM MC BEARING RATS

Bone marrow cells, obtained 14 days (solid line) and 28 days (broken line) after tumor transplantation, were incubated for 40 minutes with the GGT reaction mixture. Ordinate indicates the relative number of cells; approximately 20,000 cells were analyzed.



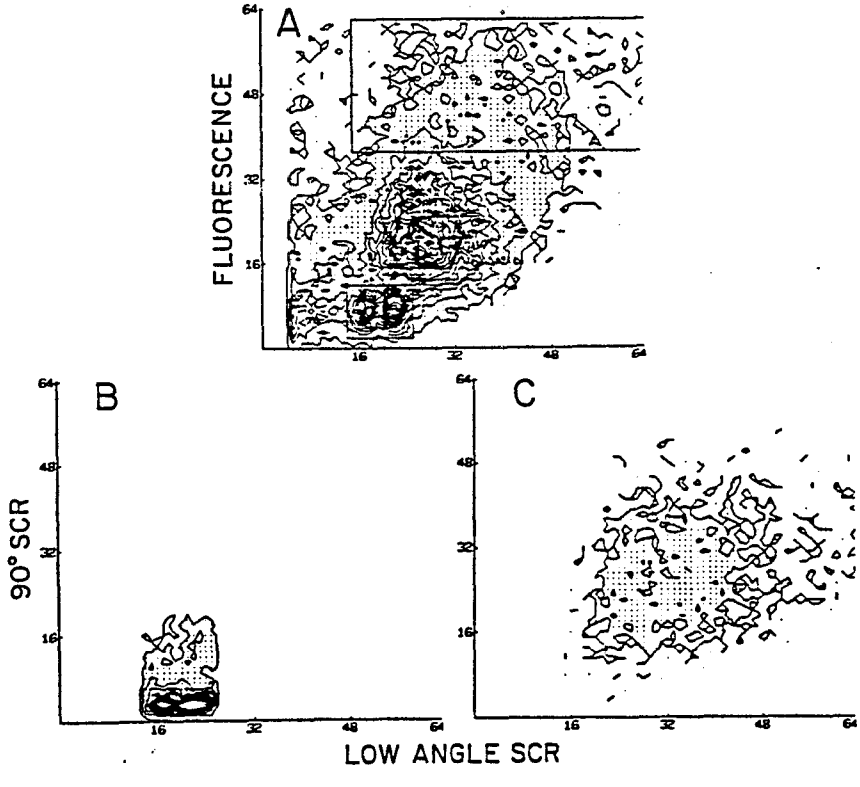
regions with high and low fluorescence (see boxes in Figure V-7A) were displayed against scatter parameters in Figure V-7B and 7C ,respectively. The more intense 90 degree and low angle scatter of the population in Figure V-7C than in 7B again suggests that granulocytic cells are the ones exhibiting the higher GGT activity. Their size, however, (cf. scatter intensity in Figure V-7C with Figure V-5C) appears to be smaller than for circulatory granulocytes. This, in particularly the relatively low 90 degree scatter of GGT rich bone marrow cells suggests that in the bone marrow not only the mature but also the as yet incompletely differentiated granulocytes are subject to progressive, tumor-induced increase in GGT activity. To determine the stages of maturity represented by these cells, and also to characterize of the lower size range population in Figure V-7B) (which presumably includes lymphocytes as well as other cells) the flow cytometer was programmed to sort the two gated regions. However, no reliable conclusions could be arrived at because the crystals of the GGT reaction product, which became larger with time, damaged the bone marrow cells and precluded their microscopic identification. (This problem, though to a much lesser extent, can also arise with peripheral leukocytes sorted with the present method).

As the multiparameter flow cytometry of blood granulocytes (Figure V-5), so that of bone marrow, revealed the presence of two distinct cell populations at 28 days

FIGURE V-7

CONTOUR DISPLAY OF GGT FLUORESCENCE AND LIGHT SCATTER FOR RAT BONE MARROW CELLS 28 DAYS AFTER MAMMARY CARCINOMA TRANSPLANTATION

The 256 channels for each parameter are compressed to make a 64x64 array for each 2 parameter display. Approximately 20,000 cells were analyzed after incubation with the GGT reaction mixture. GGT fluorescence versus low angle scatter is displayed in A; "gated" analysis of the highly fluorescent cells (see upper box in A) and less fluorescent cells (see lower box in A) are displayed in C and B, respectively. Shaded areas and sequential contours represent large numbers of cells; dots are displayed for all channels containing 2 cells, while contour lines are shown for 5 or more cells.



(but not yet at 14 days) after MC transplantation. As seen from Figure V-8B, distribution along the fluorescence axis was discontinuous; there are two major cell clusters showing moderate and very high GGT fluorescence, respectively, with essentially no cells in between them. The low angle scatter intensity in Figure V-8A and 8B indicates that, while there has been a shift to larger cell sizes, the cell size range of the GGT-rich population at 28 days after transplantation is virtually the same as the cell size range in the other major cluster present at this time (i.e., in the population with the lower GGT fluorescence).

Human Leukocytes

Light scatter (i.e., low angle and 90 degree) parameters alone can discriminate between some blood cell types. Red blood cells (Figure V-9A) display small low angle scatter and 90 degree scatter due to their small size and absence of a nucleus, while greater scatter intensities are seen for lymphocytes (Figure V-9B). Granulocytes (Figure V-9C) show even more low angle and 90 degree scatter (due to greater size and nucleus); the wide distribution in 90 degree scatter is due to the heterogeneity of nuclear segmentation in the mature granulocyte population. ALL lymphoblasts and non-blastic lymphocytes (Figure V-9D) could not be separated on the basis of light scatter profiles alone.

Qualitative analysis of intact ALL lymphoid cells

FIGURE V-8

TWO PARAMETER PULSE HEIGHT DISTRIBUTION OF BONE MARROW CELLS FROM MC BEARING RATS

The relative number of rat bone marrow cells 14 days (A) and 28 days (B) after MC transplantation are shown as a function of GGT fluorescence and low angle scatter. Approximately 20,000 cells were analyzed.

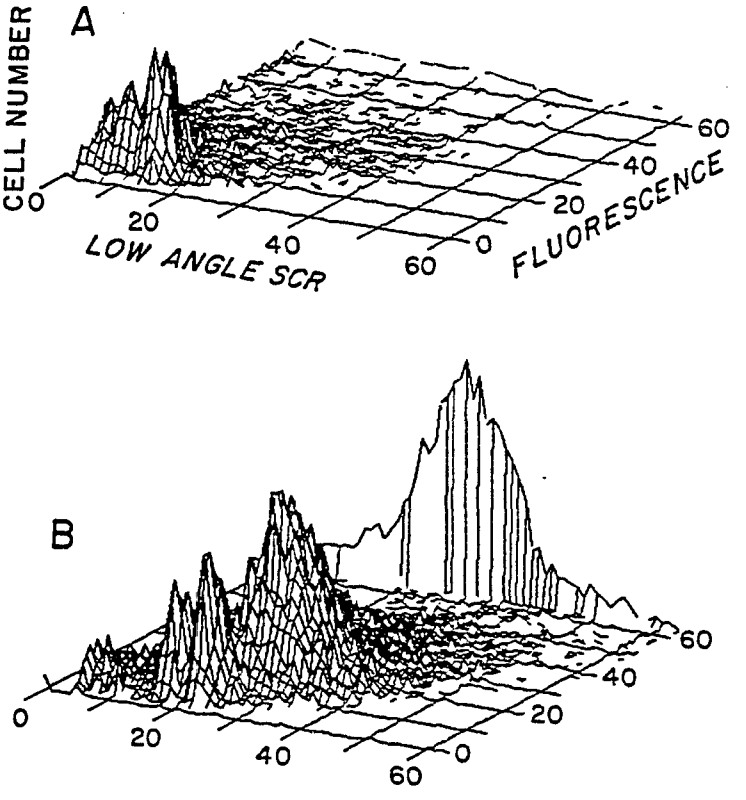
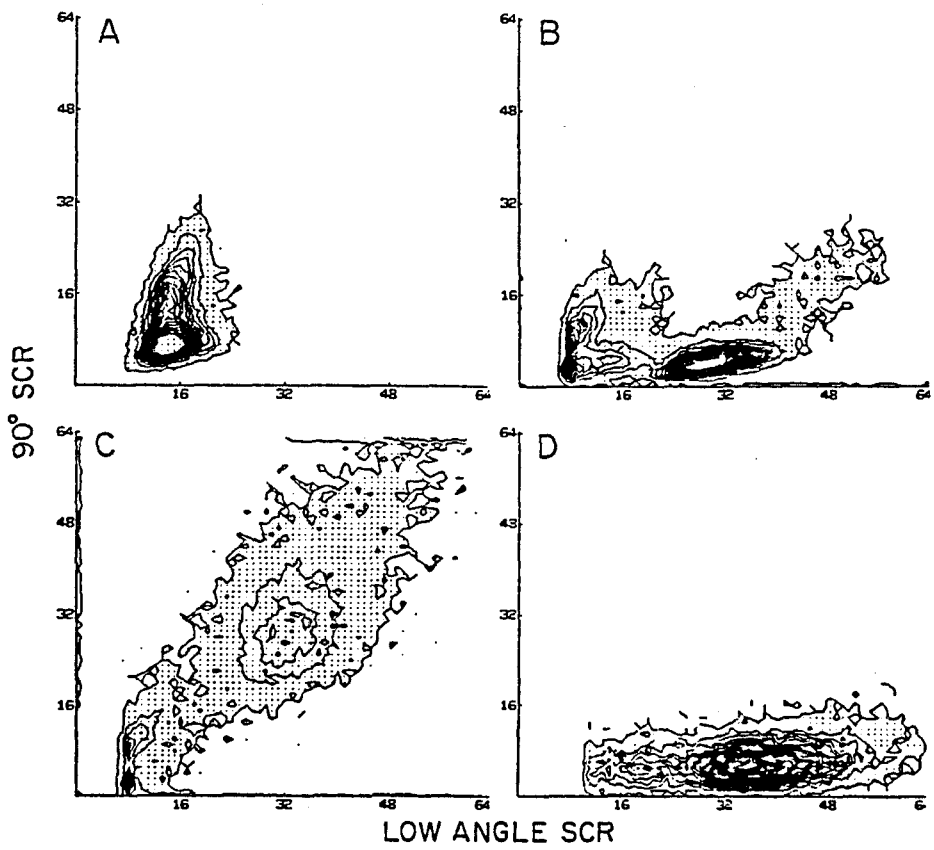


FIGURE V-9

CONTOUR LIGHT SCATTER DISPLAYS OF HUMAN BLOOD CELLS

90 degree versus low angle scatter is shown for normal human red blood cells (A), lymphocytes (B), granulocytes (C) and ALL lymphoid cells (D). The 256 channels for each parameter are compressed to make a 64x64 array for each 2 parameter display. Approximately 20,000 cells were analyzed. Shaded areas and sequential contours represent increasing number of cells; dots are displayed for all channels containing 2 cells, while contour lines are shown for 5 or more cells.



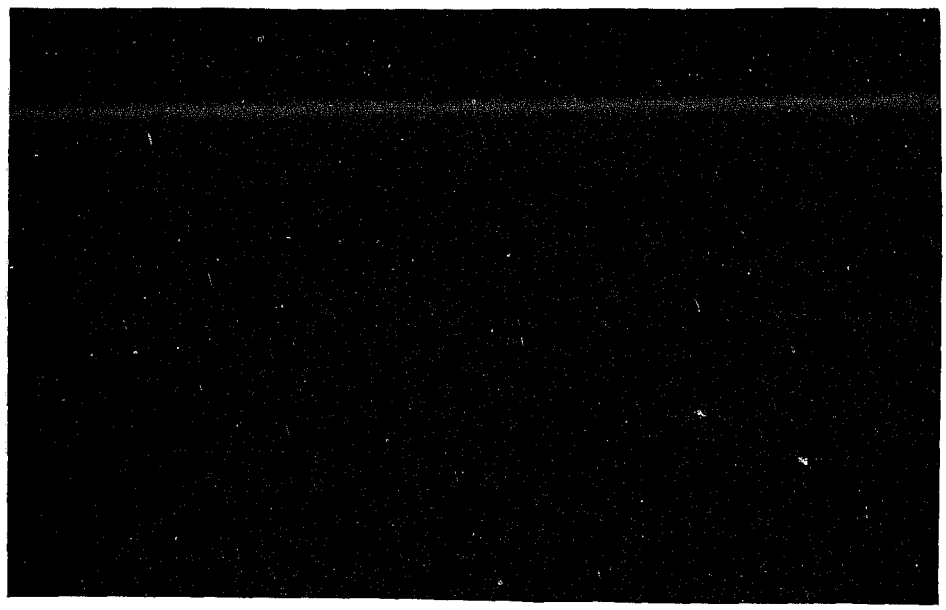
(Figure V-10A) with the GGT fluorescent probe, characterized "blastic" cells as having light pericellular staining indicative of cells with minimal GGT activity, while the other lymphocytic cells appeared more heavily stained. In most ALL cases analyzed for GGT fluorescence by flow cytometry, this reaction was not sensitive enough to distinguish the lower GGT fluorescence of blast cells from non-blastic lymphocytes (Table IV-3). The result did reveal, however, the generally lower GGT fluorescence of ALL cells from normal (Figure V-10A, 10B and 11). Also, in a few ALL cases, the distribution of GGT fluorescence among lymphocytes (Figure V-12) indicated the presence of subpopulations with relatively low and higher GGT activity.

FIGURE V-10

FLUORESCENT MICROSCOPE VISULIZATION OF GGT IN NORMAL AND ALL LEUKOCYTES

Leukocytes from ALL (A) and normal (B) subjects were stained for 30 minutes with methoxynaphthylamide substrate and nitrosalicylaldehyde coupling agent. Standard hematological analysis of the ALL blood smear indicated 37% blasts and 54% lymphocytes. Lightly stained cells show a pericellular localization of yellow-orange fluorescent MNA-NSA, while heavily stained cells are filled with crystalline product. Magnification of cells is 500x.

(A)



(B)

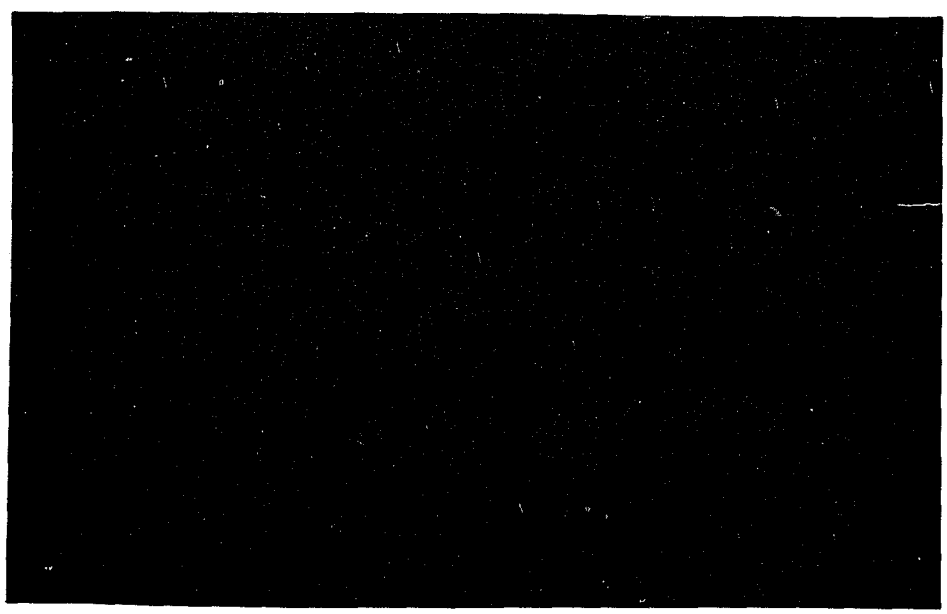


FIGURE V-11

CONTOUR DISPLAY OF GGT FLUORESCENCE AND LIGHT SCATTER FOR LYMPHOID CELLS FROM A LEUKEMIC AND NORMAL SUBJECT

GGT fluorescence versus low angle scatter is displayed for an ALL patient with 37% blasts and 54% lymphocytes (A) and for lymphocytes from a normal control (B). The 256 channels for each parameter are compressed to make a 64x64 array. Approximately 20,000 cells were analyzed, after incubation with GGT reaction mixture. Shaded areas and sequential contours represent increasing number of cells; dots are displayed for all channels containing 2 cells, while contour lines are shown for 5 or more cells.

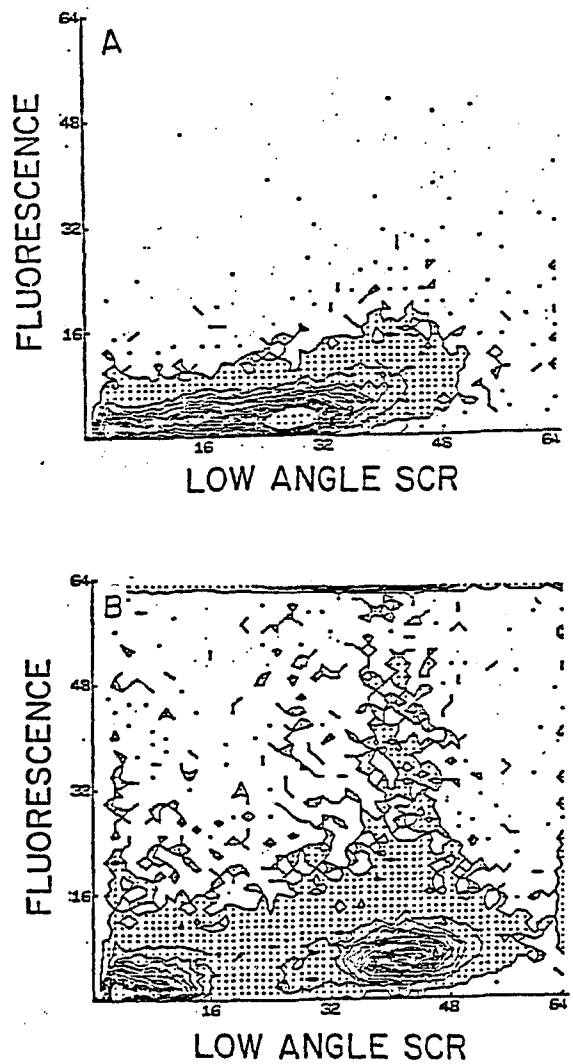
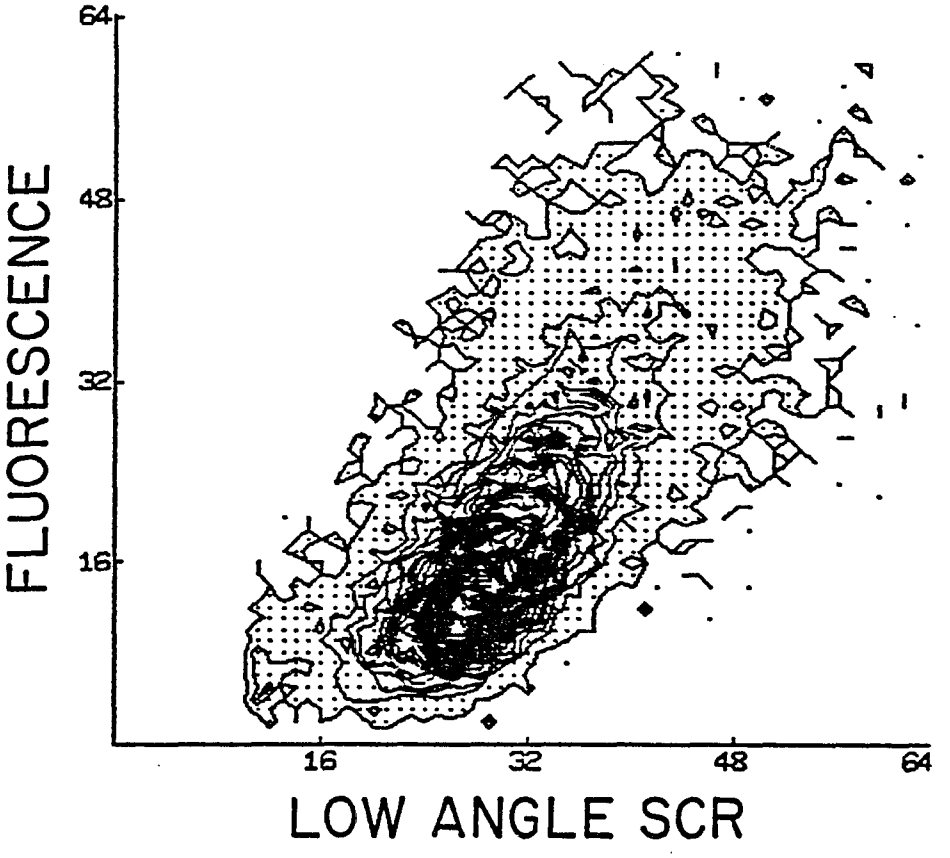


FIGURE V-12

CONTOUR DISPLAY OF GGT FLUORESCENCE AND LIGHT SCATTER FOR ALL LYMPHOID CELLS

GGT fluorescence versus low angle scatter is displayed for an ALL patient with 30% blasts and 45% lymphocytes, as determined by standard hematological parameters. The 256 channels for each parameter are compressed to make a 64x64 array. Approximately 20,000 cells were analyzed, after incubation with GGT reaction mixture. Shaded areas and sequential contours represent increasing number of cells; dots are displayed for all channels containing 2 cells, while contour lines are shown for 5 or more cells.



DISCUSSION

The possible involvement of GGT in leukocytopoiesis was previously suggested by the observation that DNA synthesis in lymphocytes exposed to pokeweed mitogen was preceded by a rise in GGT activity (Novogradsky et al., 1976). Previous results (Koss and Greengard, 1982), measured in homogenates of fractionated leukocytes from rats bearing a mammary carcinoma 5A (MC) indicated the relevance of GGT in granulocytosis; 10 times higher than normal levels of this enzyme were found in the granulocyte (but not lymphocyte) fraction of circulating WBCs. While measurements of GGT activity in these cells following total cell disruption provided the necessary quantitative data on whole cell populations, it was incapable of assessing any heterogeneity within them with respect to the cells' GGT activity. Using this model system (i.e., lymphocyte and granulocyte fractions from animals bearing the MC tumor) we tested, therefore, whether the fluorimetric method of Dolbeare and Smith, 1977, which reavealed the GGT activity of hepatocytes in hyperplastic areas of the liver in carcinogen treated animals (Vanderlaan et al., 1979) was sensitive enough to separate granulocytes from lymphocytes on the basis of their different GGT activity. The answer obtained after appropriate adaptation of the method was positive. Flow cytometric analysis and sorting of leukocytes identified the lower GGT fluorescent cells as lymphocytes, while those rich in GGT fluorescence as

granulocytes. In addition, it revealed an unexpected heterogeneity in the peripheral granulocytes, dividing these GGT rich cells into two distinct subpopulations.

The advantage of simultaneous assessment of GGT fluorescence with low angle and 90 degree scatter is that more distinct subpopulations can be identified from a heterogeneous sample than revealed by one parameter measurement. This is demonstrated by the detection of three distinct cell clusters of rat blood leukocytes (Figure V-5F) and bone marrow cells (Figure V-8B) by combination GGT fluorescence and low angle light scatter measurements, instead of only two populations by either fluorescence analysis alone (Figures V-2 and V-6, respectively) or by cell size distribution analysis (Figure V-5A-C). Not only qualitative differences, such as the presence or absence of GGT fluorescence, but also relatively small quantitative differences of enzyme activity in a given cell cluster can be evaluted (Figure V-5E). The location of each cell cluster in these three-dimensional displays under standardized conditions seems to be highly characteristic for given subpopulations of cells. Almost identical distributions were obtained for peripheral blood and bone marrow cells from an animal bearing a large tumor (c.f. Figure V-5F and V-8B). While sorting (Figure V-4) of GGT rich and poor subpopulations permitted the identification of cells, extension of analysis to three parameters (fluorescence, low angle scatter and 90 degree

scatter) (Figures V-3 and V-7) can confirm the identity of the cells without sorting.

As described in the previous Chapter, we found that the lymphocyte fraction from ALL subjects had diminished GGT activity. The few cases in which it was possible to isolate blasts from this fraction showed that the blasts had barely detectable activity. In addition, in several cases the GGT activity of the non-blastic lymphocytes was shown to be subnormal.

Normal human blood cells (red blood cells, lymphocytes and granulocytes) can be distinguished from one another using light scatter profiles alone. The profiles do not distinguish however, non-blastic lymphocytes from blasts, due to the overlap in cell size of the leukemic population. The use of the GGT reaction as a fluorescent probe should not only achieve this distinction, but also that between lymphocytes with normal as opposed to subnormal GGT activity. This test would be an ideal aid in the detection of maldifferentiated, morphologically normal leukemic lymphocytes, thus providing the clinician with an objective parameter for assessing residual leukemic cells during remission and subsequent relapse of patients. While flow cytometry did reveal the generally lower GGT fluorescence of cells in the whole lymphocyte fraction from ALL than from normal children, this reaction was not sensitive enough in most cases to separate from one another blasts, lymphocytes with a normal GGT content, and non-

blastic leukemic lymphocytes, and thus can not be used for the above purpose. In future studies of the relationship of GGT expression to cell heterogeneity and differentiation, it may be necessary to use fluorescent antibody against GGT (Chen and Haskill, 1984) for multiparameter flow cytometric analysis and sorting of lymphocytes from ALL patients.

CHAPTER VI

MITOGEN ACTIVATION OF LYMPHOCYTES FROM ALL SUBJECTS

INTRODUCTION

Studies under Chapter III and IV showed that in the blood lymphocyte fraction of children with ALL the specific activity of thymidine kinase (TK) is significantly higher, while that of γ-glutamyltranspeptidase (GGT) is lower, than in control subjects. The deviant concentrations of these enzymes were also apparent in lymphocytes physically separated from the blasts, and in the blood lymphocyte fraction of relapsed or untreated ALL patients whose circulation (unlike bone marrow) showed no or minimal blast count. It is of interest, therefore, to ask whether ALL lymphocytes, despite morphological normalcy, exhibit signs of functional impairments.

One approach to this question, examination of the effect of plant-derived mitogens <u>in vitro</u>, has been profitably used in studies of chronic lymphocytic leukemia (CLL) (Lamberson et al., 1984; Johnstone et al., 1982). The results (detailed under Discussion) revealed diminished or delayed mitogen response in terms of blastic cell formation, antibody production, and thymidine incorporation into DNA. In the investigations on numerous ALL subjects (Zusman and Nesbit, 1979; Debiec-Rychter, 1983), however, thymidine incorporation was the only parameter measured, so that whether their lymphocytes do or do not show a normal response to mitogen stimulation was not clear. The present study is the first to employ several criteria for comparing the response that mitogens evoke in lymphocytes from ALL

and normal subjects.

RESULTS

A 1976 study from Meister's laboratory disclosed a new manifestation of mitogen action; they report that concanavalin A (Con A), and to a lesser extent other mitogens, stimulate GGT expression in normal lymphocytes as well as in some leukemic cell lines. This phenomenon, hitherto not tested for in primary cultures of cells from subjects with lymphoproliferative diseases, was of particular interest in view of our aforementioned studies on GGT deficiency in lymphocytes from ALL subjects.

Incubation of normal lymphocytes for 3-4 days with (as compared to without) optimal amounts of pokeweed mitogen (PWM) resulted in increased GGT activity (Table VI-1). The values, already elevated at 48 hour but not yet at 24 hours (Figure VI-1), showed no further rise after longer (4-6 days) incubation. Lymphocytes from ALL subjects did not show this GGT response at any time point, while their TK activity (like that of normal lymphocytes) was increased appreciably (Table VI-1).

Figure VI-1 shows that the increase in normal lymphocyte TK (like in GGT) activity and thymidine incorporation (Tinc) became evident between 1-2 days of incubation with PWM, while the percent of blastic cells formed was already maximal (49%) by 24 hours.

At the time of taking blood samples from the ALL subjects studied in this chapter, there was a high blast count (over 25%) in their bone marrow. In the blood,

TABLE VI-1

GGT AND TK ACTIVITIES IN NORMAL AND ALL LYMPHOCYTES IN THE ABSENCE AND PRESENCE OF POKEWEED MITOGEN

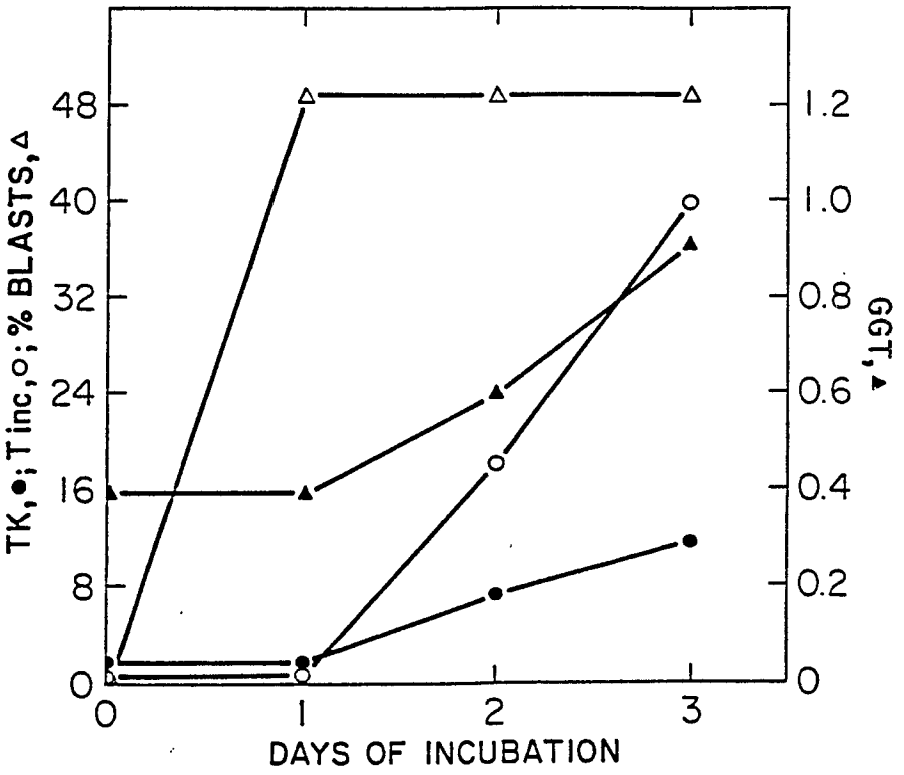
	GGT		TK	
	Normal	ALL	Normal	ALL
-PWM	0.6±0.1(3)	0.2±0.1(5)	1.7±0.3(3)	3.0±1.7(5)
+PWM	1.1±0.5(3)	0.2±0.1(5)	11.8±2.3(3)	15.8±15.2(5)

The indicated enzymes were assayed after 3 days incubation in the absence (-) and presence (+) of PWM. Means±SD (with the number of individuals in parentheses) are expressed in units per 10^6 cells.

FIGURE VI-1

EFFECT OF POKEWEED MITOGEN ON NORMAL LYMPHOCYTES

GGT activity (▲), TK activity (●), (^{3}H) thymidine incorporation (Tinc) (○), and % blasts formed (△) were determined in the same lymphocyte fraction (from a normal subject) after 0-3 days of incubation with PWM. Enzyme activities are expressed in units per 106 cells, Tinc is given as cpm per 106 cells.



however, the percent of blasts was negligible in 3 out of the 5 cases (Table VI-2, column 4). At the beginning of incubation of isolated lymphocyte fractions, or after 1-4 days of incubation without PWM, no blastic cells were present; blast counts were 35-80% in 4 out of 5 cases, when incubated 3 days in the presence of PWM, and this percentage (as indicated by cases 1 and 2) was already present at 24 hours.

The control values for thymidine incorporation showed wide individual variations but increased considerably in each case during incubation with PWM. At 3 days the average value, $36.3 \pm 22.6(5)$, was similar to that for normal lymphocytes, $39.8 \pm 37.6(3)$. Its variation among ALL subjects did not seem to bear a quantitative correlation to TK. For example, thymidine incorporation values on day 3 would clearly rank the 5 ALL individuals in a different order than did the increase in TK activities measured in the same incubates (cf. columns 6 and 7, Table VI-2). Also, TK increased considerably in Case 2 on the 3rd day of incubation with PWM, whereas thymidine incorporation did not.

The study showing the ability of mitogens to stimulate lymphocyte GGT expression (Novogrodsky et al., 1976) suggested that Con A is more effective in this respect than other mitogens. Our results on normal lymphocytes were not significantly differnt from those obtained with PWM (cf. Table VI-1 and VI-3). They showed, however, that Con A

TABLE VI-2

TK ACTIVITY, (^{3}H) THYMIDINE INCORPORATION, AND % BLASTS FORMED IN ALL LYMPHOCYTES CULTURED IN THE ABSENCE AND PRESENCE OF POKEWEED MITOGEN

ALL Cases	WBC per ml	Lymph %	Blasts %	PWM	TK	Tinc	% Blasts Formed
1	6.1	46	<1	-	5.8	4.0	0
				+(1d)	7.5	16.5	80
				+(3d)	36.9	29.7	ND
2	3.1	57	<1	-	2.6	0.2	0
				+(1d)	0.6	0.3	61
				+(2d)	2.6	5.3	55
				+(3d)	26.4	2.3	ND
3	26.6	30	58	-	2.7	19.2	61
				+(3d)	8.8	78.8	50
4	10.2	66	<1	-	1.3	0.2	0
				+(3d)	4.3	44.6	35
5	2.7	82	10	-	2.7	0.6	0
				+(3d)	2.4	26.1	ND

WBC (per ml blood x 10^6) and % of Lymph (Lymphocytes) and of Blasts, were determined by standard hematological methods on one portion of each blood sample. From the remainder of each sample, the lymphocyte fraction (containing lymphocytes plus blasts, if any) was separated, and incubated with PWM. TK activity, thymidine incorporation (Tinc), and % blasts formed were determined after 1, 2, and 3 days of incubation without (-) and with (see plus sign and number of days in parentheses) of PWM. In the absence of PWM, the values were the same after 0, 1, 2 or 3 days of incubation. Units of TK activity and Tinc (cpm) are expressed per 10^6 cells.

(unlike PWM) did cause some stimulation of GGT expression in lymphocytes from ALL subjects (Table VI-3).

The time course of increase in GGT, TK, thymidine incorporation, and blastic cell formation, in normal lymphocytes incubated with Con A (Figure VI-2) were indistinguishable from those shown in Figure VI-1; blast formation was again the earliest event. Corresponding information for lymphocytes from ALL subjects incubated with Con A is shown in Table VI-4. The ALL cases are listed in the order of decreasing TK activity on day 3, and it is again apparent that this order is not identical to that based on thymidine incorporation determined in the same culture. The mean values in ALL lymphocytes for thymidine incorporation (116.2±85.3(6)) as well as for TK on day 3 (115.7±176.3(6)) were higher than the normal means (91.8±94.8 and 27.5±6.4, respectively) (and also higher than for ALL lymphocytes incubated with PWM, see Table VI-1). Individual variations in Con A stimulated thymidine incorporation were wide; it may be seen however, that the values for 3 out of the 6 patients were much higher than the mean for normal lymphocytes, 91.8±94.8(3), (see also Figure VI-2).

TABLE VI-3

GGT AND TK ACTIVITIES IN NORMAL AND ALL LYMPHOCYTES IN THE ABSENCE AND PRESENCE OF CONCANAVALIN A MITOGEN

	GGT		TK	
	Normal	ALL	Normal	ALL
-Con A	0.6±0.1(3)	0.4±0.2(6)	1.7±0.3(3)	2.7±1.7(6)
+Con A	1.4±0.4(3)	1.0±0.5(6)	27.5±6.4(3)	115.7±176.3(6)

The indicated enzymes were assayed after 3-4 days of incubation in the absence (-) and presence (+) of Con A. Means±SD (with the number of individuals in parentheses) are expressed in units per 10^6 cells.

FIGURE VI-2

EFFECT OF CONCANAVALIN A MITOGEN ON NORMAL LYMPHOCYTES

The results refer to the GGT activity (▲), TK activity (●), (3H) thymidine incorporation (Tinc) (o), and % blasts formed (△) were determined in the same lymphocyte fraction (from a normal subject) after 0-3 days of incubation with Con A. Enzyme activities are expressed in units per 106 cells, Tinc is given as cpm per 106 cells.

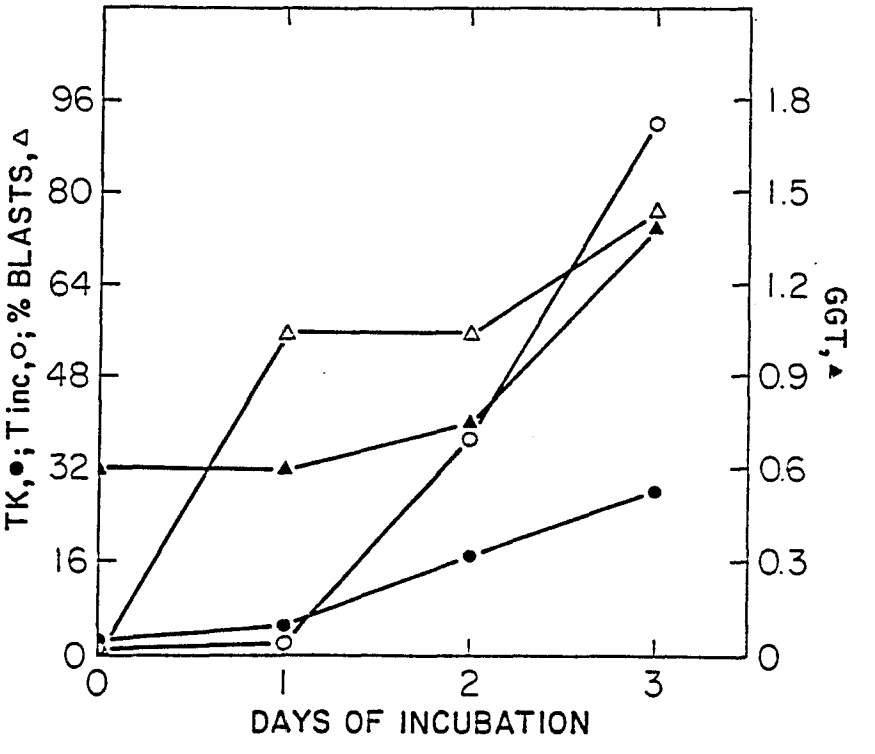


TABLE VI-4

TK ACTIVITY, (^{3}H) THYMIDINE INCORPORATION AND % BLASTS FORMED IN ALL LYMPHOCYTES CULTURED IN THE ABSENCE AND PRESENCE OF CONCANAVALIN A MITOGEN

ALL Cases	WBC per ml	Lymph %	Blasts %	Con A	TK	Tinc	% Blasts Formed
6	1.8	-	<1	-	1.1	0.2	0
				+(3d)	471.3	181.4	95
3	26.6	30	58	-	2.7	19.2	61
				+(3d)	89.7	223.8	90
1	6.1	46	<1	-	5.8	4.0	0
				+(1d)	24.7	55.8	85
				+(3d)	51.6	65.7	94
4	10.2	66	<1	-	1.3	0.2	0
				+(3d)	47.5	95.4	95
7	13.1	9	<1	-	2.5	15.0	0
				+(2d)	25.4	202.7	69
2	3.1	57	<1	-	2.6	0.2	0
				+(1d)	0.3	0.4	61
				+(2d)	16.7	17.6	55
				+(3d)	8.8	14.7	ND

TK activity, thymidine incorporation (Tinc), and % blasts formed were determined after 1, 2, and 3 days of incubation without (-) and with (see plus sign and number of days in parentheses) of Con A. For further details see Table VI-2, legend.

DISCUSSION

CLL, despite the accumulation of large numbers of lymphocytes in the blood, is accompanied by decreased immune competence (Miller and Karnofsky, 1961). Since the consequent recurring infections represent a major problem in the clinical management of the disease, efforts have been made to devise methods whereby the impaired lymphocyte function can be assessed *in vitro*. The most frequent approach has been to examine the response of CLL lymphocytes to incubation with plant derived mitogens, phytohemagglutinin (PHA) and PWM. The blast like cells formed were reported to be fewer than in the case of normal lymphocytes (Bernard et al., 1964; Lamberson et al., 1984), especially in CLL subjects with high WBC count (Rabinovitz et al., 1970). In more recent investigations, the effect of PHA and PWM was judged by thymidine incorporation into DNA; in CLL lymphocytes the magnitude of this response was reported to be similar to normal lymphocytes, but with a somewhat delayed onset (Davis and Rambotti, 1980). This delay was confirmed by a 1982 study of Johnstone et al., which also showed that the maximal thymidine incorporation attained varied considerably among different subjects and correlated inversely with the individuals' blood lymphocyte concentration *in vivo*.

In ALL, the condition that our investigations focussed on, less is known about mitogen stimulation than in CLL. Indications have been obtained that PHA or PWM has little

effect on lymphoid cells from ALL subjects with high WBC count in vivo (De Vaan et al., 1975; Debiec-Rychter, 1983), but normal controls were lacking. According to another study (which did include such controls), mitogens were without effect on the leukemic (i.e., blastic) cells themselves (Zusman and Nesbit, 1979), and the magnitude of their effect on the blood lymphoid fraction from different ALL individuals was directly proportional to the log of the non-blastic lymphocyte number (per ml) in their circulation in vivo. Whether these lymphocytes show an entirely normal response to mitogen stimulation was, however not clear. For thymidine incorporation has been the only criterion by which the stimulation was judged in the above and all other previous studies on ALL. It remained unknown, for example, whether the microscopically normal lymphocytes from the blood of such patients would be transformed to blast-like cells. The present results show that this transformation does take place to the same extent as in normal lymphocytes. However, stimulation of GGT expression failed to occur in most cases and the extent of the increase in TK and thymidine incorporation showed wide individual variations. Simultaneous assessment of several such changes, revealing deviant patterns of mitogen response, could thus provide additional means of evaluating the functional defects that morphologically normal lymphocytes from ALL subjects may exhibit.

CHAPTER VII

GENERAL DISCUSSION

Acute lymphocytic leukemia is the most frequent neoplastic disorder in children. It is characterized by abnormal lymphocyte proliferation in the bone marrow; a higher than the normal portion (over 50%) of the clonal progency remain stem cell in nature, which leads to progressive expansion of the population and to the appearance of blastic cells in the circulation.

At present 50% or more of the children with ALL will have prolonged remission and are potentially cured. Prolonged remission does not necessarily mean, however, that all cancer cells have been eradicated; relapse may occur after the termination of apparently successful chemotherapy. These children might be saved by resumption of treatment prior to clinically evident relapse, however, there are no means at present by which to detect the presence of residual cancer cells. Despite former assumptions of the uniqueness of certain antigens to leukemic cells, ALL markers expected to be leukemia specific are now known to be expressed in both normal and malignant cells. Another factor that prevents the cure of many ALL children is the emergence of a drug resistent cell population. This problem can sometimes be overcome by change to a more effective drug or combination of drugs. But again, by the time of perceiving the need for such a change it is usually too late, and relapse during treatment is almost always fatal. The realization that many of these children could be saved, and the availability of several

different treatment modalities, is fairly recent. It is not surprising therefore, that there has been relatively little success as yet it designing refined methods for early diagnosis and for monitoring treatment response.

In the last 20 years, with extensive comparison of different normal and abnormal tissues and more precise knowledge about the function of genes, it has become clear that the kinds and amounts of gene products are the primary determinants of the structural and functional characteristics of each tissue. It follows from this primacy that morphological abnormalities are always associated with deviations from the normal pattern of specific proteins or enzymes, but that the converse is not necessarily true: deviations in this pattern can occur without, or prior to, any change visible under the light or electron microscope. Therefore, biochemical analyses can provide the earliest indication of imminent disease, of changes in its course, and of individual variations in its manifestation. It has provided, for example, means for detecting precancerous changes in several organs and for monitoring the progression gastrointestinal and kidney cancers. A premise of the present investigation is that biochemical analysis of leukocytes from ALL subjects is essential not only for gaining insights into mechanism of malignancy but, that deviations in the quantitative pattern of enzymes thus identified would offer simple tests for changes in the patient's condition and for predicting

disease outcome.

ALL, like many other malignant diseases, can still not be diagnosed until the appearence of clinical or hematological signs. Biochemical studies on already manifest ALL, concerned primarily with identifying high or low risk groups, succeeded in devising immunological tests for specific cell surface markers (e.g. those for T-, B-, and pre-B-cell ALL) that predict disease heterogeneity inherent in differences of cell type. Quantification of appropriate proteins or enzymes (if exhibiting wide and meaningful variations) should have greater resolving power since, by definition, tests for cell type markers limited to "plus" or "minus" answers cannot reveal heterogeneity in disease manifestation that exists between ALL subjects of the same cell type class. Nor have the above qualitative markers been found useful for detecting changes in the conditions of the same individual. Objective assessment of this condition is thus restricted to microscopic criteria. In fact, a single criterion, the presence or absence of an appreciable number of blast cells, is routinely used for conclusive assessment of treatment response. This requires bone marrow aspiration, because the circulation of ALL subjects with active disease may be devoid of blasts and because, as is fully recognized, microscopy fails to detect the small blastic cells or maldifferentiated lymphocytes to be present to varying extents in ALL subjects.

Study of these cells would provide further insight

into mechanisms operating in leukemia but, no methods are available for their isolation. A small step in this direction was made by the present discovery of lymphocytes which, despite their morphological normalcy, exhibited GGT deficiency. There was no published method, however, for assay of GGT in intact leukocytes. A method designed for hepatocytes was, therefore, modified and adapted for applicability to study of leukocyte abnormalities evoked by a rat mammary carcinoma. Flow cytometric analysis and sorting confirmed the difference in GGT content between two rat leukocyte populations and revealed the existence of a hitherto unsuspected subpopulation. For the purpose of sorting human leukocytes the procedure requires further refinements. It confirmed, however, on whole cells the generally low GGT content of the lymphocyte fraction from ALL subjects. Also, the heterogenous distribution among the cells supported the contention that, in addition to blasts with minimal GGT activity, there is a morphologically normal lymphocyte subpopulation with partial GGT deficiency.

In view of the limitations of the morphological approach, efforts have been made to assess the integrity of lymphocytes by functional tests, comparing, for example, their response to mitogens with that of normal cells. Our studies in this connection provided new information about lymphocytes from normal subjects as well. We found that, unlike suggested in a report based on limited data,

stimulation of GGT expression (by PWM or ConA) does not precede (but probably follows) DNA synthesis, and the formation of blast like cells is an even earlier event. In lymphocytes from ALL subjects thymidine incorporation has been the only parameter studied in the past. The fact that they can give rise to blast like cells, (in as large number as do normal lymphocytes) is thus one new finding. The other - that PWM mitogen causes no or little stimulation of GGT expression in ALL lymphocytes - is a further indication of the defective nature of these lymphocytes. The magnitude of mitogen-evoked increase in TK and thymidine incorporation did not correlate with each other. Both varied widely among individuals, so that their measurement (together with GGT) after incubation with mitogen may reveal prognostic differences between individual ALLs of the same cell type class. The number of cases we subjected to mitogen study, however, was too small to establish correlations with clinical status.

When averaged over the whole ALL population, the specific activity of TK (per mg protein) in the blood lymphoid fraction showed a statistically significant correlation to the number (per ml blood) of blasts. The closer correlation it showed, however, to the number of lymphocytes (which include the small blasts cells whose number reflect proliferative rate in the bone marrow) suggests that the rise in TK may signal subsequent blast crisis. The ratio of TK1 to TK2 (though its declined during

remission and rose at relapse) showed no close correlation to hematological parameters and encompassed a narrow range. Therefore, this isozyme ratio, though of general importance to the understanding of gene expression in actively dividing cells, is of little practical value to assessing disease state as it varies during treatment or among different ALL subjects at diagnosis. On the other hand, simple quantification of TK (i.e., with only ATP as the phosphate donor) appears to constitute a valuable diagnostic tool. Simultaneous measurement of GGT (which shows an inverse quantitative correlation to TK) would, of course, render any conclusion more reliable. Observations on relapsed patients (judged as such by blasts in the bone marrow) in whom the blood lymphocyte TK rose and GGT declined before the appearence of blasts in the circulation, suggests that these enzymes are sensitive indicators of relapse. Whether they predict relapse early enough for successful change to alternative therapeutic agents, and whether they obviate the need for bone marrow aspiration, remain to be established.

We do not know whether the enzymes showing deviant concentrations in ALL are regulated by defective genes; one cannot even assume that the lymphocytes exhibiting these deviations are derived from the leukemic clone. For the conditions prevailing in the leukemic bone marrow may well affect normal progenitor cells, altering their rate of maturation or proliferation, which can result in the

release to the circulation of leukocytes with incomplete biochemical differentiation. Indeed, even the granulocytes of ALL subjects showed significantly diminished GGT activity. This deficit, restored during remission and present again at relapse, varied in magnitude. In about one-third of the newly diagnosed or relapsed pre-B ALL children, the circulatory granulocytes' GGT activity was only 10-20% of normal. It seems, therefore, that the presence or absence of this sign of functional maldifferentiation in granulocytes is a factor in the heterogeneity of disease manifestation among subjects with apparently the same type of ALL, and that measurement of GGT in the circulatory granulocytes, as well as lymphocytes, may be useful for monitoring the efficacy of chemotherapy.

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