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CHARACTERIZATION OF A NOVEL MYELOID DIFFERENTIATION ANTIGEN ASSOCIATED WITH ACUTE MYELOGENOUS LEUKEMIA

By

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ABSTRACT

A monoclonal antibody has been developed that detects a unique cell surface antigen (NHL-30.5) with a molecular weight of 180,000 expressed on the human acute promyelocytic cell line HL-60. In addition to HL-60 and other AML cell lines, the antibody reacts with a significant proportion of hemopoietic cells from 40/48 patients with acute myeloid leukemia (AML), and on a variety of other hematologic disorders characterized by the presence of immature myeloid blast cells. In contrast it does not react with normal mature hemopoietic cells, including lymphocytes, monocytes, granulocytes, erythrocytes, platelets, and splenocytes. Only one of 15 acute lymphoblastic leukemias has demonstrated reactivity (weakly) and all lymphoid cell lines tested have been uniformly negative. Reactivity with cells from patients in the chronic phase of chronic myeloid leukemia (CML) is also rare (7/26) and the number of NHL-30.5 positive cells is low (<20%). The acute phase of CML is strongly NHL-30.5-positive if the blast crisis is of the myeloid variant but is clearly negative in lymphoid blast crisis.

Analysis of normal differentiating bone marrow cells and mature peripheral blood mononuclear cells stained indirectly with the NHL-30.5 monoclonal antibody and FITC-second antibodies did not reveal a distinctly positive population. However, the cells with the highest fluorescence intensities (comprising 5% of the total population) sorted on a fluorescence activated cell sorter were highly enriched in both erythropoietic (CFU-E/BFU-E) and granulopoietic (CFU-C) progenitors. It therefore appears that the NHL-30.5 antigen is not an AML-associated marker but rather a normal myeloid differentiation antigen that is expressed on immature myeloid cells. Consistent with this hypothesis is the observation that a number of AML-

derived cell lines that are blocked at an early stage of maturation lose NHL-30.5 expression when they are induced to terminally differentiate. These results support the concept that undifferentiated myeloid progenitors accumulate in AML patients due to a block in their capacity to differentiate into the stages characterized by loss of the NHL-30.5 antigen. The NHL-30.5 monoclonal antibody identifies a previously undescribed progenitor cell antigen and is potentially a useful reagent to differentiate myeloid leukemias from lymphoid leukemias, particularly in the acute phase of CML.

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LIST OF ABBREVIATIONS

ALL - Acute lymphoblastic leukemia
AML - Acute myelogenous leukemia
BFU-E - Burst forming unit-erythroid
BM - Bone marrow
BPA - Burst promoting activity
BSA - Bovine serum albumin
CALLA - Common acute lymphoblastic leukemia antigen
CFU-C - Colony forming unit-culture
CFU-E - Colony forming unit-erythroid
CFU-GM - Colony forming unit-granulocyte/macrophage
CFU-GEMM - Colony forming unit-granulocyte erythrocyte magakaryocyte macrophage
CFU-S - Colony forming unit-spleen
CLL - Chronic lymphocytic leukemia
CMML - Chronic myelomonocytic leukemia
CML - Chronic myelogenous leukemia
CR3 - Complement receptor 3
CSF - Colony stimulating factor
CTL - Cytotoxic T lymphocyte
DMEM - Dulbecco's modified Eagle's minimal essential medium
DMSO - Dimethylsulfoxide
DTH - Delayed type hypersensitivity
EBSS - Earl's balanced salt solution
EBV - Epstein Barr virus
EDTA - Ethylenediaminetetraacetic acid
Epo - Erythropoietin
ET - Essential thrombocytosis
FAB - French American British leukemia study group
FACS - Fluorescent activated cell sorter
FCS - Fetal calf serum
FITC - Fluorescein isothiocyanate
GaMIg - Goat anti-mouse immunoglobulin
G-CSF - Granulocyte colony stimulating factor
GM-CSF - Granulocyte macrophage colony stimulating factor
G6PD - Glucose-6-phosphate dehydrogenase
HAT - Hypoxanthine aminopterin and thymidine
HBSS - Hanks balanced salt solution
HBSS-Ca-Mg - HBSS minus calcium and magnesium
4-HC - 4-hydroperoxycyclophosphamide
HLA - Human Leukocyte Antigen
IL-3 - Interleukin 3
LAA - Leukemia associated antigen
LCM - Leucocyte conditioned medium
LFA-1 - Lymphocyte function antigen-1
LIA - Leukemia inhibitory activity
Mac-1 - the mac-1 antigen
MF - Myelofibrosis
MLC - Mixed lymphocyte culture
MPD - Myeloproliferative disease
My-10 - the My-10 antigen
NB-2 - monoclonal antibody against the transferrin receptor
NBT - Nitrobluetetrazolium

LIST OF ABBREVIATIONS CONTINUED

NHL-62.14 - monoclonal antibody against the transferrin receptor
NK - Natural killer
PBS - Phosphate buffered saline
PB - Peripheral blood
Ph^1 - Philadelphia chromosome
PHA - Phytohemagglutinin
PV - Polycythemia vera
RA - Retinoic acid
RaMIg - Rabbit anti-mouse immunoglobulin
Rh - Rhesus antigen
SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T200 - High molecular weight (200,000) leucocyte-common antigen
TdT - Terminal deoxynucleotidyl transferase
TPA - 12-O-tetradecanoylphorbol 13-acetate

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Darien and Ken:

"Think where man's glory most begins and
ends,
and say my glory was I had such friends"

W.B. Yeats (1865-1939)

What am I, Life? A thing of watery salt
Held in cohesion by unresting cells,
Which work they know not why, which never
halt,
Myself unwitting where their Master dwells?

John Masefield (1878-1967),
Sonnets, 14

C H A P T E R I

THE HEMOPOIETIC SYSTEM

1) OVERVIEW OF THE HEMOPOIETIC SYSTEM

The fully differentiated hemopoietic cells in the peripheral blood have a finite lifespan and are incapable of self-renewal. Since hemopoiesis must occur throughout the lifespan of an individual, continual replacement of these relatively short lived mature cells is maintained by a population of less differentiated hemopoietic cells. The hemopoietic system is thus comprised of a hierarchy of a diverse range of cells at different stages of differentiation. The developmental sequence of these cells has been known for some time and is based largely on the fact that the majority are in the later stages of maturation and are therefore morphologically recognizable as precursors of the granulocytic, megakaryocytic, or erythroid lineages. In normal adults, the primary sites for active hemopoiesis are restricted to bone marrow in the vertebrae, ribs, sternum, pelvis, scapulae, skull and extreme proximal portions of the long bones. The hemopoietic tissue within the marrow is compartmentalized by plates of bony trabeculae protruding into the marrow cavity, although under conditions of severe hematologic stress expanded output can be accomplished by increasing the proportion of hemopoietic tissue at the expense of the fat containing areas.

In order to maintain homeostasis the hemopoietic system must have the capacity to self-renew, and this ability resides in a population of hemopoietic stem cells. This stem cell pool provides cells for differentiation at variable rates according to the demand for functional blood cells, and at the same time maintains a relatively constant reserve of

stem cells. Stem cells thus possess two important features: a) an unrestricted differentiation potential, i.e. the capacity to produce cells representative of all blood cell lineages (1), and b) an extensive self renewal capacity, i.e. the ability to give rise to new stem cells that are also pluripotent (2).

Early evidence for the existence of these primitive stem cells came from studies showing that mouse bone marrow contained a class of cells capable of repopulating lethally irradiated mice with a functional hemopoietic system (3,4). Furthermore, Barnes et al (5) reported that in some mice recovering from sublethal doses of irradiation, virtually all dividing cells in hemopoietic tissue (including lymphoid tissue) possessed the same unique radiation-induced chromosomal marker. Since these chromosomal abnormalities are generated randomly in single cells, the identification of the same marker in differentiated cells of a number of lineages suggested that the markers arose in a pluripotent stem cell. Further evidence that these stem cells, or at least a subpopulation of stem cells, possess both myeloid and lymphoid (T and B) differentiation potential has been inferred from several studies in the mouse system (6).

The existence of a stem cell common to both the myeloid and lymphoid lineages in man is supported from studies of clonal neoplastic disorders, particularly chronic myelogenous leukemia (CML) (7). Approximately 90% of patients with CML contain the Philadelphia (Ph^1) chromosome in their dividing marrow cells. The identification of this marker in red cell precursors, granulocytes, platelets and some B-lymphocytes (8,9) suggested that these cells were descendants of a pluripotent stem cell. This is further supported by studies with the X-linked glucose-6-phosphate dehydrogenase (G6PD) marker (see section on CML). Female patients with CML who are heterozygous at this

locus show only a single enzyme type in their hemopoietic cells (10,11), an observation that further documents the monoclonality of this disorder. Similarly, one patient with sideroblastic anemia was demonstrated to express a single G6PD isoenzyme in both T and B lymphocytes in addition to myeloid cells (12).

Since no morphological, cytochemical, or antigenic markers are known that are exclusively restricted to hemopoietic stem cells, the identification of these cells has relied heavily upon recognition of their progeny. Progress in the development of clonal assays for these primitive cells over the past 20 years has greatly facilitated analysis of the organization of the hemopoietic system and the factors that regulate hemopoietic stem cell differentiation. These assays are reviewed in the following two sections and their contribution to our current understanding of differentiation in the various hemopoietic lineages is summarized in Figure I (13).

2) ASSAYS

(A) In Vivo: The Spleen Colony Assay

In 1961 Till and McCulloch (14) described the first assay for the quantitative detection of primitive hemopoietic cells. This assay is based on the observation that when hemopoietic cells are injected intravenously into lethally irradiated syngeneic mice, a population of cells within the initial inoculum is capable of forming macroscopically visible nodules on the recipient spleen. The cell initiating these spleen colonies was termed CFU-S (colony forming unit-spleen). The widespread use of this assay as a method of measuring the size of the stem cell compartment is based on observations that the CFU-S appears to fulfill the criteria by which a cell is classified as a hemopoietic stem cell: a) Proliferative capacity: The size of the

individual colonies (approximately 10^5 to 10^7) reflects the extensive proliferative capacity of the cell that gave rise to the colony. b) Differentiation potential: Histological examination of individual spleen colonies revealed the presence of erythroid, granulocytic, and megakaryocytic lines of differentiation suggesting that the CFU-S was indeed a multipotent cell (1). Early studies indicated that some CFU-S may have lymphoid in addition to myeloid differentiative potential (15-18), although this has never been clearly established. The CFU-S is currently viewed as a stem cell restricted to myelopoiesis. c) Clonality: Evidence for the monoclonal origin of these colonies was obtained by generating spleen colonies with cells containing radiation-induced chromosomal markers. Unique karyotypes were observed in a number of colonies and the marker was found in a very high proportion of the metaphases obtained from each colony (19). These results were also confirmed using stem cell deficient (W/W^V) mice as recipients (1). d) Self renewal capacity: Analysis of CFU-S by serial transplantation studies has demonstrated that some of the cells within spleen colonies are themselves capable of spleen colony formation (2).

There are some limitations associated with the use of the CFU-S assay as a quantitative measure of stem cells however. Only a fraction of the colony-forming cells injected into an irradiated recipient will actually settle in the spleen (2), the remaining cells either dying or growing in the bone marrow. This problem is further compounded with the observation that more than one spleen colony may occasionally arise from a single CFU-S (5). There is also accumulating evidence that the CFU-S population, as defined by the spleen colony assay, is a heterogeneous population that can vary in both self-renewal capacity and differentiation potential (20).

In summary, although there is evidence to support the existence of a common lympho-myelopoietic stem cell, the precise location of the CFU-S within the hierarchy of hemopoietic cell differentiation has not been established. The CFU-S is clearly a cell with multipotent myeloid differentiation potential, but there is as yet no definitive evidence implicating this cell in lymphopoiesis.

(B) In Vitro: Assays for Clonogenic Progenitors

The advent of in vitro colony assays has provided much information about a population of cells that are intermediate between the stem cell and maturing effector cell compartments. Members of this intervening population are termed progenitors and they appear to differ from stem cells in that they lack self-renewal capacity and are restricted in their differentiation potential. Due to the considerable heterogeneity of all hemopoietic cell populations, the initial development of an agar cloning system capable of supporting the growth of granulocytic or macrophage colonies (21,22) was a major technical achievement that provided an in vitro system in which individual cell populations could be analyzed under controlled conditions. The assay involves the immobilization of hemopoietic cells in semi-solid medium containing appropriate nutrients, serum and growth factors. The proliferation of a single progenitor cell is therefore able to give rise to a clone of mature descendants sufficiently localized to be identified as a colony. The progenitors giving rise to colonies of granulocytes and macrophages were initially termed CFU-C (colony forming unit in culture) and more recently CFU-granulocyte/macrophage (CFU-GM). Subsequently the culture conditions that allow the proliferation of what appear to be the earliest committed cells in each myeloid differentiation lineage have since been

described (23). These studies have demonstrated considerable heterogeneity in each pathway of differentiation.

Colonies of erythroid cells were first documented using the plasma clot system (24). Subsequently the fibrin clot (25), methylcellulose (26), and agar systems have been used, although agar does not appear to support the later phases of erythroid differentiation very well (27). When an appropriate combination of nutrients and stimuli are provided, these semi-solid media are able to support the growth of colonies of hemoglobin synthesizing erythroblasts. One of the features characteristic of the colonies derived from the more primitive of these erythroid progenitors is their organization into discrete clusters. The migratory abilities of these progenitors during early cell divisions is lost when they enter the terminal stages of erythroid differentiation and this contributes to the formation of a multiclustered colony. The term BFU-E (burst-forming unit-erythroid) is used to designate the cell of origin. The definition of sequential stages of erythropoietic differentiation in the progenitor compartment is based on the decreasing proliferative capacity and the loss of migratory ability that accompanies the differentiative divisions. Different sized erythroid colonies have therefore been shown to detect progenitors in at least three stages of differentiation along the erythroid pathway (28,29). The most primitive BFU-E is the precursor of the large multiclustered colonies (>8 clusters) that consist of many erythroblasts (>1000). The more mature BFU-E give rise to smaller bursts containing 3-8 clusters of erythroblasts (30), and single or paired clusters of erythroblasts originate from a more differentiated cell type designated as the colony forming unit-erythroid (CFU-E). These CFU-E were the first erythroid colonies to be cultured in vitro and they are easily recognized as 1-2 tight clusters containing 8-50 cells.

All erythroid progenitors assayable in these clonal cultures appear to represent a continuum of different stages of differentiation. Further support for this concept of an erythropoietic hierarchy comes from studies demonstrating differences in the properties of cells that generate these different sized erythroid colonies. These include: differences in progenitor cell size, density, cell surface antigen expression, sensitivity to erythropoietin and leukocyte-derived factors, and normal cycling status (31).

The granulocyte progenitor, known as the CFU-C, is also the precursor for macrophages and is commonly referred to as the CFU-granulocyte/macrophage (CFU-GM) (23). Heterogeneity in this precursor population is well established and major subpopulations have been identified that differ in their buoyant density, proliferative capacity, cell cycle status, sensitivity to pathway-specific regulatory molecules (32), and cell surface antigenic phenotype (33). Since the proliferative activity of CFU-GM reflects the concentration and type of colony stimulating factor(s) present (34), difficulties are sometimes encountered when trying to subclassify these progenitors into different stages of maturation. Similar progressions of cell types of decreasing proliferative capacity have been identified for the megakaryocyte progenitor cell compartment (35).

Several of the more direct lines of evidence indicating that the myeloid colonies described above are derived from single colony-forming cells include:

1. microculture of single cells leading to colony formation (36).
2. male/female mixing experiments that produce colonies containing either a male or female karyotype (37).
3. Demonstration of a single G6PD isoenzyme in bone marrow colonies from a G6PD heterozygote (38).

The colonies described above detect progenitors that are restricted to a particular myeloid lineage. Culture techniques that permit the clonal growth of hemopoietic progenitors with multilineage differentiation potential have since been described by several groups of investigators (39-42). Colonies containing hemopoietic cells of several lineages were first described using cultures of fetal liver cells in agar (39). Notably these mixed erythroid colonies grew in the absence of detectable erythropoietin, although it is now known that the conditioned medium from the pokeweed mitogen-activated spleen cells used in these cultures contained interleukin-3 (IL-3). Since most studies report these colonies to contain granulocytes, erythrocytes, macrophages and megakaryocytes the cell of origin has been termed a CFU-GEMM. The presence of mast cells and eosinophils in clonally derived mixed colonies have also been reported (43). The lymphopoietic potential of CFU-GEMM is supported by a number of studies indicating the presence of lymphoid cells bearing either B or T cell markers in mixed colonies (44-48). G-6PD analysis of the myeloid and T lymphoid cells within these colonies has established that the colonies originate from a single lympho-myelopoietic progenitor (rather than more than one clonogenic cell) (46). Recently, Nakahata et al (49) documented the single cell origin of human mixed colonies that express various combinations of myeloid cell lineages (neutrophil, erythrocyte, macrophage, megakaryocyte, eosinophil, and basophil). These colonies contained combinations of cells in 2-5 lineages, demonstrating that there is considerable heterogeneity in the progenitor cell compartment that gives rise to human mixed colonies. These results are in agreement with studies of the single-cell origin of colonies expressing various combinations of cell lineages in the mouse system (50).

The identification of a progenitor capable of mixed colony formation in vitro raised the question of the precise relationship between this CFU-GEMM and the CFU-S in the hierarchy of hemopoietic differentiation (Figure I). A number of studies now suggest that the CFU-GEMM and CFU-S may represent partially overlapping populations: both possess similar buoyant densities and each acquire the characteristic of increasing density and smaller size associated with development (51-54). Similarities also exist in the proliferative state of CFU-S and CFU-GEMM (54-56) and there is a significant correlation between the number of spleen colonies and the number of mixed colonies obtained from individual spleen colonies (54). Direct evidence for this overlap has come from cell separation experiments where fetal liver CFU-S have been fractionated into two populations and the majority of the mixed colony forming cells segregated into only one of these fractions (57). The ability of CFU-GEMM to undergo self-renewal has also been documented (58). Evidence for a more direct relationship between CFU-GEMM and CFU-S was obtained by the demonstration that mixed colonies in vitro from both fetal liver (59) and adult bone marrow (60) contain CFU-S.

The existence of an in vitro colony forming cell more primitive than the CFU-GEMM has been suggested from the identification of unique colonies composed primarily of undifferentiated blast cells in the mouse (61). The progenitors of these colonies were designated S-cells ('stem' cells) and appeared to be more primitive than CFU-GEMM since replating of primary stem cell colonies revealed a high incidence of secondary stem cell colonies in addition to GEMM colonies. The human equivalent of the S-cell has been reported to exist in umbilical cord blood (62) but its presence in adult hemopoietic tissue has not been documented. This may reflect the inability of present culture conditions to stimulate the growth of the human S-cell.

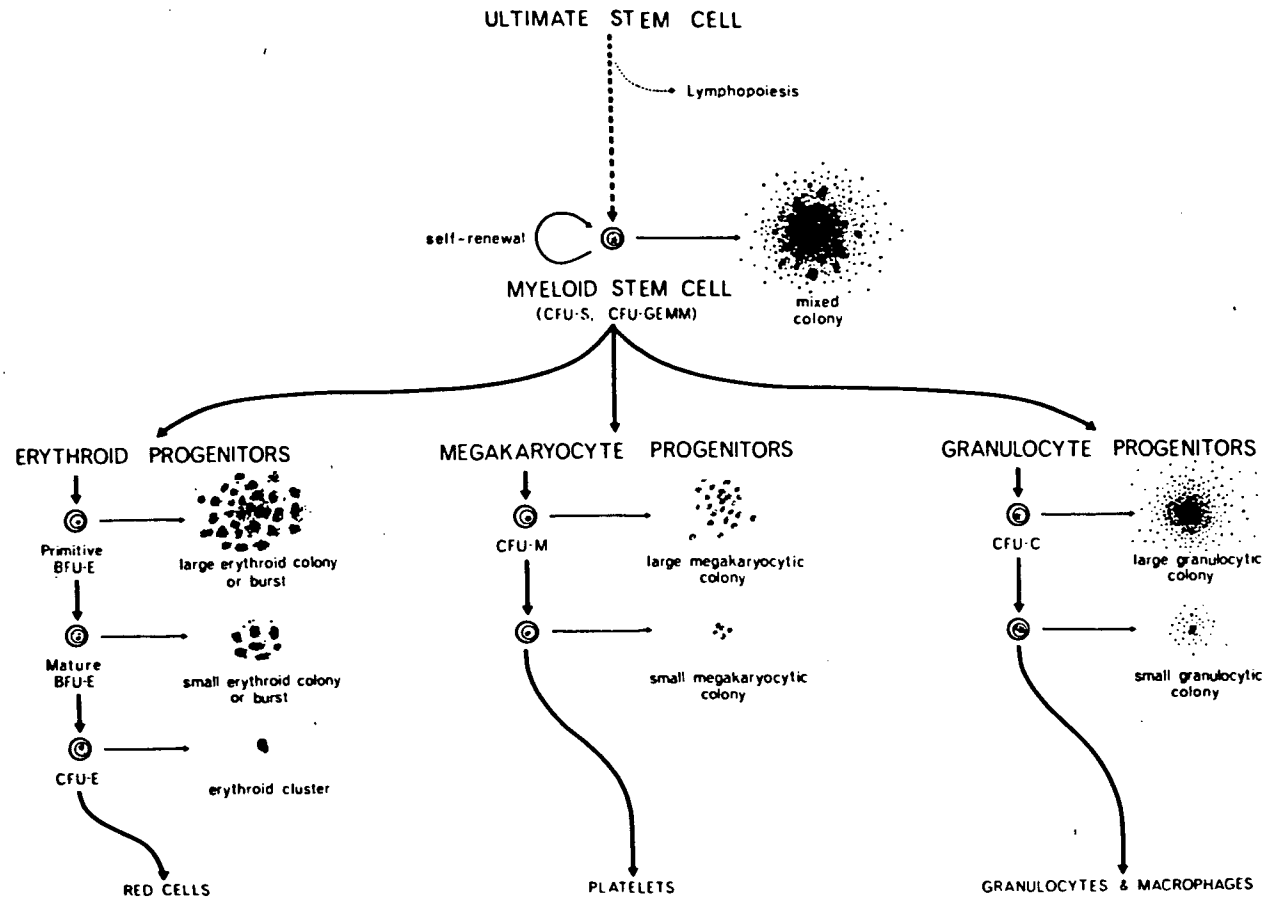

FIGURE I Diagrammatic representation of the hierarchy of hemopoietic progenitor compartments currently identified by colony assay procedures. From ref (13).

Further evidence supporting the existence of a stem cell more immature than the CFU-GEMM comes from studies with the long term bone marrow culture system. Successful long-term cultures were generated from marrow suspensions depleted of Ia-positive cells suggesting that, in contrast to CFU-GEMM, the cells initiating long-term cultures are Ia-negative (63-65). Successful long-term cultures can also be generated from marrow cells treated with the alkylating agent 4-hydroperoxy-cyclophosphamide (4-HC). Since CFU-GEMM are known to be highly sensitive to this drug (66,67), it appears that another stem cell (not represented by CFU-GEMM) is resistant to 4-HC, presumably because it is not in active cell cycle (66). This drug has also been used in the in vitro purging of bone marrow during autologous bone marrow transplantation. Bone marrow treated with 4-HC (at levels that are lethal to CFU-GEMM) can reconstitute full hemopoietic function in supralethally irradiated patients (68,69) suggesting that the stem cell responsible for hemopoietic reconstitution is 4-HC-resistant.

3) REGULATION OF STEM CELL DIFFERENTIATION

A) Regulatory Molecules

The development of in vitro assays for hemopoietic progenitor cells has provided a unique system for the investigation of the regulation of hemopoiesis. The importance of such regulatory mechanisms is particularly evident when one considers the short half lives of the circulating peripheral blood cells. The hemopoietic system is a cell renewal system which balances cell proliferation and differentiation at rates proportional to the demand for these fully differentiated cells and is therefore responsible for maintaining the number of circulating cells within a narrowly defined range. The precision and flexibility of the marrow in meeting ongoing blood cell

requirements and in responding to specific hemostatic stress implies that the proliferation of this tissue is modulated by sensitive biological control mechanisms. The regulation of this developmental process is thought to reside in a group of hemopoietic growth factors and some ill-defined interactions with marrow stromal cells. Those growth factors involved in the regulation of the early stages of hemopoiesis stimulate the proliferation and differentiation of cells capable of forming more than one blood cell type and have been termed multilineage growth factors. Those controlling the later stages act on cells with a more restricted differentiation program and are therefore considered to be lineage-specific growth factors (70).

The Colony Stimulating Factors

The identification of a factor required for the proliferation of granulocyte/macrophage progenitors in semi-solid medium provided the first candidate for a pathway-specific regulator of hemopoiesis. The proliferation of CFU-GM *in vitro* was shown to be dependent on a sufficient concentration of a protein released by feeder layer cells (21,71) or conditioned medium from cultures of these feeder layers (72,73). Further analysis of this conditioned medium showed that its biological activity resided in a family of glycoproteins termed colony stimulating factors or CSF's (74). The use of conditioned media to facilitate the *in vitro* growth of hemopoietic cells has since provided the conditions in which other myeloid progenitor cell types can be stimulated to form colonies (23). Purification of the different biological activities present in this conditioned medium has revealed a series of growth factors which are progressively restricted in their biological activity and target cells (75). Interestingly, the CSF's can be produced by most tissues but they are distinguished from each other in their unique specificity for subsets of hemopoietic cells. GM-CSF, for example is a multilineage growth

factor capable of stimulating the proliferation and development of granulocyte and macrophage progenitor cells. This factor has been isolated from both lung tissue (76) and pokeweed mitogen-stimulated spleen cells (77), although its precise role in vivo is not clearly established. For routine human cell culture purposes the usual source of this factor is leukocyte-conditioned medium (76) or placental-conditioned medium (79).

The growth factors G-CSF and M-CSF are more restricted in their activities, stimulating predominantly subsets of granulocyte (80) or macrophage colonies (81) respectively. The concept of lineage and stage-related growth factors is not absolute however, since both GM-CSF (82) and G-CSF (83) have been shown to support the early divisions of pluripotent precursors, although they lack the ability to support the terminal proliferative and differentiative events in lineages other than the granulocyte/macrophage pathway. In addition, G-CSF is considered unique among the CSF classes in its ability to induce terminal differentiation in murine myeloid leukemia cell lines (84).

It is now possible to clearly distinguish on both molecular and functional grounds, four major classes of CSF in the mouse: GM-CSF, G-CSF, M-CSF, and IL-3 (74,85). These factors have been purified to homogeneity (74,86-88) and in the case of GM-CSF and IL-3, molecularly cloned (89-91). Factors stimulating both eosinophil and megakaryocyte colony formation in agar cultures of bone marrow have also been described (92). In contrast to the murine system, human CSF's are not as well characterized, although human analogues with biological activity similar to mouse GM-CSF (93), M-CSF (94), G-CSF (95) and IL-3 (96) have now been identified and molecular cloning of these factors is expected in the near future.

Erythropoietin and IL-3

Since CFU-E show a marked dependence on the presence of erythropoietin (Epo) in the culture medium, it has long been assumed that cells at this level of erythropoietic differentiation are responsive to Epo (31). In contrast to the CSF's, however, this hormone has clearly been demonstrated to have a physiological role in stimulating erythroid differentiation in vivo (97). The major effect of Epo is generally considered to be from the mature BFU-E stage to the nondividing erythroblast stage, although it is not certain whether it acts as a mitogenic stimulus or a survival factor (31).

The search for factors other than Epo which might influence the early stages of erytropoiesis has identified a molecule (IL-3) that may act at the level of pluripotent progenitor cells (98) and early committed erythrocytic precursors (primitive BFU-E) (29). The effects of this factor appear to be additive with those of M-CSF and erythropoietin in that IL-3, in combination with Epo or CSF-1 (M-CSF) yields much larger colonies containing erythroid or monocytic cells than Epo or CSF-1 alone (70). A number of investigators have identified factors with similar biological activities and current evidence suggests that the activity of these factors resides in the same macromolecule. These factors include: IL-3 (99), burst-promoting-activity (BPA) (100), hematopoietic growth factor (HCGF) (101), P-cell growth factor (102), and multi-CSF (103). The mechanism of action of this molecule has not been identified but is an area currently under active investigation (104). For in vitro clonogenic assays the source of IL-3 is usually conditioned medium from agar or lectin-stimulated leukocyte-rich fractions of human blood, or from pokeweed mitogen-stimulated mouse spleen cells for mouse progenitors. Available evidence suggests that it is the T-lymphocytes in these tissues that produce this factor in response to either mitogens or specific antigens (105,106).

Detection of another multilineage growth factor has recently been reported. Initial studies identified a factor in conditioned medium that conferred upon very primitive cells the ability to respond to CSF-1 (M-CSF) (107). This factor, termed hemopoietin 1, has recently been purified and shown to be distinct from IL-3 (108). The effect of this factor is synergistic, requiring the presence of other growth factors and having no colony-stimulating activity by itself.

It seems likely that the control of hemopoiesis is accomplished by appropriate combinations of positive and negative influences. The possible involvement of a variety of inhibitory factors that may regulate hemopoiesis is a controversial issue and conflicting reports have been obtained. A number of potential physiologic inhibitors have been identified that are thought to act either directly on progenitor populations or indirectly by modulating CSF release. Definitive evidence that such inhibitors play a role in the regulation of hemopoiesis in vivo is still lacking however.

There can be no doubt that there are a number of distinct molecular species that are necessary for the growth and differentiation of hemopoietic cells in culture. With the exception of Epo however, the natural physiological role of these factors, both stimulatory, and inhibitory, in the regulation of the cell types that they appear to influence in vitro has yet to be established. Whether inappropriate expression of these growth factors plays a role in leukemogenesis is a question that should be answered in the near future.

(B) Regulatory Role of the Microenvironment

The intimate structural association between hemopoietic and stromal cells in the marrow suggests that the stromal cells may influence hemopoietic

development possibly by supplying the necessary cell matrix and diffusible growth factors required for hemopoiesis. The precise nature of these interactions is unknown and experimental evidence for such a regulatory role is largely circumstantial.

The initial observations that CFU-S developing in the spleen display a predominance of erythropoiesis, while those developing in the bone marrow display a predominance of granulopoiesis suggested that CFU-S differentiation is influenced by its microenvironment (109). Transplantation of marrow stroma into the spleen or the transplantation of spleen stroma subcutaneously, demonstrated that each type of organ stroma regenerated its distinctive ability to support hemopoiesis and control the differentiation of pluripotent stem cells.

The most cited example of a defect in the interaction between stroma and hemopoiesis is the genetically determined macrocytic anemia in mice carrying the Steel mutation (Sl/Sl^d). The hemopoietic abnormality in these mice appears to reflect a stromal defect rather than a stem cell defect since the anemia may be cured by the transplantation of a normal spleen but not by transplantation of marrow stem cells (110).

An approach to the analysis of stromal and hemopoietic cell interactions has been the development of a liquid culture system that supports the proliferation of CFU-S for several weeks. The ability of these long-term bone marrow cultures to maintain the self-renewal and differentiation potential of the CFU-S is thought to reflect the presence of a complex adherent layer that presumably serves as the <u>in vitro</u> equivalent of the marrow stroma (111). In confirmation of the <u>in vivo</u> studies on the Sl/Sl^d mouse, Dexter and Moore (112) demonstrated that Steel-hemopoietic cells could be supported by a normal marrow-derived adherent layer, but the Steel-derived adherent-layer was

incapable of supporting long term hemopoiesis. Recent studies of CFU-S stimulatory and inhibitory activities in the hemopoietic tissues of Sl mice have suggested that the Sl defect may reside in the production of a proliferation stimulator from an as yet unidentified regulator-producing cell (113).

For the purpose of this thesis it is sufficient to say that there is now an accumulating body of evidence to support the regulatory role of the microenvironment on hemopoiesis. Although the nature of these interactions is largely unknown, it is believed that close range cellular interactions, the elaboration of the previously discussed growth factors, and perhaps a host of short range stromal cell-derived factors that are as yet unidentified, may all be involved. Characterization of the cells comprising the stroma and its in vitro analogue (114) will be important in helping to elucidate the mechanism of these interactions.

(C) Theories of Stem Cell Commitment

Two theories are commonly cited to explain how stem cell self-renewal and differentiation is regulated. The first model, proposed by Curry and Trentin (109,115) is based on histological studies of the composition of spleen colonies and proposes that commitment of pluripotent stem cells is a result of specific inductive microenvironmental signals surrounding the stem cells. This theory finds support in studies demonstrating a regulatory role of the microenvironment on hemopoiesis (see previous section on the microenvironment). It is also possible that diffusible growth factors such as the CSF's may influence these commitment decisions. In experiments using paired daughter cells of individual granulocyte-macrophage progenitors, Metcalf showed that GM-CSF can directly influence both the rate of

proliferation and the differentiation pathway entered by the progeny of these granulocyte/macrophage precursor cells (116).

The second theory, developed by Till et al (117) proposes that the decision between self-renewal and commitment to differentiate is a stochastic event, and that only the probabilities of such events can be influenced by environmental factors. This model was initially developed to explain the marked variation in the distribution of CFU-S, CFU-C, BFU-E, and CFU-E in spleen colonies (2). Such variation is consistent with various determination events occurring at random. An extension of this model proposes that stem cell commitment is governed by progressive and stochastic restriction in the differentiation potential of stem cells (118). This concept is supported by the identification of bipotent progenitors such as erythroid-eosinophil (119), granulocyte-macrophage (22), erythroid-megakaryocyte (60,120), neutrophil-erythroid (121), and oligopotent progenitors containing two or three lines of differentiation (43,50,122).

Further support for the stochastic concept comes from analysis of the differentiation of hemopoietic colonies derived from the two progeny of a single parent progenitor cell. The single progenitors used in these studies were isolated using a micromanipulation technique from blast cell colonies cultured from the spleens of 5-fluorouracil-treated mice (61). Eighteen to 24 hours later the paired progenitors were separated, replated, and the two colonies derived from each paired progenitor were analyzed for the presence of various combinations of lineages. The results clearly documented dissimilar patterns of differentiation in the two daughter cells, providing strong evidence for the stochastic model of stem cell differentiation (123). An extension of these studies also revealed disparate differentiation in colonies derived from paired progenitors that were replated sequentially (124).

Although these results are consistent with the basic principle of a stochastic process of differentiation, some skewing in lineage expression was noted and this might reflect the influence of some extrinsic factor. In the human system, paired daughter cells from My-10 antigen-positive cord blood cells were also recently shown to produce colonies consisting of dissimilar lineage combinations, thus supporting the studies in the mouse (125).

Of course these two models are not necessarily mutually exclusive. One can combine elements of both models and suggest that the decision between stem cell self-renewal and differentiation is basically a stochastic process that can be modulated by the hemopoietic microenvironment.

4) NEOPLASTIC DISORDERS OF MYELOPOIESIS

(A) The Chronic Myeloproliferative Diseases and the Acute Leukemias

As outlined in the previous sections the hemopoietic system can be represented by a lineage diagram illustrating the differentiation pathways that begin at the pluripotent stem cell level and end at the level of the mature effector cell (Figure I). A variety of neoplastic disorders affect the hemopoietic system and they manifest themselves by various degrees of impairment of this differentiation scheme. The broadest classification of the leukemias contains two groups: the acute and chronic leukemias. Each of these is in turn categorized as either lymphoid or myeloid and further subdivision involves a more detailed analysis of the leukemic cell phenotypes.

The chronic myeloproliferative disorders (MPD's) are a group of hematologic neoplasms characterized by expansion of all three myeloid compartments in the bone marrow. In the peripheral blood however there is usually a predominance of only one lineage and this contributes to the clinical presentation. Granulocytes are increased in chronic myelogenous

leukemia (CML), red cells are increased in polycythemia vera (PV), and platelets are increased in essential thrombocytosis (ET). The use of G6PD isoenzyme analysis has demonstrated that all of the MPD's involve the clonal expansion of a neoplastic stem cell which has retained a variable capacity for granulocytic, monocytic, erythrocytic, and megakaryocytic differentiation (126). Accumulating evidence also suggests that the neoplastic stem cell in CML, PV, and ET is capable of differentiation along the B cell lineage (127). Although all myeloid elements at the time of diagnosis are of the abnormal clone, the ability of these cells to continue to differentiate into functional end cells explains why the chronic course of these disorders can be maintained for several years with minimal chemotherapy. The natural progression of CML is somewhat more aggressive than the other MPD's in that transition to an acute phase occurs on average three years following initial diagnosis. This conversion is thought to involve the clonal evolution of a cell within the malignant clone that ultimately leads to the rapid accumulation of nondifferentiating blast cells that possess characteristics of either acute myelogenous leukemia (AML) (60% of cases) or acute lymphoblastic leukemia (ALL) (30% of cases) (128). It is important to distinguish between myeloid and lymphoid blast crisis since the latter often responds to chemotherapy with vincristine and prednisone and the former is largely refractory to treatment (129). Transition to an acute phase also occurs in PV, ET, and MF although less frequently (less than 10% of cases) (124).

In contrast to the chronic leukemias, the acute leukemias encompass a group of related disorders that are all associated with replacement of normal bone marrow by nondifferentiating, and consequently nonfunctional, cells that appear blocked at a particular level of maturation. Failure of normal hemopoiesis is thus the most serious pathophysiologic consequence of acute

leukemia. The identification of a 'preleukemic' phase has been identified in some older patients and this is characterized by chronic marrow insufficiency evolving over time into acute leukemia (131). The precise frequency of the preleukemic phase is unknown since many patients that present with an acute leukemia may have previously experienced asymptomatic hematologic abnormalities.

The excessive production of nonfunctional immature cells in AML is in direct contrast to CML where a large population of fully differentiated cells is produced. Since CML ultimately evolves into an AML-like leukemia, the abnormal myelopoiesis characteristic of these two disorders will be considered in further detail.

(B) Chronic Myelogenous Leukemia (CML)

Level of Stem Cell involvement

The most compelling evidence that CML is a clonal disorder of hemopoeitic stem cells comes from the analysis of G6PD heterozygotes with this disease. The genetic locus for G6PD is on the portion of the X-chromosome that is randomly inactivated early on in embryonic development (132). The G6PD gene has several alleles that specify isoenzymes that can be electrophoretically distinguished. Since only one G6PD gene is active in each somatic cell, normal cells in females who are heterozygous for the A and B alleles consist of two populations, one synthesizing isoenzyme A and the other isoenzyme B. On the other hand, tumours that originate in a single cell would express only a single enzyme phenotype. The clonal theory of CML is strongly supported by studies with this enzyme marker. Females who are heterozygous for G6PD and who have CML express double-enzyme phenotypes in nonhemopoietic tissues but only one type of enzyme in CML granulocytes (133). Although it is conceivable

that many cells were altered initially, only one clone is evident by the time the disease is clinically overt. This leukemic population thus originated from a single cell that possessed a proliferative advantage.

Cytogenetic studies have also been used to infer the monoclonality of CML. The identification of the Ph^1 chromosome in 90-100% of dividing marrow cells has been taken as evidence for a single cell origin. This type of evidence is less convincing than isoenzyme studies because it relies on the identification of a chromosomal abnormality that is very specific for CML. Indeed, there is evidence to suggest that the early events in the pathogenesis of CML predispose to the development of the Ph^1 chromosome and that the acquisition of the Ph^1 leads to a further growth advantage (134).

Isoenzyme and cytogenetic studies have been used to determine the differentiation potential of the cell that initially acquired the selective growth advantage. Identification of the Ph^1 chromosome or a monoclonal pattern of G6PD isoenzyme expression in granulocytes, monocytes, macrophages, erythrocytes, megakaryocytes, eosinophils, and basophils has demonstrated that CML is a clonal disorder of a pluripotent hemopoietic stem cell common to these pathways (8,10,135-137). Notably, the Ph^1 chromosome is absent from bone marrow fibroblasts and other mesenchymal tissues (138) and these cells express a double isoenzyme phenotype (133).

This type of analysis has been combined with <u>in vitro</u> assays for clonogenic progenitors and has demonstrated that CFU-C, BFU-E, CFU-E and CFU-GEMM-derived colonies in CML patients possess both the Ph^1 chromosome (139-141) and a single G6PD enzyme identical to their circulating myeloid cells (142-144).

CML thus appears to involve a multipotent myeloid stem cell. That this stem cell is capable of lymphoid as well as myeloid differentiation is

consistent with several clinical observations. The identification of the Ph^1 chromosome (9) and the detection of a single isoenzyme pattern (145) in the B-lymphocytes of patients with CML provides strong support for the involvement of a substantial proportion of the B-lineage, although not all B cells arise from the CML clone (134). In confirmation of the involvement of the B-lineage, Epstein-Barr virus transformed B-lymphoblastoid lines were established from a single patient with CML and all cell lines that were Ph^1 chromosome positive also expressed a monoclonal pattern of the G6PD enzyme type present in the leukemic myeloid cells (134).

The extent to which T-lymphocytes are involved in CML has historically been more difficult to ascertain. Attempts to demonstrate the Ph^1 chromosome in peripheral blood T-cells from CML patients have generally been unsuccessful and the majority of the T-cells appear to be polyclonal by G6PD analysis, although T-cells in patients with poorly controlled disease may be members of the neoplastic clone (145). It is possible that small numbers of Ph^1-positive T-cells are present but they are undetectable due to the predominance of normal long-lived T-cells or T-cell restricted stem cells that antedated the development of the disease. Consistent with the hypothesis that CML results from the transformation of a stem cell with both myeloid and lymphoid differentiation potential is the existence of myeloid and lymphoid blast crisis in the terminal phase of CML. Although the vast majority of lymphoid blast crises possess a preB phenotype (including immunoglobulin gene rearrangement) (146,147), several cases of T-lymphoid blast crises have recently been reported (142-144) suggesting that, at least in some cases the disease may involve a stem cell capable of T-lymphocyte differentiation.

Etiology and Pathogenesis

That CML arises from a primitive progenitor that retains the capacity to produce and replace differentiated myeloid and possibly lymphoid cells is now well established. The precise nature of the initial transformation event that has such a marked effect on myelopoiesis remains obscure. It is known that CML may develop following exposure to radiation (151) although in the vast majority of cases no environmental factors have definitely been implicated. Recently it was reported that the Ph^1 chromosome recurred in donor cells following an allogeneic bone marrow transplantation (152). This would suggest persistence of the factor that initially induced the disease, thus reinducing leukemia in the donor cells possibly by the transfection of oncogenic material from residual leukemic cells to the donor cells. Donor-cell relapse following allogeneic bone marrow transplantation is a rare event however (153), suggesting that if transfection is indeed the mechanism by which leukemogenesis is initiated in donor cells, the efficiency of this process must be very low.

Genetic Instability and the Philadelphia chromosome

The likelihood that tumour-associated chromosomal changes play an important role in leukemogenesis is highly suggested by their nonrandom nature. CML was the first human neoplasm to be associated with a specific and reproducible chromosomal abnormality, the Philadelphia (Ph^1) chromosome. Originally described by Nowell and Hungerford (154), the Ph^1 chromosome is now known to typically result from a translocation involving chromosomes 9 and 22 (155). The presence of this marker in at least 90% of patients with CML (156) suggests that the acquisition of the Ph^1 may represent an important event in the pathogenesis of CML. This reciprocal translocation has been shown to involve two oncogenes. C-sis, the cellular homologue of the Simian sarcoma

virus transforming gene is normally located on chromosome 22, and c-abl, the cellular homologue of the murine Abelson leukemia virus oncogene is located on chromosome 9. The Ph^1 translocation moves the c-abl locus from chromosome 9 to chromosome 22 and the c-sis locus moves in the opposite direction (157,158). The most consistent finding in CML, including those cases that involve complex translocations, is the movement of material from 9 to 22 (159). In addition, this c-abl-containing region always appears to join chromosome 22 at a highly specific breakpoint cluster region (bcr) (160), suggesting that the c-abl locus may play the most important role in the development of the disease.

The translocated c-abl gene is rearranged and amplified in the CML cell line K562 (161), and an anomalous 8kb transcript has been detected in both K562 cells and hemopoietic cells from all Ph^1-positive CML patients thus far examined (162,163). This CML-specific 8kb RNA has been shown to be a fused transcript of the abl and bcr genes (164). The c-abl gene product produced by these Ph^1-positive cells also appears to be abnormal in that it possesses a tyrosine kinase activity similar to the v-abl protein (the normal cellular gene product has no detectable tyrosine kinase activity) (165). Presumably this protein is a hybrid protein translated from the anomalous RNA fusion product. Together these results suggest that translocation of the c-abl locus may lead to rearrangement and amplification of the gene resulting in the production of a hybrid protein that has kinase activity characteristic of its transforming counterpart in the Abelson leukemia virus. The abnormal abl transcript has been reported to be absent in Ph^1-negative CML (166), although one patient with apparently Ph^1-negative CML recently showed rearrangement of the bcr locus resulting from a joint translocation of bcr and c-abl to chromosome 12 (167). It would therefore appear that the proximity of the

c-abl gene to the bcr locus is an important step in the pathogenesis of CML, although presumably other mechanisms are involved in cases of Ph^1-negative CML where no rearrangements of the abl and bcr genes are detected. Interestingly, a preliminary report has suggested that the Ph^1 chromosome of childhood ALL may actually be distinguished from that of CML by the absence of breakpoints within the bcr region (168). Since normal maturing myeloid cells express c-abl, the presence of an altered c-abl oncogene product in CML cells is an intriguing observation, although why this alteration has such a drastic effect on myelopoiesis is not yet known. The transfer of material in the reverse direction from chromosome 22 to 9 is not associated with the transcriptional activation of the c-sis gene, and therefore it is unlikely that this gene is involved in CML (158)

An important question regarding the etiology of CML is whether the acquisition of the Ph^1 chromosome is a primary or secondary event. Compatible with the view that it is secondary are reports of occasional patients with CML whose marrow cells are Ph^1-negative at presentation but later become Ph^1-positive (169-171). Fialkow et al (134) provided more direct evidence by demonstrating the existence of Ph^1-negative cells derived from the leukemic clone using a combination of G6PD and cytogenetic analysis. In these studies the B-lymphocytes from a patient with CML were transformed with the Epstein-Barr virus (EBV) and a number of B-cell lines, some Ph^1-positive and some Ph^1-negative, were established. Of the Ph^1-negative lines only those expressing the type B isoenzyme characteristic of the patients leukemia were genetically unstable. Ph^1-negative cell lines that expressed the type A enzyme did not have karyotypic abnormalities. These results suggest that the formation of the Ph^1 chromosome is not the sole event in the pathogenesis of CML. It may well be that at least two steps are required in the evolution of

CML; the first involving a heritable change at the pluripotent stem cell level and the second inducing the Ph^1 chromosome in the descendants of this cell (134). The initial transformation event may in fact predispose a cell to the subsequent acquisition of the Ph^1 chromosome and this may in turn confer a proliferative advantage over both normal stem cells and neoplastic stem cells that are Ph^1-negative.

The chronic phase of CML is unstable and at some point the disease progresses to an aggressive acute leukemia that is rapidly fatal. Transformation to the acute phase is usually accompanied by the development of additional chromosomal abnormalities superimposed on the Ph^1 chromosome. The mechanism underlying this progressive genetic instability is unknown but it has been suggested that activation of a genetic transposition system may account for the accelerating chromosomal instability characteristic of this and many other neoplastic disorders (172).

Differentiation of the Neoplastic Clone

Leukemic myeloid cells appear to mature normally during the chronic phase of CML, although subtle abnormalities in granulocyte (173,174) and platelet (175) function have been reported. Normal differentiation is also observed *in vitro* where clonally-derived Ph^1-positive progenitors are capable of differentiating into colonies that are morphologically indistinguishable from colonies derived from nonclonal progenitors (176).

Despite the normal morphological appearance of CML colonies a number of abnormalities are evident. Studies have demonstrated that the frequency of all classes of circulating progenitors is greatly elevated in CML (177-180) and this increase represents expansion of the leukemic clone. Although the cardinal feature of CML is an increase in the number of circulating granulocytes, erythrocytic and megakaryocytic progenitor compartments are

enlarged to the same extent as the granulocyte progenitor compartment (several thousand fold) suggesting that there is no preferential commitment to the granulocyte lineage in CML (177). Similarly, there appears to be no lineage specificity in the cycling status of CML progenitors (181). It has been reported that 70% of the CFU-C from CML patients have a density < 1.062 g/cm^2 whereas only 5% of control CFU-C have this density (176). It is not known if this low density is intrinsic to the CML progenitors (126).

The ability of CML progenitors to execute their normal differentiation programs is not complete however, since a number of CML patients have been shown to exhibit a variable capacity for Epo-independent erythroid differentiation in vitro (182). Terminal erythropoietic differentiation may also be adversely affected since many patients are anemic in spite of the elevated levels of erythroid progenitors (181). This might reflect a defect intrinsic to the progenitors or a secondary effect of the leukemia.

Suppression of Normal Hemopoiesis

Ph^1-negative cells have been found to persist in the marrow of patients with CML but their growth is apparently suppressed by the malignant clone in vivo (183,184). Several investigators have explored the possibility that leukemic cells may suppress the proliferation of normal stem cells and thus confer a growth advantage upon the leukemic progenitors. Thymidine suicide experiments have demonstrated that the circulating progenitors in CML are in active cell cycle whereas in normal individuals very few are cycling (181,185). This suggests a defect in CML that prevents the progenitors from leaving the cell cycle and entering G_0. It is likely that the overall proliferative advantage of the leukemic clone reflects a combination of the expanded progenitor compartment, the cell cycle activity of these progenitors, and suppression of normal hemopoiesis by leukemia-derived inhibitory factors.

The role of known inhibitors in the observed suppression of normal hemopoiesis is unclear, although defects in a variety of regulatory networks that may involve chalones, cell-associated inhibitors, isoferritins and prostaglandins have been proposed (173,186-188).

(C) Acute Myelogenous Leukemia

Level of Stem Cell Involvement

In AML the neoplastic cells are unable to follow normal differentiation pathways although some features of granulopoietic differentiation are usually evident in the leukemic blast cells. A number of nonrandom chromosomal abnormalities have been identified in AML cells and although these changes are not as specific as the Ph^1 is for CML, the data within a given patient indicate that the disease is clonal at the time of study. Studies of G6PD heterozygotes with AML confirm these observations of clonality (189). Moreover, the identification of a single G6PD isoenzyme in platelets, erythrocytes, and leukemic blasts suggests that the blast population in AML originates from a multipotent stem cell common to these lineages (189). In younger patients the disease appears to be expressed in cells with differentiation restricted to the granulocyte-macrophage pathway. On the basis of these findings Fialkow et al have suggested that there is heterogeneity in the stem cell origin of AML: involvement of a multipotent stem cell in elderly patients and a granulocyte-macrophage restricted stem cell in children. This was based on a small number of patients however and may not necessarily reflect true age-related differences in the disease.

Further evidence was recently obtained for the heterogeneity of AML in two adult AML patients who expressed a single G6PD isoenzyme in their monocytic blasts (190). All other myeloid cell populations expressed a double

enzyme phenotype, suggesting that in these patients transformation occurred at the level of a progenitor with differentiation restricted to monocytic differentiation. Immunoglobulin heavy chain gene rearrangements were recently reported in the blast cells from two of fourteen children with AML, suggesting that, at last in some cases, AML may also involve stem cells capable of B lymphoid differentiation (191). More convincing evidence for B-cell involvement is a recent report of two AML patients, one with restricted (granulocytic) and the other with multipotent (erythrocytic, granulocytic, and megakaryocytic) differentiative expression of the involved stem cells (192). G-6PD analysis of EBV-transformed B-cells from the patient whose abnormal clone showed multipotent expression showed involvement of the B-lineage. In contrast, evidence for B cell involvement was not detected in the patient whose abnormal clone showed restricted expression. These findings underscore the heterogeneity of stem cell involvement in AML. An alternative explanation is that the disease always originates in a multipotent stem cell, and that in some cases it is not capable of differentiating down one or more lineages. Nevertheless, the discrimination between AML with restricted or multipotent differentiative expression may reflect differences in etiology and pathogenesis, and may therefore have future prognostic and therapeutic implications.

Etiology and Pathogenesis

The abnormal blast cell population that characterizes AML is unable to complete normal myeloid differentiation programs, and its accumulation rapidly leads to failure of normal hemopoiesis. As in the case of CML the nature of the transforming event(s) that initiates the disease remains obscure, although its association with agents known to cause DNA damage such as radiotherapy and chemotherapy used in the treatment of other malignancies (193,194) and carcinogens such as benzene (195) is well documented.

The disease may arise de novo or it may be preceeded by syndromes of hemopoietic dysfunction collectively referred to as preleukemia. The preleukemic phase generally occurs in older individuals, and AML evolving from this phase has a poorer prognosis than AML arising with no recognized preleukemic phase (131). Treatment of the disease is designed to reduce the leukemic population by courses of chemotherapy until the leukemic cells are essentially undetectable. In contrast to CML, G6PD studies have shown that cytotoxic therapy allows normal granulopoiesis to re-emerge (189), although not in all cases (196). The leukemic clone ultimately regains dominance during relapse.

Chromosomal Abnormalities

A number of nonrandom chromosomal changes have been associated with AML although none of them are as consistent as the Ph^1 chromosome in CML. Abnormal karyotypes have generally been reported in 50% of AML patients using conventional banding techniques (156), although recent studies using high-resolution chromosome techniques suggest that abnormalities may be present in cells from all AML patients (197). Nonrandom changes include trisomy 8, monosomy 7, and other abnormalities in chromosomes 5, 17, and 21. In aggressive disease these nonrandom changes may occur in a background of multiple chromosome rearrangements suggesting that they are the primary change and other abnormalities reflect clonal evolution (198). Probably of greater biological importance are rearrangements that consistently associate with a particular subclassification of AML. These would include the translocations involving chromosomes 8 and 21 in M2-AML and chromosomes 15 and 17 in M3-AML. Although the meaning of these consistent translocations is yet to be elucidated, their association with cells that are blocked at the myeloblast (M2-AML) or promyelocyte (M3-AML) stage of differentiation is intriguing. An

interesting report of an association between an inversion of chromosome 16 and abnormal eosinophils in M4-AML has also been documented (199).

The Ph^1 translocation has been demonstrated in a number of patients with AML (200). Some of these cases probably represent CML in blast crisis, although in other cases it appears to be distinct from CML in that complete remission is associated with loss of the Ph^1 chromosome (200,201).

In some cases a genetic predisposition to DNA damage or chromosomal instability has been implicated in the genesis of AML. Such conditions include Down's syndrome (202), ataxia-telangiectasia (203), congenital agranulocytosis (204), celiac disease (205), Bloom's syndrome (206), Fanconi's anemia (207), Wiscott-Aldrich syndrome (208) and von Recklingshausen's neurofibromatosis (209). Associations with immune deficiency states (210) and immunosuppressed transplant recipients (211) have also been reported. As with most cancers, AML probably arises from a complex interplay between host and environmental risk factors.

Role of Cellular Oncogenes

Identification of the constant chromosome regions that are involved in myeloid hemopoietic malignancies is of considerable importance in view of the increasing evidence implicating cellular oncogenes in normal myeloid differentiation. Chromosomal aberrations have the potential to cause changes in normal oncogene regulation and thus contribute to leukemic myelopoiesis.

The myb gene is the cellular homologue of the transforming gene of a virus that is know to cause AML in chickens (212). The c-myb gene is specifically expressed in hemopoietic cells and appears to be tightly regulated during myeloid cell differentiation (213). This oncogene was recently reported to be amplified in a case of AML (214). Interestingly, the c-myb gene codes for a protein that has some degree of structural homology to

the c-myc gene product (215). The c-myc gene has also been reported to be amplified in AML (216,217). The transcription of c-myb and c-myc in myelogenous leukemia cell lines is lost when these lines are induced to differentiate (218,219), suggesting a possible role in the normal differentiation process.

The fps/fes oncogene may also prove important in this regard since its expression has been shown to be largely confined to normal hemopoietic tissue (220) and hematologic malignancies (221). This gene becomes transcriptionally less active in myeloid leukemia cell lines only when these cells are induced to undergo monocytic differentiation (222).

Also notable is the demonstration that the N-ras gene is altered in some cases of AML and that this altered gene is capable of transforming NIH 3T3 cells or rat-1 fibroblasts (223-226). An amino acid substitution at codon 13 of the N-ras gene has recently been implicated in the conversion of this gene into one with transforming activity (227).

Differentiation of the Neoplastic Clone

In the case of AML the leukemic blast cells are identified by their lack of differentiated characteristics. The most common method for the classification of AML involves the use of morphological and cytochemical criteria to categorize the leukemic blast cell population into subgroups that appear to correlate with different stages of normal hemopoietic cell differentiation (228). Specific subtypes are therefore defined by the direction of differentiation and the degree of cellular maturation. Six subgroups are currently recognized in the FAB classification (Table I).

Two main models have been advanced to explain the existence of the predominating blast population. The first model proposes either a block in a normal myeloid differentiation pathway or an uncoupling between proliferation

TABLE I

French-American-British (FAB) classification of acute myelogenous leukemia. A classification scheme based on morphological and cytochemical criteria (228).

M1	Myeloblastic
M2	Myeloblastic with evidence of maturation
M3	Promyelocytic
M4	Myelomonocytic
M5	Monocytic
M6	Erythroleukemia

and differentiation (229,230). The second model is based on the observation that despite their general adherence to normal myeloid differentiation programs, leukemic blasts may occasionally express markers of differentiation that are out of context for a normal cell at the equivalent stage of differentiation. The latter model proposes that the blast population represents a novel lineage consisting of components of normal differentiation assembled abnormally (231). This hypothesis finds support in the identification in single cells of markers of more than one lineage that are not normally expressed coincidentally. These would include the coexpression of lymphoid and myeloid markers (232-239), as well as the coexpression of markers characteristic of different myeloid lineages (233,240). Such observations support the concept of aberrant gene expression in the leukemic cells, although an alternate explanation is that in some of the cases the leukemic cells are arrested at a developmental stage characterized by multilineage marker expression.

Autoradiographic studies have shown that the majority of AML blast cells are nondividing cells (241). It is interesting to note however, that the differentiation potential of many of these abnormal end-cells does not appear to be irreversibly blocked (229,242). It is now evident that fresh AML cells or cell lines derived from patients with AML will undergo various degrees of differentiation in response to a number of physiological (229,243,244) and nonphysiological (222,245) inducers of differentiation (see Chapter IV).

Blast Cell Progenitors

Several assays have been described that support the growth of blast cell progenitors in AML. Culture conditions that favour the growth of these cells include the addition of PHA-stimulated leukocyte-conditioned medium as a stimulatory factor (246) and the removal of T cells from the cell suspension

to be assayed (247). In these assay systems the majority of the leukemic blasts fail to proliferate but their progenitors are able to undergo 1-5 cycles of cell division to form clusters of poorly differentiated cells. The cells within these colonies are morphologically similar to, and possess the same chromosomal aberrations (248) and G6PD isoenzyme (7), as blast cells in direct marrow preparations from the patient. Analysis of the replating efficiency of these colonies demonstrated that some of the blast progenitors possessed the stem cell characteristic of self-renewal (249). Presumably the capacity for self-renewal in these cells is biologically relevant since this property would allow clonogenic blast progenitors to maintain an independent leukemic population in vivo. Considerable heterogeneity in self-renewal capacity is observed between patients but this property appears to be a stable characteristic in individual patients (250) and may in fact be negatively correlated with prognosis (251).

The clinical significance of these blast progenitors is further exemplified by the finding of a significant correlation between their numbers and the blast count (252) and the observation that, in contrast to the blast cells, they are actively cycling (253,254). A distinction between the blast progenitor population and the total blast population resides in their respective sedimentation and cell surface antigen profiles (255,256).

The ability of the leukemic clone to suppress normal hemopoiesis is evident in AML patients that present with anemia, granulocytopenia, and thrombocytopenia (257). Whether or not this reflects physical exclusion by the expanding mass of leukemic cells or leukemia-derived inhibitory activities is uncertain. An acidic isoferritin termed leukemic inhibitory activity (LIA) has been reported in the serum of AML patients and was shown to inhibit normal granulopoiesis in vitro (188). Unfortunately, the lack of

culture conditions capable of supporting early stem cell growth prevents the analysis of inhibition at stages prior to the progenitor level.

In contrast to CML, complete remission in AML appears to represent the reestablishment of normal hemopoiesis. In an AML marrow that is in clinical remission only normal karyotypes are found (140), all lineages express both forms of G6PD (258), the progenitor levels approach normal dimensions (23), and LIA can not be detected (188). An exception to this was recently reported in an AML patient who entered a complete remission characterized by morphologically and karyotypically normal hemopoietic cells but marrow progenitors were nonetheless clonally derived (196).

5) ANTIGENIC ANALYSIS OF LEUKEMIC MYELOPOIESIS

(A) Tumour Antigens

Analysis of cellular differentiation relies on the identification of specific gene products that are associated with various stages of maturation. Within the field of experimental hematology extensive use has been made of morphological and cytochemical techniques to identify the developmental sequence of the terminal stages of hemopoiesis. Many of these features have been shown to exist in leukemic as well as normal cells and this provides a basis for current classification schemes.

Heterogeneity in the clinical features and responses to treatment in patients with AML has been recognized for a long time and underlies the necessity for new classification systems. One such approach has been the identification of gene products whose expression is correlated with either a specific level of differentiation or the malignant phenotype in general. For example, a number of biochemical markers have been identified that may be useful in defining patterns of hemopoietic cell differentiation and

potentially in the accurate diagnosis of leukemias and lymphomas (259). One notable example is the enzyme terminal deoxynucleotidyl transferase (TdT) which appears to be a useful marker for the initial diagnosis and subsequent monitoring of lymphoid neoplasms (259,260).

A second approach is the immunological analysis of the cell membrane using monoclonal antibodies. Embedded in the lipid bilayer of the cell membrane are proteins that mediate specific cellular functions. Numerous membrane abnormalities have been identified in neoplastic cells (261) and presumably these alterations may influence the neoplastic behaviour of a tumour cell by modulating cellular interactions, interactions with the extracellular matrix, and cellular responses to growth factors and hormones. The search for cell surface correlates of malignancy using immunological approaches has therefore been a major thrust of research into normal and leukemic hemopoietic cell differentiation. Immunophenotyping of normal and leukemic cell populations has resulted in the identification of two major types of cell surface antigens: 1) putative tumour antigens and 2) differentiation and lineage-specific antigens.

Tumour Antigens: an overview

For a tumour to generate specifically sensitized lymphocytes and stimulate antibody production, it must express antigens that are immunogenic in the host. The demonstration of tumour antigens, and assessment of their relevance as targets in transplantation rejection has been largely restricted to studies of chemically and virally-induced neoplasms in animals. These tumour specific transplantation antigens (TSTA's) are classically defined by the rejection of tumours that are injected into syngeneic mice previously immunized with the same tumour (262). Chemically induced tumours generally possess an individually specific rejection antigen that does not cross-react

with rejection antigens expressed on other tumours induced by the same carcinogen. Virus-specific surface antigens characteristic of DNA viruses can also act as TSTA's although in this case immunization confers immunity to all tumours induced by the same virus. In some cases the inappropriate expression of embryonic antigens (263) or the modification of histocompatibility antigens (264) on tumour cells may also provide a basis for transplantation rejection. In contrast to virally and chemically-induced tumours, spontaneously arising tumours in rodents are less frequently capable of eliciting a host rejection response (265) and this must be kept in mind when studying the human situation.

A variety of studies in humans have demonstrated that the immune system is capable of mounting a response against autologous tumour cells (266). Although these findings support the contention that some human tumours express cell surface components that are immunogenic, almost nothing is known about the molecular nature of these antigens. Despite the extensive efforts to identify human tumour-specific antigens, the difficulties associated with demonstrating that a tumour antigen is entirely absent from any normal tissue has led to the term tumour-associated antigen. An antigen detectable only in embryonic cells and tumour cells would thus be operationally defined as a tumour-associated antigen.

Leukemia-Associated Antigens Defined by the Patient's Immune Response

A number of studies have implied the existence of leukemia-associated antigens (LAA's) by demonstrating that the sera of patients with AML contain antibodies that are directed toward autologous and/or allogeneic leukemic cells (267). Some of these antibodies were shown *in vitro* to be cytotoxic to host AML cells with the addition of complement or autologous effector cells (268,269). Interestingly the observed cytotoxicity was low at presentation,

peaked at the onset of clinical remission, and then decreased during continued therapy with the lowest levels being observed at relapse (269). The identification of antibody bound to leukemic cells in vivo and the demonstration that some of the serum-derived antibodies are capable of blocking the ability of host lymphocytes to respond to autologous blasts in vitro, lend further support for the existence of leukemia antigens that can elicit an immune response in vivo (270).

Cell mediated immunity is generally believed to play a major role in the host resistance against tumour cells. The presence of cellular immunity is demonstrated in vitro in a mixed lymphocyte culture assay (MLC) and in vivo by delayed-type hypersensitivity reactions (DTH). A number of investigators have utilized the MLC assay and reported that remission peripheral blood lymphocytes will respond in vitro to autologous blast cells (271-273), as will the normal lymphocytes from haploidentical siblings (274). Similarly, studies utilizing membrane preparations of autologous blast cells demonstrated positive DTH skin reactions in a number of AML patients during both remission and relapse (275). These results are not always confirmed however (276), and there is variation between assays (277). It is possible that these different assays (MLC, cytotoxicity, and skin testing) coupled with the source of antigen (whole blast cells, irradiated blast cells, mitomycin C-treated blast cells, or blast cell extracts) are actually measuring different phases of the immune response against different antigens (278).

Unfortunately the specificity of all these immune reactions is unknown and it has been suggested that the putative tumour antigens may not be true neo-antigens but rather normal antigens which are usually restricted to infrequent precursor cell populations (279).

Leukemia-Associated Antigens Defined by Xenoantisera to Leukemic Cells

A variety of heteroantisera aimed at detecting leukemia-associated antigens have been reported and have demonstrated varying degrees of specificity for AML cells. Unfortunately the preparation of antisera to leukemic cells is subject to a number of technical difficulties that limit their clinical usefulness. To render these antisera antigen specific, extensive absorptions are required with other hemopoietic cell populations and consequently the absorbed reagents are generally of low titer. Other problems reflect the small volumes of antisera available and the lack of reproducible specificity between and within laboratories. Nevertheless, antigens associated with AML have been identified using antisera derived from several sources. Mice rendered tolerant to AML remission cells and then challenged with blast cells from the same patient produced antisera that reacted with leukemic myeloblasts (280,281). This antiserum has been successful in the early diagnosis of relapse in AML before the appearance of morphologically detectable myeloblasts in the bone marrow (282). An antiserum raised in rabbits against membrane components of a Burkitt's lymphoma cell line showed specificity for acute leukemia cells (lymphoid and myeloid) and this antiserum did not require prior absorption to prevent reactivity with normal cells (283,284). Other antisera raised in rabbits (285,286), nonhuman primates (287-289), and patients receiving immunotherapy with leukemia cell vaccines (290,291) have shown specificity for AML cells.

Nonimmunological approaches have also been used to identify cell surface leukemia markers. These techniques involve the radiolabelling of cell surfaces followed by either one-dimensional or two-dimensional polyacrylamide gel electrophoresis (PAGE). AML blast cells have been analyzed by the one-dimensional (292) and 2-dimensional techniques (293) and both methods have

revealed maturation and leukemia-associated differences in the electrophoretic mobilities of ectoproteins. One of these proteins isolated by the two-dimensional ectolabelling procedure, was preferentially associated with leukemic myeloblasts and had approximately the same localization on 2D gels as one would expect the AML antigen defined by a recently described anti-AML antiserum (286). This antigen has a molecular weight of 68,000 and an isolelectric point of 7.1 (294).

Other studies have shown that AML blast cells cultured in vitro continuously shed surface components into supernatant medium (295). Characterization of the glycoproteins shed from AML blasts by gel chromatography, isoelectric focussing, immunoprecipitation and PAGE has defined characteristic profiles that differ quantitatively and qualitatively from compounds shed from normal or other leukemic cells (296). These observations are clinically relevant since leukemia-associated antigens that are shed into the host circulation may facilitate the escape of the malignant cells from immune destruction (297).

If antibodies are being formed in vivo against circulating leukemia-associated antigens that are shed from leukemic cell surfaces then one would expect antigen-antibody complexes to be formed. A number of studies have detected these immune complexes in the sera of patients with acute leukemia (298-300). In one study of AML the levels of immune complexes correlated significantly with clinical course and prognosis (298). Unfortunately the nature of the antigens that are present in these immune complexes is not known. Molecular analysis of these antigens would provide a way to identify leukemia-associated antigens that are immunogenic in the host.

Example of a Leukemia-Associated Antigen: CALLA

The detection of the common acute lymphoblastic leukemia antigen (CALLA) on leukemic cell populations has proven to be very useful in the diagnosis and subclassification of ALL (301). The antigen was initially defined by heterologous polyclonal antisera (302) and later using a murine monoclonal antibody (303) as a glycoprotein with a molecular weight of 100,000. Initial studies did not detect this molecule on normal hemopoietic cells but it was clearly highly expressed on lymphoblasts from patients with ALL. The molecule was therefore designated a leukemia-associated antigen. Subsequent studies revealed the expression of the antigen on a small population of mononuclear cells in normal marrow (304) suggesting that CALLA is not leukemia specific but rather a normal differentiation antigen. CALLA-related surface antigens have now been identified on fibroblasts and mature granulocytes (weakly) (305), and renal proximal tubule epithelium and breast myoepithelium (306). Within the hemopoietic system the anatomical distribution of CALLA-positive cells in normal lymphatic tissue and lymphomas (307), and the identification of CALLA-positive mononuclear cells in normal marrow that co-express cytoplasmic μ, the B1 differentiation antigen, TdT, and Ia (308) support the concept that CALLA is an early lymphoid differentiation antigen. ALL has thus been suggested to represent the clonal expansion of a cell that normally expresses this molecule.

Data regarding the CALLA antigen is an example of the accumulating evidence that suggests that if one looks hard enough, and with optimally sensitive reagents, most leukemia-associated antigens will be found to be present on normal cells. Detailed scrutiny of all the human leukemia-associated antigens reported to date has provided no convincing evidence for the existence of leukemia-specific antigens. The association of

these antigens with the leukemic phenotype is thought to reflect a) quantitative differences between tumour cells and normal cells, b) the inappropriate expression of a normal tissue antigen in another lineage or tissue, or c) the expression of a normal antigen at an inappropriate level of differentiation (309). The possibility of a true tumour antigen may exist in the identification of rearranged oncogene products or products of mutated normal genes. Of course putative tumour antigens that are ultimately shown to be normal antigens under rigorous testing may still be clinically useful.

The immunoglobulin idiotype on the surface of a monoclonal B cell malignancy provides the best example of an operationally defined tumour-specific antigen (279). Although immunoglobulin is a normal gene product of a B cell, malignancies of B cell origin express cell surface immunoglobulin with a unique V-region characteristic of the particular B cell clone from which the tumour was derived. This idiotypic determinant may thus be defined as a unique tumour specific marker.

(B) Normal Myeloid Differentiation

Cell Surface Phenotyping of Mature Myeloid Cells

Membrane glycoproteins may be common to a variety of cell types, an example being the transferrin receptor that is associated with proliferating cells (310). Other proteins are unique to a specific differentiated cell as a result of distinct patterns of gene expression characteristic of a particular developmental pathway. Molecules with such lineage-restricted expression may mediate important functions characteristic of that lineage. Many of these embedded proteins are exposed on the cell surface and are thus amenable to immunological analysis. The hybridoma technique developed by Kohler and Milstein (311) has revolutionized the immunological analysis of hemopoiesis by providing a method for the production in virtually unlimited

amounts of monoclonal antibody with defined class, avidity and specificity. These monospecific antibodies do not require extensive absorption procedures, are available in large quantities either as hybridoma supernatant or ascites fluid, and their specificity does not vary between batches.

Monoclonal antibodies have now been raised against every hemopoietic lineage. Although the greatest amount of work has centered on the immune system, analysis of myeloid differentiation using monoclonal antibodies is now leading to the definition of myeloid subpopulations on the basis of their surface phenotypes. Antigenic expression may be restricted to one or more myeloid lineages or to morphologically and functionally distinguishable subsets along the myeloid differentiation pathways.

An example of a tissue-specific antigen in the hemopoietic system is the leukocyte common antigen (T200), an antigen that is widely expressed on all leukocytes (T and B lymphocytes, thymocytes, macrophages, and granulocytes) but not on other tissues (312). This antigen comprises a family of highly conserved, structurally related molecules, that show molecular weight heterogeneity (180,000-220,000) and antigenic differences that are associated with distinct cell types and the state of maturation (313). The suggestion that T200 mediates important functions at the leukocyte cell surface is supported by the ability of anti-T200 antibodies to block NK-mediated cytolysis (314), B cell differentiation (315), and CTL differentiation and cytolytic activity (316,317). The recent observation that the rat T200 molecule spans the lipid bilayer and contains a large cytoplasmic domain of 705 amino acids makes this antigen a prime candidate for membrane-cytoskeleton interactions (318).

The identification of maturational and functional subsets of the monocytic differentiation pathway is of considerable interest in view of the

major role of these cells in the immune response. A large number of monoclonal antibodies have now been raised against cells of this lineage, some of which are specific for monocytes while others may also react with cells of another myeloid lineage (319-321). A number of studies have demonstrated functional heterogeneity of macrophage populations (322,323) and the cell surface phenotyping of these macrophages offers a unique approach to the classification of these subpopulations (324,325).

Monoclonal antibodies have been instrumental in defining the structural and functional relationships of a family of high molecular weight surface glycoproteins termed LFA-1, mac-1, and p150,95 that are found on leukocyte cell surfaces (326). These molecules are now known to share a common β-subunit and are distinguished by their noncovalent association with a distinct α-subunit. Of particular relevance to the macrophage lineage is the mac-1 glycoprotein that was initially identified as a myeloid differentiation antigen (327). This molecule is present on monocytes and granulocytes but is absent from T and B lymphocytes. Subsequent studies have identified mac-1 as the receptor for the third component of complement (CR3), mediating the adherence and phagocytosis of C3b-coated particles by granulocytes and macrophages. Interestingly, a clinical syndrome characterized by recurrent bacterial infections is associated with a congenital deficiency of this molecule (328).

Monoclonal antibodies raised against myeloid cells have also identified cell surface antigens that are restricted to mature granulocytes (321,329-332). The mature neutrophil plays a central role in the host defense against bacterial infections and presumably some of these cell surface glycoproteins are intimately involved in effecting this function. A number of studies have described the ability of certain monoclonal antibodies

to inhibit neutrophil chemotaxis and degranulation (331), oxidative metabolism (333), antibody-dependent cellular cytotoxicity (334), and phagocytosis (335) suggesting that the cell surface components defined by these antibodies may play a role in mediating each of these functions. The significance of neutrophil surface glycoproteins is exemplified by the clinical disorder characterized by an inherited deficiency of the mac-1, LFA-1 and p150,95 glycoprotein family (328). The adverse effect of this deficiency on adhesion-dependent functions of myeloid cells (such as attachment and spreading, agglutination, antibody-dependent cellular cytotoxicity, and CR3-mediated adherence and phagocytosis) is thought to account for, at least in part, the recurrent bacterial infections encountered in this disease (328). The observation that many granulocyte antigens are conserved among the major primate families further attests to the functional importance of these structures (336,337).

Antibodies that have been found to be specific for the erythroid lineage include those directed at glycophorin (338), the Rh blood group antigen (339), band-3 (340), and two erythroid precursor antigens termed Ep-1 and Ep-2 (341). Another erythroid membrane antigen with a molecular weight of 37,000 was shown to be expressed on all hemopoietic cells but arranged in the erythrocyte membrane in a lineage-specific fashion (342). Other monoclonal antibodies that react with erythroid cells appear to be less specific for this lineage (343).

Analysis of the normal cellular distribution of myeloid cell surface antigens is a powerful technique to study gene products associated with myeloid cell differentiation. Although the majority of these antigens have unknown functions, we are now beginning to elucidate the functions of some of these molecules, the LFA-1 family being a prime example. The broad

distribution of some of these antigens on many myeloid cells is itself indicative of an important role (344). Antigens that possess a lineage-restricted pattern of expression, such as band-3 on erythroid cells (340), probably mediate lineage-specific functions, and those that define subpopulations of cells within a lineage may identify functional subgroups of cells. Further development of functional assays for myeloid cells and their precursors should facilitate functional analysis.

Cell Surface Phenotyping of Stem Cell Populations

The development of hybridoma technology, in conjunction with clonal assays for hemopoietic progenitors, has allowed the generation of monoclonal antibodies selectively reactive with populations of myeloid cells at specific stages of differentiation. It is now possible to enrich for progenitor populations by taking advantage of differences in cell surface antigen expression between progenitor cells and their mature progeny (345,346). Two techniques are commonly used to define the antigenic phenotype of the numerically infrequent progenitor cells found in hemopoietic tissue. The first technique involves the incubation of bone marrow cells with the monoclonal antibody in question followed by the addition of complement. Stem cells expressing specific antigen are therefore killed and subsequent colony assays demonstrate a decreased plating efficiency. A major limitation of this assay is that antigens expressed at low density may not render the target cell susceptible to complement-mediated killing. Nevertheless, this technique has been particularly useful in the HLA-typing of human hemopoietic progenitor populations.

The second technique involves the use of the cell sorting capabilities of a fluorescence activated cell sorter (FACS). Fluorescent-positive and negative cells are separated by this instrument and the respective fractions

can be assayed in clonogenic assays. The obvious advantage of this technique is that it separates viable populations rather than selectively killing a population with antibody and complement.

Combinations of these techniques have been used to demonstrate the expression of class II histocompatibility antigens (Ia-like) on the major progenitor cell classes (347). Although the exact proportion of Ia^+ erythroid colony-forming cells has been somewhat controversial, recent studies suggest that if the conditions of the complement-mediated cytotoxicity are sufficiently optimal one can detect Ia antigens on the vast majority of CFU-GM, BFU-E, and CFU-E (348,349). One study reported that DC antigens (DC being a subregion of the HLA-D locus) are not detectable on progenitors but the significance of this observation is not known (349). In contrast to the immune system the function of class II antigens on hemopoietic progenitors is not clear, although there is some evidence to suggest a role in hemopoietic suppression (350-352). The observation that lymphocytes express Ia molecules with a unique 'lymphoid' epitope that is not detectable on the Ia molecules of monocytes or hemopoietic progenitors, illustrates lineage specific variation in Ia expression and is a potential basis for selective compartmentalization and regulation of Ia-associated function (353).

While most evidence suggests that committed myeloid and erythroid progenitor cells are Ia^+, the more primitive CFU-S in the mouse may not express these antigens (354), and studies with human long term bone marrow culture have suggested that the putative multipotent stem cell that is detected by this assay is also Ia^- (63-65). In contrast to this data it was recently shown in vivo, in a canine autologous transplant model, that Ia^+ bone marrow cells are essential for the successful engraftment of

transplanted marrow (355). It should be noted however that it is not known whether these Ia-bearing cells are pluripotential stem cells or essential accessory cells.

Using these techniques it has been possible to define cell surface changes during the course of differentiation from the committed progenitor compartment to the fully differentiated effector cell compartment. Difficulties of analysis have prevented definitive statements as to the antigenic characteristics of the more primitive stem cells. Changes in the antigenic pattern on the cell surface during differentiation may reflect membrane alterations that mediate cellular responsiveness to extrinsic regulatory signals. These may be quantitative changes in antigen density but this is difficult to assess due to the infrequency of progenitor cells. Several patterns of antigen expression are evident: Some antigens are not expressed on progenitors, appearing only with terminal differentiation (356,357). Of the antigens shown to be expressed on progenitor populations, the vast majority also react with differentiated cells (347,358-360). Others are expressed on progenitors and their differentiating progeny, but absent, or weakly expressed on fully mature cells (360).

Central to the immunological analysis of hemopoiesis has been the search for progenitor or stem cell specific monoclonal antibodies that would provide a one step purification procedure for these cells. Very few of these monoclonal antibodies have been described. Since many leukemic cell lines display immature characteristics, attempts have been made to use these lines as target cells to raise progenitor-specific monoclonal antibodies. Two such monoclonal antibodies possessing narrow specificity were selected from a panel of monoclonal antibodies raised against the K562 erythroleukemia cell line (361). These antibodies reacted with <3% of normal bone marrow cells,

and had low or absent binding to mature peripheral blood cells. The ability of these antibodies in the presence of complement to reduce colony formation suggests that they recognize antigens specific to undifferentiated cells. The HEL cell line (362) is also an erythroleukemia cell line that is considered to be blocked at an early stage of differentiation. The characterization of a series of monoclonal antibodies against this cell line identified a protein with a MW of 24,000 that was present on 4-8% of bone marrow cells including the majority of BFU-E, CFU-E, and CFU-E (363). This determinant was also expressed on platelets and megakaryocytes and can not therefore be considered truly progenitor-specific. Bodger, et al described a monoclonal antibody, RFB-1, which reacts with hemopoietic progenitor cells (364), including CFU-GEMM. RFB-1 is expressed on approximately 30% of normal bone marrow and weakly on mature peripheral blood T cells. Although the specificity of this antibody for progenitor populations is not complete, this antibody has been useful in enriching CFU-GEMM up to 150-fold using a combination of light scatter and fluorescence intensity of the RFB-1 antigen in the FACS (365).

The best example of a cell surface antigen that is selectively expressed on hemopoietic progenitor cells is Civin's My-10 (366). The anti-My-10 monoclonal antibody recognizes a cell surface antigen with a MW of 115,000 which is expressed on hemopoietic progenitors and is undetectable on maturing myeloid and lymphoid cells. Less than 2% of normal bone marrow cells express this antigen, and these My-10-positive cells are blast like in morphology. The 3C5 monoclonal antibody recently described by Katz et al (367) may recognize a similar structure to My-10, although a direct biochemical comparison has not yet been reported.

(C) Leukemic Myeloid Differentiation

Differentiation of malignant cells has been characterized primarily by morphologic, cytochemical, and to some extent functional criteria. Attempts to define the lineage affiliations and maturational level of leukemic cells has been greatly improved by the development of monoclonal antibodies that react with normal hemopoietic differentiation antigens. This approach is based on the assumption that the leukemic blast cells, although abnormal in many respects, continue to express cell surface antigens characteristic of the lineage from which they were derived.

AML has long been recognized as a heterogeneous disease that shows morphological features of different stages of normal myeloid differentiation. The FAB classification of AML is based largely on these morphological observations and it currently subdivides AML into six categories (Table I). There do not appear to be major prognostic differences among these subtypes although longer remissions in M3 patients (368), and a somewhat poorer prognosis in M5 patients (368,369) have been reported. As more and more chemotherapeutic regimens are being developed, it is becoming increasingly important to define useful classification schemes that can correlate different clinical features with specific subtypes. One approach is the immunological analysis of the leukemic cell surfaces. Monoclonal antibodies reactive with lineage-restricted antigens have clearly been shown to be useful in the diagnosis of ALL and such analyses have provided prognostically useful information (370-372). Since the optimal therapy for ALL and AML is accomplished with different chemotherapeutic agents (and allogeneic bone marrow transplantation is the treatment of choice in AML) the distinction between these diseases is very important. Such a distinction is not always apparent using morphological and cytochemical criteria, but monoclonal

antibodies to myeloid and lymphoid antigens have been quite successful in these situations (373). Detailed analysis of lymphoid leukemias with a panel of monoclonal antibodies and other markers has shown that leukemic lymphoid cells express a conservative framework of normal differentiation markers with minimal deviations (230). These consistent features of leukemic phenotypes in relation to normal hemopoietic differentiation have been taken to reflect the imposition of maturation arrest in the leukemic cells.

The conservation of differentiation-associated phenotypes has also been reported in AML. In one study a series of monoclonal antibodies reactive with normal myeloid cells at different stages of maturation (anti-My-4, My7, My8, Mo1, and Ia) were used to classify 70 patients with AML into four phenotype groups, each corresponding to a normal immature myeloid cell (374). Group I corresponded approximately to the normal CFU-C (21%), Group II to the myeloblast (26%), group III to the promyelocyte (8%), and group IV to the promonocyte (45%). Each group contained more than one morphologic type indicating that the level of differentiation on the cell surface may not always parallel morphology. Correlations between cell surface phenotype and differentiation status have also been reported in studies utilizing other panels of monoclonal antibodies (375,376). Not all the cells within a given leukemic population express a particular antigen however, and the relative proportion of these antigen-positive and antigen-negative cells varies considerably between patients (377). It is likely that the extent to which AML blasts are reported to adhere to normal differentiation programs is a reflection of which gene products are analyzed. Clearly leukemic cells express a large number of normal cell surface antigens but it is not known whether the observed antigenic heterogeneity represents disorganized gene expression or merely the lack of synchronization in the total blast population.

As previously mentioned a proportion of the blast population is capable of forming colonies in methylcellulose assays. These colony-forming cells are presumed to be responsible, at least in part, for maintaining the blast population in vivo. Immunophenotyping of the clonogenic cells in AML has shown that the cell surface profile of the total population does not necessarily reflect the phenotype of the clonogenic cells (256). Furthermore, a comparison of the surface phenotype of the clonogenic cells with normal myeloid progenitors identified three patterns: (a) a phenotype similar to late CFU-GM, (b) a phenotype similar to early CFU-GM or CFU-GEMM, and (c) a composite phenotype of early and late CFU-GM. The clonogenic cells were also reported to have a relatively consistent phenotype in contrast to the total population (256).

The leukemic myeloid cells in the chronic phase of CML are capable of differentiating into mature granulocytes and so the cell surface phenotype of CML cells generally reflects the dominant population of granulocytes and to a lesser extent their differentiating precursors (378). When blast crisis occurs the identification of the lymphoid variant is clinically important since this group of patients frequently respond to vincristine and prednisone (129). Several surface antigens have been identified which appear to be of value in this regard. Lymphoid antigens such as CALLA (379), B-cell antigens such as B1 (380) and B4 (381), and T-cell antigens such as T101 (378). Two thirds of blast crises are of the myeloid variant. These are felt to represent AML but cytochemical stains are often negative or inconclusive (378). A number of myeloid specific monoclonal antibodies have been useful in these equivocal situations, particularly My7 and My9. Panels of these monoclonal antibodies have been used to classify CML blast crisis into six immunologically defined subgroups: Myeloid, lymphoid, erythroid, megakaryocytic, undifferentiated, and mixed (378).

Therapeutic Potential of Monoclonal Antibodies

The use of monoclonal antibodies in the treatment of hematologic neoplasia is still in its infancy. Although monoclonal antibodies that are truly leukemia-specific would be ideal for these purposes, many studies indicate that tumour-specific antigens may not exist or that it may prove impossible to obtain monoclonal antibodies against them. On the other hand they may not be required if the antigen is tumour-specific within the context of the hemopoietic system or if quantitative differences can be taken advantage of.

A number of studies have demonstrated that the passive administration of monoclonal antibodies reacting with leukemic cells can be efficient in the treatment of selected animal leukemias (382). The first monoclonal antibody serotherapy trial in humans utilized an antibody directed against a lymphoma-associated antigen in the treatment of a B-cell lymphoma (383). Subsequent trials have emphasized the use of monoclonal antibodies against the CALLA antigen in the treatment of ALL (384), against T-cell differentiation antigens in T-cell leukemia or lymphoma (385,386) and against the T101 antigen in B-chronic lymphocytic leukemia (387,388). Unfortunately these clinical trials have reported only limited responses to the infused antibody. Problems encountered generally include the presence of circulating antigen which binds the injected monoclonal antibody, antigenic modulation of the tumour cell surface as a direct consequence of antibody binding (particularly true for CALLA) resulting in antigen-negative tumour cells, and finally the immune response against the infused mouse antibodies. Since the process of antigenic modulation depends on the binding of bivalent antibody, Cobbold and Waldmann (389) reported that monoclonal antibodies that are made monovalent are no longer capable of eliciting antigenic modulation and may therefore have increased therapeutic potential.

The most cited example of successful serotherapy was a report of the use of anti-idiotypic monoclonal antibody to treat a patient with a B-cell lymphoma (390). This patient had entered an accelerated phase of the disease and was no longer responsive to conventional therapy. Following eight i.v. infusions with the anti-idiotypic antibody the patient entered a complete remission and 30 months later remained free of detectable disease (278).

One of the difficulties associated with autologous bone marrow transplantation is the high risk of contamination of the transplanted remission marrow by residual leukemic cells (278). The goal is to therefore effectively eliminate neoplastic cells ex vivo while sparing the hemopoietic stem cells that are vital for engraftment. Clinical trials are now using monoclonal antibody and exogenous complement for these purposes (391-393). Monoclonal antibody 'cocktails' containing several antibodies have been particularly effective (394), especially when combined with a chemotherapeutic agent (395).

The preparation of immunotoxins may also prove useful in the immunotherapy of leukemia. Monoclonal antibody has been successfully coupled to powerful toxins such as diptheria and ricin toxin in the hope of specifically targeting these compounds to leukemic cells (396). Encouraging results have been reported in animal studies (396,397) but the extreme toxicity of these immunoconjugates will necessitate further research before clinical trials can be initiated in humans.

6) THESIS OBJECTIVE

An increasing number of laboratories have developed monoclonal antibody reagents that recognize antigens on the surfaces of normal myeloid hemopoietic cells and on myeloid leukemic cells at various levels of maturation. A comparison of the cell surface antigenic profiles of normal differentiating myeloid cells with leukemic blast cells has revealed patterns of antigen expression consistent with models of leukemic hemopoiesis that postulate a block in normal differentiation programs as a mechanism to explain the elevated proportion of these poorly differentiated cells in AML. Although the surface antigen profiles of the relatively mature myeloid cells has been extensively studied, only a limited number of antigens which are restricted in their expression to myeloid precursor populations have been characterized. In view of the fact that AML blast cells are thought to represent the leukemic counterpart of myeloid precursors, it is particularly interesting to identify those antigens that are specific to normal precursor populations since they may have important regulatory roles in early myelopoiesis.

As part of a study to identify cell surface antigens characteristic of the more immature stages of myelopoiesis a number of monoclonal antibodies were raised against an AML cell line, HL-60, that is blocked at an early stage of myeloid differentiation. One of these antibodies, NHL-30.5, was initially identified by its inability to bind HL-60 cells following the induction of granulocytic differentiation in this cell line. Subsequent studies showed the antibody to possess considerable specificity for hemopoietic cells from patients with hematologic disorders characterized by the presence of immature myeloid cells, in particular AML. These observations indicated that the NHL-30.5 monoclonal antibody might define an early myeloid differentiation antigen that may be of use in the cell surface phenotyping of myeloid

leukemias. The identification of the antigen defined by this monoclonal antibody, and the characterization of its cellular distribution is the subject of this thesis.

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C H A P T E R II

MATERIALS AND METHODS

1) PRODUCTION OF THE NHL-30.5 MONOCLONAL ANTIBODY

A female BALB/c mouse was immunized twice with the acute promyelocytic leukemia cell line HL-60 by intraperitoneal injection over a period of several weeks. Three days following a final intravenous injection the immune spleen cells were fused with the NS-1 myeloma cell line according to the method of Kohler and Milstein (1). The NS-1 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 15% FCS. The immune splenocytes and NS-1 cells were fused in a 50% solution of polyethylene glycol (PEG; Baker Chemical, NJ) in DMEM at a ratio of approximately 10^8 spleen cells to 10^7 NS-1 cells. The fused cells were resuspended in 50 ml of DMEM + 15% FCS and a one ml volume was plated in each well of a 48-well tissue culture dish (Flow laboratories, Virginia) containing a feeder layer of irradiated 3T3 fibroblasts ($\sim 2 \times 10^5$ fibroblasts/well). The day following the fusion, half of the medium was aspirated from the wells and 0.5 ml of DMEM containing 15% FCS, 13 µg/ml hypoxanthine, 0.19 µg/ml aminopterin, and 3.9 µg/ml thymidine (HAT medium) was added to each well. This procedure was repeated three and five days later.

Hybridoma supernatants from 96 wells were screened for reactivity against HL-60 cells in an indirect binding assay (see below) using ^{125}I-rabbit $(Fab')_2$ anti-mouse immunoglobulin as second antibody. Fifty of the 96 wells gave positive results against HL-60 and the cells from some of these

cultures were subsequently cloned in soft agar. The cloned hybridomas were again screened for reactivity with the HL-60 cell line and 34 positive clones were grown up in DMEM + 15% FCS. To enhance the selection of antibodies that might identify antigens associated with early myeloid cell differentiation the initial selection procedure identified monoclonal antibodies that reacted with leukemic specimens containing immature myeloid blast cells. One of the antibodies, NHL-30.5, reacted with HL-60 cells but this ability was lost when the cells were induced to differentiate. This hybridoma was subsequently recloned twice and a more detailed analysis of its cellular distribution was undertaken.

The NHL-30.5 antibody-producing hybridoma cells were injected into pristane-primed BALB/c mice and several weeks later the ascites fluid was harvested from these mice. The pooled ascites was precipitated with ammonium sulphate (50% saturation), dissolved in 20 mM phosphate buffer (pH 8.0) and loaded onto a P100 column equilibrated with the same buffer. The OD_{280} of each fraction was measured and the first peak pooled and loaded onto a DEAE-affigel blue column. Fractions from the DEAE-affigel-blue column were tested for activity in the binding assay and for purity by SDS-PAGE, and the peak was again pooled.

2) CELL LABELLING PROCEDURES

Binding Assay

Binding of the antibody to a panel of cell lines and fresh leukemic tissue was tested by an indirect binding assay using radioiodinated $F(ab')_2$ fractions of rabbit anti-mouse Ig antibodies (RaMIg). One million target cells were incubated with 50 µl of hybridoma supernatant in a 96-well microtitre plate for one hour at 4°C. After washing twice with 200 µl Earle's balanced salt solution containing 0.5% BSA, 0.1% NaN_3, and 10 mM

HEPES buffer, the cells were incubated with 50 µl (10^5 cpm) of ^{125}I-RaMIg (1-3 x 10^7 cpm/µg) and further incubated for one hour at 4°C. Cells were washed again three times, transferred to gamma-counter tubes, and counted on a Beckman biogamma counter.

Antigen Estimation

A modification of the binding assay was used to estimate the number of NHL-30.5 antigens on various cell types. Twenty µl of chloramine T (0.5 mg/ml) was added to 25-50 µg (in 25-50 µl) of purified NHL-30.5 antibody followed by the addition of 1 mCi ^{125}I-sodium iodide (Amersham, Canada). The reaction was stopped after 15 minutes with the addition of 50 µl of sodium metabisulfite (2 mg/ml) and the iodinated antibody was loaded onto a small P10 sizing column. The antibody peak was pooled and this stock was used in a direct binding assay. Various concentrations of the iodinated antibody (1-3 x 10^7 cpm/µg) were titrated with different numbers of target cells to determine saturation levels, and the specific binding was calculated by subtracting the amount of radioactivity bound to the cells in the presence of an excess of cold antibody from the amount bound at saturation in the absence of cold antibody. An estimate of antigen density was then obtained using the following calculation:

$$\#\ \text{molecules/cell} = \frac{\text{specific binding (cpm bound/cell)}}{\text{specific activity of antibody (cpm/mole)}} \times 6.02 \times 10^{23}$$

FACS Analysis

Cells (1-2 x 10^6) were washed with RPMI 1640 containing 10% FCS, 0.1% NaN_3, and 10mM HEPES buffer, and incubated for one hour at 4°C with 50 µl of undiluted hybridoma culture supernatant in a 96-well microtitre plate. Media

alone, or an unrelated monoclonal antibody raised against mouse lymphocytes was added to the control cells. The cells were washed twice and resuspended in 50 µl of FITC-conjugated rabbit or goat $(Fab')_2$ antibodies to mouse Ig. After an additional incubation for one hour at 4°C, the cells were incubated with propidium iodide (25 µg/ml) for five minutes before the final three washes. Dead cells could then be gated out from the analysis on the basis of propidium iodide fluorescence. If the cells were not going to be analyzed the same day they were washed three times in phosphate buffered saline (PBS) and fixed for 30 minutes on ice with 1% paraformaldehyde in PBS.

In situations where the fluorescence profiles of the media control and the test sample demonstrated a clear crossover point when overlapped, the percent positive cells was calculated by subtracting the background staining in the media control (at the crossover point) from those reacting with the test sample and dividing by the number of cells analyzed (10,000 or 20,000). If no distinct crossover point was evident the top 5% fluorescent cells in the media control was subtracted from the number of fluorescent cells in the test sample at the same gates.

Flow Cytometry

The FACS system (Becton Dickinson, CA) was used to measure the size and fluorescence intensity of hemopoietic cells labelled with various monoclonal antibodies. This is accomplished by passing a stream of cells, in single file, through the focussed beam of a high power laser (in this case a Spectra Physics Argon laser at a power setting of 400 mW) that is coupled with a set of highly sensitive detectors (Figure II). These photodetectors translate optical signals, emitted by individual cells as they pass through the laser beam, into electrical impulses that are then stored for display and analysis. The intensity of the light scatter signal depends to a large extent on cell

size, and this is one parameter routinely displayed by the instrument; the larger the cell, the greater the light scatter. Light scatter measurements are also useful to discriminate between viable and nonviable cells.

If the cells have been tagged with a fluorescent probe (such as antibody coupled to a fluorescent dye), the fluorescence intensity of each cell when excited by the laser can be analyzed separately from light scatter measurements. The fluorescence detectors are covered by filters that block the wavelength of light emitted by the laser and this reduces background signals.

When the sorting capabilities of the FACS are utilized, the stream of cells is subjected to an ultrasonic vertical vibration of ~40,000 Hz that breaks the stream into small droplets containing, on average, one cell for every eight droplets (2). The optical measurements made on each cell are compared to preset parameter levels before the cell reaches the stream tip. If the criteria for inclusion into a sorted population are met, then the droplet containing the cell of interest is electrically charged as it reaches the stream tip and can therefore be appropriately deflected as it passes through an electric field created by charged deflection plates.

Since hemopoietic cells are easily dispersed into single cell suspensions, they are ideal subjects for FACS analysis. All cells stained in this study were analyzed on either a FACS IV (Department of Neurology, University of B.C.) or a FACS 440 (Terry Fox Laboratory, B.C. Cancer Research Centre). The instruments were routinely standardized using glutaraldehyde-fixed chicken red blood cells and fluorescent microspheres. Debris was gated out on the basis of light scatter measurements.

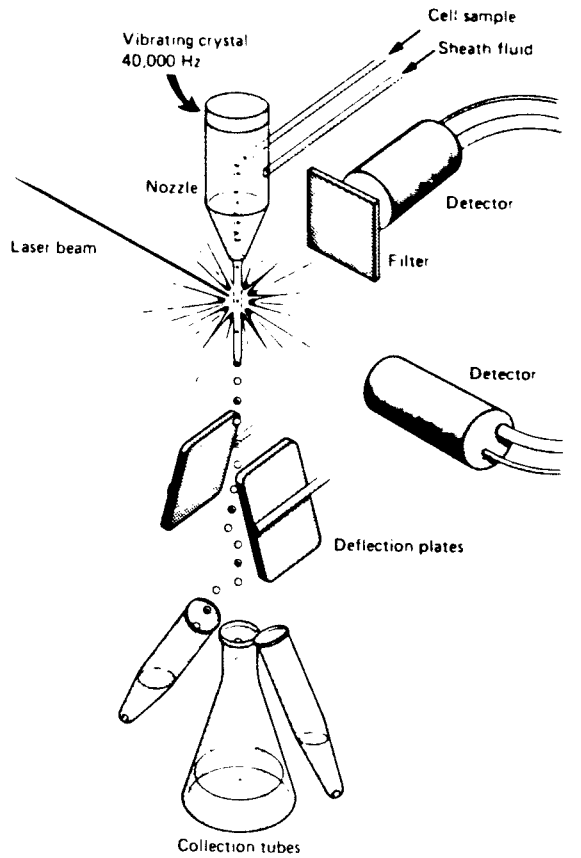

FIGURE II Schematic representation of the fluorescence activated cell sorter (FACS). From ref (2).

3) IMMUNOPRECIPITATION

Target cells (2×10^7) were washed three times with PBS and resuspended in 0.3 ml of PBS in a glass tube coated with 100 µg of iodogen (Pierce Chemical, Rockford, IL) (3). One mCi of ^{125}I-sodium iodide (Amersham, Canada) was added and the cells were incubated at room temperature for one hour while agitating. The cells were washed twice in EBSS + 0.5% BSA, 0.1% NaN_3, and 10 mM HEPES buffer, and resuspended in 1.5 ml of PBS containing 50 µl of phenylmethylsulfonylfluoride. The cells were then lysed with the addition of 0.5 ml of 2% NP40 in PBS and the lysate was incubated on ice for five minutes before microfuging (Eppendorf) for five minutes at 4°C. The lysate was incubated with 30 µl of monoclonal antibody for one hour at 4°C and subsequently with 40 µl of a 50% suspension of rabbit anti-mouse Ig-coupled sepharose beads (approximately 2 mg of anti-mouse Ig/ml of beads) overnight. The following day the beads were washed in EBSS + 0.5% NP40 five times and the bound material removed by incubating the beads with SDS-PAGE sample buffer in a boiling water bath for five minutes. The immunoprecipitate was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4) using either 10% or 7.5% gels followed by autoradiography with Kodak X-OMAT film and a Dupont Cronex intensifying screen. Molecular weight determinations were obtained by comparison to protein standards (BioRad, Canada).

HL-60 cells were metabolically labelled with ^{35}S-methionine (800 Ci/mmol) or ^{3}H-leucine (5 Ci/mmol) (Amersham, Canada) to determine if the NHL-30.5 antigen is synthesized by these cells. For ^{35}S-methionine labelling, 2×10^7 cells were washed twice in DMEM containing no methionine, and resuspended in 2 ml of methionine-free DMEM containing 10% dialyzed FCS. For ^{3}H-leucine labelling, medium containing no leucine was used. One hundred µCi of ^{35}S-

methionine or 1 mCi of ^{3}H-leucine was added and the cells were cultured for four hours at 37°C. Cells were washed three times and resuspended in lysis buffer (1% Triton X-100 in saline containing 50 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$, and 1mM $MnCl_2$). The lysate was incubated on ice for 20 minutes, and then microfuged for five minutes at 4°C. The supernatant was then incubated with lentil lectin-coupled beads (Sigma, St. Louis, MO), prewashed with lysis buffer containing 0.5% BSA, for one hour. The unbound proteins were removed in three washes with lysis buffer and the bound fraction in another 3 washes of lysis buffer containing 0.1M α-methyl mannoside. The lentil lectin-bound and unbound fractions were then immunoprecipitated with monoclonal antibodies as previously described for radioiodinated cells.

Phosphorylation of the NHL-30.5 antigen was studied by labelling HL-60 with ^{32}P. HL-60 cells (2×10^7) were washed twice in DMEM containing no phosphate and then incubated with 0.5 mCi of ^{32}P-orthophosphate (50-100 mCi/mg, New England Nuclear, Canada) in phosphate-free DMEM + 15% dialyzed FCS for three hours at 37°C. The cells were then lysed in 0.5% NP40 and immunoprecipitation was carried out as described for ^{125}I-labelling.

4) HEMOPOIETIC CELL LINES

The culture conditions adopted for the cell lines utilized in this thesis are summarized in Table II.

5) DIFFERENTIATION OF MYELOID LEUKEMIA CELL LINES

Granulocytic Differentiation

Myelogenous leukemia cell lines were induced to differentiate using a variety of inducing agents. Previous studies have outlined the optimal culture requirements for the induction of granulocytic differentiation in the HL-60 cell line using dimethylsulfoxide (DMSO) (13) or retinoic acid (13). Cells were cultured in plastic tissue culture flasks (Corning, NY) at a

TABLE II

Culture Conditions for

Human Hemopoietic Cell lines

CELL LINE	ORIGIN	CULTURE MEDIUM
HL-60 (5)	Acute promyelocytic leukemia	DMEM + 10% FCS
K562 (6)	Chronic myeloid leukemia (Ph^1-positive)	DMEM + 10% FCS
KG-1 (7)	Acute myeloblastic leukemia	α + 10% FCS
KG-1a (8)	Acute myeloblastic leukemia	RPMI + 10% FCS
HEL (9)	Erythroleukemia	RPMI + 10% FCS
U937 (10)	Histiocytic lymphoma	RPMI + 10% FCS
DHL, 1,4,6,8 and 10 (11)	B-cell lymphoma	RPMI + 10% FCS
Jurkat (12)	T-cell leukemia	RPMI + 10% FCS

All cell lines were cultured in an atmosphere of 5% CO_2 at 37°C.

The above cell lines were generous gifts from the following individuals:

HL-60: Dr. J. Levy, Dept. of Microbiology, University of B.C.
K562: Dr. BB Lozzio, Dept. of Medical Biology, University of Tennessee.
KG-1: Dr. HP Koeffler, Dept. of Medicine, University of California, L.A.
HEL/KG-1a/U937: Dr. T. Pawson, Dept. of Microbiology, University of B.C.
Jurkat: Dr. DG Kilburn, Dept. of Microbiology, University of B.C.

seeding concentration of 2 x 10^5 cells/ml in the appropriate medium (see section on cell lines) containing 10% FCS and either 1.25% DMSO (Fisher, Canada) or 1 x 10^{-6} M retinoic acid (Sigma, St. Louis, MO). Control cultures were incubated in tissue culture medium containing no inducing agent. The cultures were incubated for five days and the viability of the cells on each day was assessed by trypan blue exclusion. One, three, and five days following the addition of the inducing agent, the cells were harvested for analysis of the expression of selected cell surface antigens. Morphological and cytochemical assessment of the extent of differentiation was examined independently by a hematopathologist at the Vancouver General Hospital.

Monocytic Differentiation

Cells (HL-60, KG-1, and HEL) were cultured in 100 mm diameter tissue culture dishes at a seeding concentration of 2 x 10^5 cells/ml in appropriate medium containing 1.6 x 10^{-8} M (HL-60 or KG-1), or 10^{-6} to 10^{-7} M (HEL) 12-O-tetradecanoylphorbol-13 acetate (TPA). Since the TPA was dissolved in DMSO, equivalent amounts of DMSO were added to control cultures. Induced HL-60 and KG-1 cells were harvested on days one and two and induced HEL cells on days one, three, and five. Nonadherent cells were removed and the adherent cells were treated with saline containing 0.2% EDTA and 0.1% BSA at 4°C for about 30 minutes to facilitate the removal of the adherent cells. Strongly adherent cells were gently removed with a rubber policeman.

6) HUMAN CELL PREPARATIONS

Patient Specimens

Heparinized peripheral blood and bone marrow aspirates were obtained with informed consent from patients with various hematologic malignancies. Normal marrow controls were obtained from individuals donating their marrow for transplantation or from patients with malignancies that did not involve the

marrow. Bone marrow cells were prepared by one of two techniques. The first involved spinning at 800g for four minutes followed by the removal of the buffy coat and treating with ammonium chloride (9 volumes NH_4Cl (8.3 g/l in water), 1 volume tris base (20.6 g/l adjusted to pH 7.65 with 1M HCL), and finally adjusted to pH 7.2) to remove residual red cells. The second technique had a lower yield but enriches for immature populations by removing granulocytes by centrifugation on 1.077g/cm^3 ficoll-hypaque (LSM, Litton Bionetics, Kensington, MD) or 1.077g/cm^3 percoll (Pharmacia, Uppsala, Sweden). Peripheral blood specimens were generally obtained by ficoll-hypaque preparation, although the large number of cells in some patient specimens (particularly CML) made it easier to prepare a buffy coat. All specimens for sorting experiments (Chapter 5) were isolated on ficoll-hypaque (1.077g/cm^3) gradients.

Cell Fractionation

Granulocytes and erythrocytes were purified from the peripheral blood of healthy volunteers using a one-step ficoll-hypaque (1.114 g/cm^3) sedimentation procedure (Mono-Poly Resolving Medium, Flow Laboratories, Inglewood, CA). Purity of the enriched populations was assessed morphologically. For monocyte purification (15), the light density fraction of ficoll-hypaque (1.077g/cm^3) separated cells were washed and resuspended at 5 x 10^5 cells/ml in RPMI 1640 with 10% FCS and placed in 100 mm diameter tissue culture dishes. Following an incubation period of 90 minutes at 37°C, the adherent cells were washed five times with RPMI 1640 containing 10% FCS and treated for one minute with normal saline containing 0.2% ethylenediaminetetraacetic acid (EDTA) and 0.1% bovine serum albumin (BSA). The adherent cells were then gently removed with a rubber policeman and shown to be >80% positive for nonspecific esterase.

Platelets were obtained from heparin or citrate anticoagulated blood of pooled donors from the Canadian Red Cross (Vancouver, B.C.). Platelets from individual donors were prepared by spinning 10 ml of peripheral blood at 190 x g for 20 minutes at room temperature and removing the supernatant. This platelet-rich plasma was then spun at 2,500 x g for five minutes at room temperature and the pelleted platelets were resuspended in binding assay medium adjusted to pH 6.5 with 1M citric acid.

Normal bone marrow fibroblasts from three separate donors were obtained by placing 2-3 x 10^6 nucleated marrow cells from each donor in 8 ml of medium (α + 20% FCS) in 60 x 15 mm tissue culture dishes (Falcon, California). The cells were cultured in an atmosphere of 5% CO_2 at 37°C, and five days later the nonadherent cells were discarded and replaced with fresh medium. When confluent, the fibroblast cultures were washed twice in saline followed by the addition of 5 ml of a 0.25% trypsin solution (in citrated saline). Ten minutes later one ml of FCS was added to stop further trypsin action, and the adherent cells were detached by gentle pipetting. The cells were washed in α + 20% FCS and cultures were reinitiated with 10^5 cells/dish. After one or two such subcultures the enzymatic detachment of the adherent cells for FACS analysis was accomplished using bacterial collagenase type I (200 units/mg, Sigma, MO) (16) instead of trypsin to prevent digestion of cell surface antigens. The collagenase was dissolved in calcium and magnesium-free Hank's balanced salt solution (HBSS-Ca-Mg). Immediately prior to use, FCS was added to give a final concentration of 20% FCS and 0.1% collagenase. Confluent cultures were drained of medium, washed twice in HBSS-Ca-Mg, and cultured in 10 ml of the collagenase solution for three hours in an atmosphere of 5% CO_2 at 37°C. Adherent cells were again removed by gentle pipetting.

For PHA-stimulation of lymphocytes, the mononuclear fraction from ficoll-hypaque ($1.077g/cm^3$) separated peripheral blood was cultured in plastic tissue culture flasks at a concentration of 10^6 cells/ml in RPMI 1640 containing 10% FCS and 1% phytohemagglutinin (PHA; Gibco, Calgary, Alberta). The cultures were incubated in an atmosphere of 5% CO_2 and the cells were harvested after three days. Viability was assessed by trypan blue exclusion and stimulation was confirmed by pulsing the cells with ^3H-thymidine and counting in a scintillation counter (17).

7) CELL SORTING

Normal peripheral blood, normal marrow, and CML marrow cells were separated by ficoll-hypaque ($1.077g/cm^3$) and stained for sorting as previously described under 'FACS Analysis' using sterile conditions and medium containing no NaN_3. Cells were sorted into positive and negative fractions depending on their reactivity with the NHL-30.5 monoclonal antibody according to the following criteria: In cases where less than 5% of the cells were positive (seen with all normal bone marrow and peripheral blood specimens) the sort gates were adjusted so that cells with the highest fluorescence intensity, comprising 5% of the total population, were included into the positive fraction, and cells with the lowest fluorescence intensity (the remaining 95%) were sorted into the negative fraction. In situations where greater than 5% of the cells were NHL-30.5-positive (seen only with ficoll-hypaque separated CML peripheral blood) then all positive cells were sorted into the positive fraction. The sorted fractions were then counted and plated in standard methylcellulose assays to determine the distribution of the various myeloid progenitor classes within each sorted population. As a control some specimens were stained with media or an isotype-matched monoclonal antibody (IgG1) specific for phycoerythrin, and the top 5% again sorted and assayed for progenitors.

8) ASSAYS FOR CLONOGENIC MYELOID PROGENITORS

Cells were plated in standard 0.8% methylcellulose culture medium containing 30% FCS, 1% deionized BSA, 10^{-4}M 2-mercaptoethanol, 3 units per ml of human urinary erythropoietin (100-300 units/mg) (18), and 9% human leukocyte conditioned medium (19) prepared by a modification of the standard agar-serum overlay procedure (20). Normal unsorted bone marrow cells were plated at 1 x 10^5 cells per ml and peripheral blood at 4 x 10^5 cells per ml. Unsorted CML cells and all sorted cells were also plated at multiple lower cell concentrations to ensure obtaining a condition where colony numbers were sufficient but not excessive. Cultures were scored using an inverted microscope and the data obtained consistent with a linear cell dose-colony yield relationship. Adequate burst and granulocyte colony formation by separated cells was achieved by the presence of leukocyte conditioned medium. Colonies derived from CFU-E (1-2 clusters of erythroblasts) and mature BFU-E (3-8 clusters of erythroblasts) were counted on day 10, and from primitive BFU-E (>8 clusters of erythroblasts) and CFU-C (>20 granulocytes and/or macrophages) on day 18, according to established criteria (21).

In order to determine whether binding of the NHL-30.5 monoclonal antibody to progenitor cell surfaces could inhibit or stimulate colony formation, 1-10 µg/ml of purified NHL-30.5 monoclonal antibody was included in the assay medium. Assays both with and without erythropoietin and leucocyte conditioned medium were used in these experiments. For inhibition studies, monoclonal antibodies against the transferrin receptor (NHL-62.14, and NB-2) were used as positive controls, and purified IgG and an anti-LFA-1 monoclonal antibody were used as negative controls.

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C H A P T E R III

NHL-30.5: A MONOCLONAL ANTIBODY DEFINING AN ACUTE MYELOGENOUS LEUKEMIA (AML)-ASSOCIATED ANTIGEN

1) INTRODUCTION

Heterogeneity in the clinical features and responses of patients with AML has been recognized for a long time and underlies the need for new approaches to disease classification. The most common method involves the use of morphological and cytochemical criteria to categorize leukemic blast cell populations into subgroups that appear to correlate with different stages of normal hemopoietic cell differentiation. In the last several years, a new method for analyzing hemopoietic cell differentiation events using monoclonal antibodies (1) has emerged, and a large number of myeloid surface antigens have now been identified by this technique (2-5). Some of these are restricted in their expression to cells at various stages of maturation within a particular lineage while others may be found on cells of a number of different hemopoietic lineages (Chapter I). The surface phenotypes of AML blast cells include some of these antigens and, in general, the pattern of antigen expression is consistent with the view that the blasts maintain a cell surface profile characteristic of a normal myeloid cell at an equivalent level of differentiation (3,6), although some exceptions to this have been reported (7-9).

To obtain further information about the nature, control, and distribution of phenotypes in blast cell populations from different AML patients, we initially embarked on a program to develop new monoclonal

antibody reagents with greater specificity for cells with immature myeloid characteristics. Preliminary studies identified one such antibody, NHL-30.5, which showed an apparent specificity for hemopoietic cells from patients with AML. The identification of the antigen defined by this monoclonal antibody, and analysis of its cellular distribution in normal and leukemic hemopoietic tissue is the subject of this Chapter.

2) RESULTS

Reactivity of NHL-30.5 Monoclonal Antibody With Various Human Cells

The production of monoclonal antibodies reactive with HL-60 cells is described in Chapter II. One of the antibodies, NHL-30.5, showed apparent specificity for AML cells and was further characterized.

The reactivity of the NHL-30.5 monoclonal antibody with a variety of normal and malignant hemopoietic cells was tested by fluorescence staining. Samples were reacted with the antibody, FITC-conjugated rabbit $(Fab')_2$ anti-mouse Ig, and then analyzed on the FACS. Figure III shows the fluorescence profiles of the reactivity of NHL-30.5 with ficoll-hypaque separated peripheral blood cells from a representative normal, AML, and CML patient. Three controls were used. The first was a media control in which cells were incubated with the second FITC-conjugated rabbit $(Fab')_2$ anti-mouse Ig without prior exposure to NHL-30.5. As a positive control we used a monoclonal antibody produced in the same fusion as NHL-30.5 that reacts with all human hemopoietic cells tested to date (except red cells), and as a negative control we used an unrelated mouse monoclonal antibody raised against mouse lymphocytes. The fluorescence profiles of the binding of NHL-30.5 to the normal and CML samples were the same as the negative controls. The profile of the AML patient, however, showed significant binding of the antibody above background.

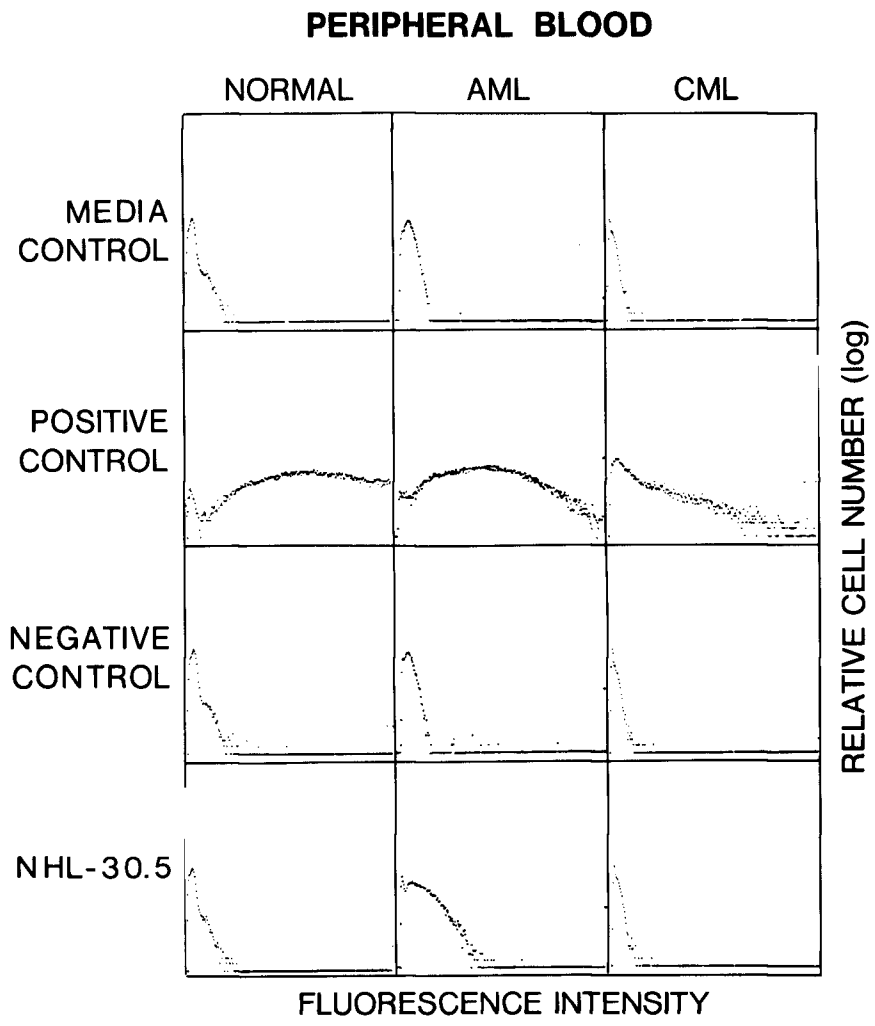

FIGURE III FACS analysis of the binding of NHL-30.5 monoclonal antibody to ficoll-hypaque-separated peripheral blood from a normal, AML, and CML patient. Cells were stained with NHL-30.5 and FITC-rabbit $(Fab')_2$ anti-mouse Ig. Three controls were used: The media control had no test antibody added, the positive control was a monoclonal antibody produced in our laboratory that has reacted with all human hemopoietic cells tested to date (except red cells), and the negative control was an unrelated monoclonal antibody raised against mouse lymphocytes.

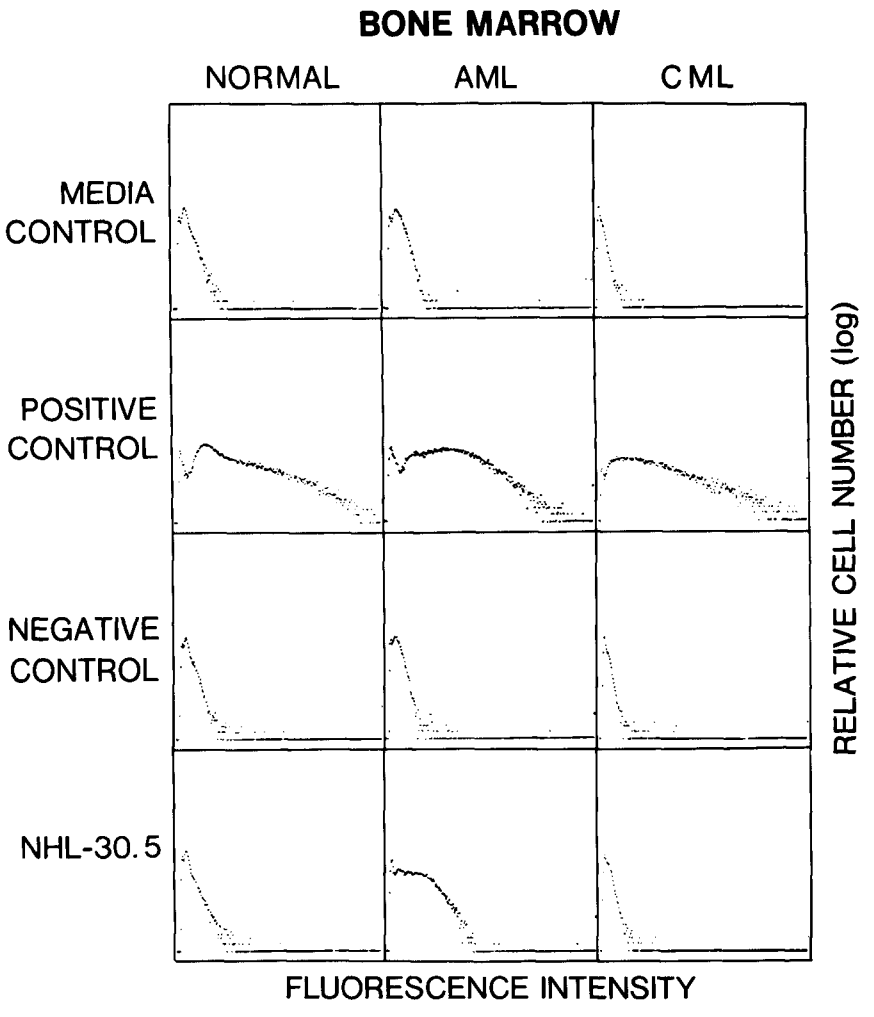

FIGURE IV FACS analysis of binding of NHL-30.5 to buffy coat samples of bone marrow from a normal, AML, and CML donor. The analysis was carried out as in Figure III.

Figure IV indicates the reactivity of the antibody with marrow cells from the same normal, AML, and CML donors used in Fig III. The only cells with detectable binding above the media and negative controls are those from the AML patient. Normal peripheral blood granulocytes (>90% granulocytes by morphology), monocytes (adherent cells, >80% positive for nonspecific esterase), lymphocytes (the nonadherent cells from the monocyte purification procedure), PHA-stimulated lymphocytes, erythrocytes (ABO), splenocytes, and platelets were also tested in this manner. None of these showed any detectable staining (Figure V).

The reactivity of NHL-30.5 was also tested in a binding assay using ^{125}I-rabbit $(Fab')_2$ anti-mouse Ig second antibodies. Table III gives the binding assay results for the first 19 AML patients tested, together with the % positive cells determined by FACS analysis and associated clinical data. Fifteen of the 19 samples tested were positive by both FACS and binding assays (patients Nos. 1-15). A value of >10% positive was considered a positive result in the FACS, and >1,000 cpm was considered positive by binding assay. Table IV lists all of the AML patients analyzed to date that have demonstrated reactivity with the NHL-30.5 monoclonal antibody. Binding assay data is not shown since these studies were not continued after analysis of the first 19 patients. AML patients that did not react (i.e. showing <10% NHL-30.5-positive cells) are listed in Table V. A total of 48 AML specimens have been studied with this antibody and 40 of them were classified as NHL-30.5-positive. Five patients with AML and related disorders were studied during the course of their disease and these results are displayed in Table VI (see discussion). A summary of all the AML patients tested with NHL-30.5 is given in Table VII. All of the M1 patients studied and most of the M2's and M4's were positive. One of two M3's and only two of six M5's were positive.

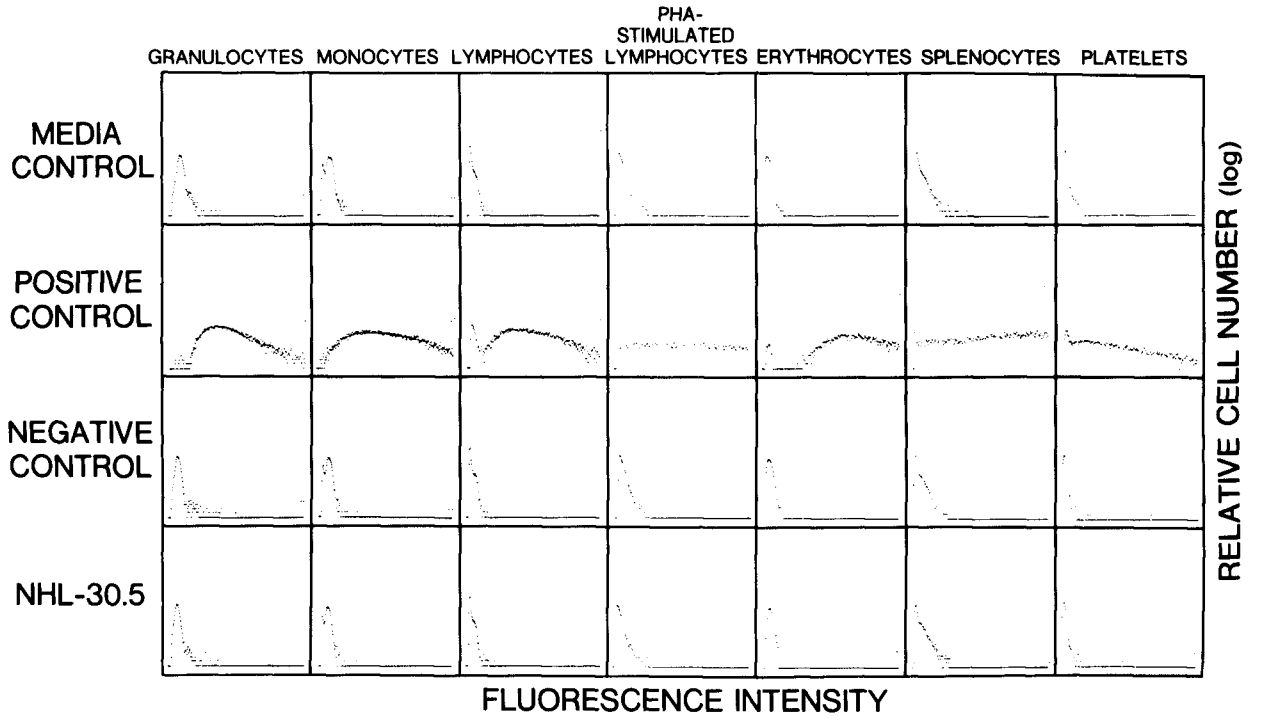

FIGURE V FACS analysis of the binding of NHL-30.5 to populations of granulocytes, monocytes, lymphocytes, PHA-stimulated lymphocytes, erythrocytes, splenocytes, and platelets from normal donors. The analysis was carried out as described in Figure III. Mouse anti-human serum was used as positive control for the erythrocytes. PHA-stimulated lymphocytes were cultured at a concentration of 10^6 cells/ml in RPMI 1640 containing 10% FCS and 1% phytohemagglutinin for three days.

TABLE III

Reactivity of the NHL-30.5 monoclonal antibody with the first 19 AML patients studied

	FAB classification	Age/sex	WBC x 10^9/l	% Blasts		FACS analysis % positive		NHL-30.5 binding (cpm)§	
				PB	BM	PB	BM	PB	BM
1	M1	70/F	282	98	97	49	51	2,029	2,033
2	M1	62/M	9.3	82	94	26	55	1,123	1,734
3	M1	67/M	17.1	63	71	16	NT	3,617	NT
4	M1	55/M	0.9	58	81	66	NT	NT	NT
5	M1	67/M	3.4	21	35	15	NT	1,327	NT
6	M2	54/F	12.1	51	60	53	46	2,574	3,726
7	M2	55/M	43.8	67	32	21	21	763	1,075
8	M2	88/M	5.3	6	20	53	11	NT	4,316
9	M2	44/F	.525	29	71	NT	12	NT	NT
10	M2	74/F	4.7	38	41	20	46	1,097	1,588
11	M2	34/F	62	86	89	42¶	NT	NT	NT
12	M4	36/F	21.9	42	47	64	26	1,014	NT
13	M4	69/M	10	8	33	26	21	672	695
14	M4	49/F	30.3	57	72	58	NT	1,407	NT
15	M4	43/M	127	94	79	70¶	NT	4,198	NT
16	M2	15/M	4	9	32	2	NT	843	867
17	M3	26/F	2.3	OCC.	9	<1	NT	NT	NT
18	M4	42/F	15.5	67	91	<1¶	NT	394	NT
19	M5	27/M	112	86	87	<1	NT	11	53

¶ Leukapheresis samples

§ Binding data represents the radioactivities of NHL-30.5-labelled cells above background levels obtained with media controls in which no first antibody was used. Media controls were <350 cpm.

NT = not tested

Patients # 5 and 9 were in relapse at testing. All others were tested at presentation and prior to treatment.

TABLE IV
A summary of AML specimens containing >10% NHL-30.5-positive cells (NHL-30.5-positive)

AML PATIENT	FAB CLASSIFICATION	% BLASTS PB	% BLASTS BM	% POSITIVE BY FACS PB	% POSITIVE BY FACS BM
1	U	45	45	58	63
2	M1	85	90	55	58
3	M1	94	92	NT	48
4	M1	11	66	9	51
5	M1	98	97	49	51
6	M1	82	94	26	55
7	M1	63	71	16	NT
8	M1	58	81	66	NT
9	M1	21	35	15	NT
10	M2	60	94	54	52
11	M2	51	60	53	46
12	M2	67	32	21	21
13	M2	6	20	53	11
14	M2	29	71	NT	12
15	M2	38	41	20	46
16	M2	86	89	42*	NT
17	M3	17	8	36	23
18	M4	4	26	22	NT
19	M4	55	48	NT	42
20	M4	51	79	23	32
21	M4	13	31	NT	47
22	M4	40	95	NT	15
23	M4	2	52	2	63
24	M4	33	66	52	NT
25	M4	78	59	85	71
26	M4	43	78	47	31
27	M4	50	31	48	NT
28	M4	61	74	80	82
29	M4	72	71	36	38
30	M4	42	47	64	26
31	M4	38	49	36	29
32	M4	8	33	26	21
33	M4	22	82	27	17
34	M4	57	72	58	NT
35	M4	88	70	70	NT
36	M4	94	79	70*	NT
37	M4	77	78	64	51
38	M5	84	82	58	29
39	M5	99	98	14	NT
40§	unclassified	38	28	NT	58

* leukapheresis samples
§ Initially diagnosed as a monosomy 7 childhood myeloproliferative disease. This patient was tested when his disease had progressed into AML.
NT = not tested, U = undifferentiated

TABLE V

A summary of AML specimens containing <10% NHL-30.5-positive cells (NHL-30.5-negative)

AML PATIENT	FAB CLASSIFICATION	% BLASTS		FACS ANALYSIS % POSITIVE	
		PB	BM	PB	BM
1	M2§	9	32	2	NT
2	M3	0cc.	9	<1	NT
3	M4	67	91	<1*	NT
4	M4	88	84	<1	<1
5	M5	86	87	<1	NT
6	M5	6 (85)¶	18 (60)¶	NT	4
7	M5	62	74	<1	1
8	M5	43	50	<1	NT

* leukapheresis samples

§ Relapsed 11 months later with 46% NHL-30.5 positive cells in the peripheral blood and 18% in the bone marrow.

¶ figures in parentheses indicate % early monocytes.

NT = not tested

TABLE VI

Sequential testing of cells from patients with AML and related disorders

PATIENT	STATUS	DATE	% BLASTS		FACS ANALYSIS % POSITIVE	
			PB	BM	PB	BM
1	AML-M2					
	presentation	Oct 1/1982	11	32	2	NT
	treated	Oct 20/1982	<1	<1	NT	<1
	remission	Nov 10/1982	<1	3	NT	2
	relapse	Aug 22/1984	15	65	46	18
2	AML-M1					
	presentation	Jan 12/1983	63	71	16	NT
	remission	Apr 20/1983	<1	<1	NT	<1
3	AML-M4					
	presentation	Oct 12/1983	72	71	36	38
	relapse	Dec 13/1984	84	86	43	56
4§	MONOSOMY 7	Sep 13/1984	16	17	33	NT
	MPD	Nov 30/1984	38	28	58	NT
5§	UNSPECIFIED	Sep 28/1984	11	NA	20	NT
	MPD	Dec 28/1984	53	NA	66	NT

MPD = myeloproliferative disease

§ Patients #'s 4 and 5 were being monitored for progression of their disease into AML.

NT = not tested

Marrow cells from patients with various other malignant hemopoietic diseases were also examined for their reactivity with NHL-30.5 by FACS analysis and/or binding assay. The results of these tests are summarized together with the AML data in Table VII. Five non-AML samples reacted with the antibody. These were a chronic myelomonocytic leukemia (CMML) (1/1), a myelfibrosis (MF) (1/2), an acute lymphoblastic leukemia (ALL) (1/15), seven chronic myeloid leukemias (CML) (7/26), and three blast crises of chronic myeloproliferative disorders (3/6, Table VIII). Reactivity with cells in the acute phase of CML was restricted to one patient with myeloid blast crisis and a biphenotypic variant containing both a myeloid and a lymphoid blast population (Table VIII). All three lymphoid blast crises tested were uniformly negative. In addition, one patient with an unspecified myeloproliferative disorder entered an unusual blast crisis characterized by the presence of a CALLA-positive, HLA-DR-positive, TdT-negative, and NHL-30.5-positive blast population.

Differentiation of HL-60

Approximately 70-90% of cultured HL-60 cells showed reactivity with NHL-30.5. When the cells were induced to differentiate by incubating in the presence of DMSO, the fluorescence profiles of the induced cells became identical to those of the media and negative controls (Figure VI), indicating that a dramatic decrease in antigen expression had occurred. Binding assay data confirmed these results. Control cultures bound >1,900 cpm and DMSO-treated cells <100 cpm. Following exposure of the cells to the DMSO, >80% of the cells showed morphological evidence of differentiation beyond the promyelocyte stage.

Immunoprecipitation of the antigen

The antigen defined by NHL-30.5 monoclonal antibody was immunoprecipitated from the surface of iodinated HL-60 cells as described in Chapter II. SDS-PAGE of the precipitate showed the antigen to have a molecular weight of 180,000 under reducing conditions (Figure VII). Immunoprecipitation of iodinated fresh AML cells from a specimen containing 77% blasts revealed a protein with approximately the same molecular weight on SDS gels (Figure VIII).

3) DISCUSSION

In this chapter, the isolation and preliminary characterization of a murine IgG1 monoclonal antibody, NHL-30.5, that reacts with a significant proportion of hemopoietic cells from newly diagnosed or relapsed AML patients is described. The NHL-30.5 producing hybridoma was derived from a fusion between an NS-1 myeloma cell and a spleen cell from a BALB/c mouse immunized with HL-60 cells. The antibody is not cytotoxic for HL-60 cells in a direct ^{51}chromium release assay. The apparent molecular weight of the antigen on cultured HL-60 cells is 180,000, where it is expressed on 70-90% of the cells grown under standard culture conditions. The antigen also appears to have the same molecular weight on fresh leukemic cells derived from a patient with M4-AML (Figure VIII).

Forty of 48 AML samples tested thus far have been found to contain detectable numbers (>10% above background) of NHL-30.5-positive cells. Greatest consistency was seen between NHL-30.5 positivity and the M1 classification, although the vast majority of the M2's and M4's were also positive. Notably, one of the two M3 patients (acute promyelocytic leukemia) did not have detectable NHL-30.5-positive cells, even though this antibody was initially raised against an antigen on leukemic promyelocytic cells (HL-60).

TABLE VII

Summary of results of testing blood (ficoll-hypaque-separated) and/or marrow cells (buffy coat-separated) from various patient categories with the NHL-30.5 monoclonal antibody

	NUMBER TESTED	NUMBER POSITIVE§	
AML - undifferentiated	1	1	40/48 (83%)
AML - M1	8	8	
AML - M2	8	7	
AML - M3	2	1	
AML - M4	22	20	
AML - M5	6	2	
AML - unclassified	1	1	
Chronic myelomonocytic leukemia	1	1	
Myelofibrosis	2	1*	
Chronic myeloid leukemia (chronic phase) (for acute phase see Table VIII)	26	7	
Acute lymphoblastic leukemia	15	1	
AML in remission	5	0	
Acute lymphoblastic leukemia in remission	3	0	
Polycythemia vera	1	0	
Multiple myeloma	1	0	
Myelodysplasia	3	0	
Chronic lymphocytic leukemia	2	0	
Normal bone marrow	10	0	

* A 66-year-old male who presented with myelofibrosis and a peripheral leucocyte count of 2,700 with 19% blasts. Six months after testing, his peripheral counts rose to 123,000 with 50% blasts, and 2 months later he developed myeloid skin infiltrates and died unresponsive to chemotherapy.

§ positive: >10% NHL-30.5-positive cells

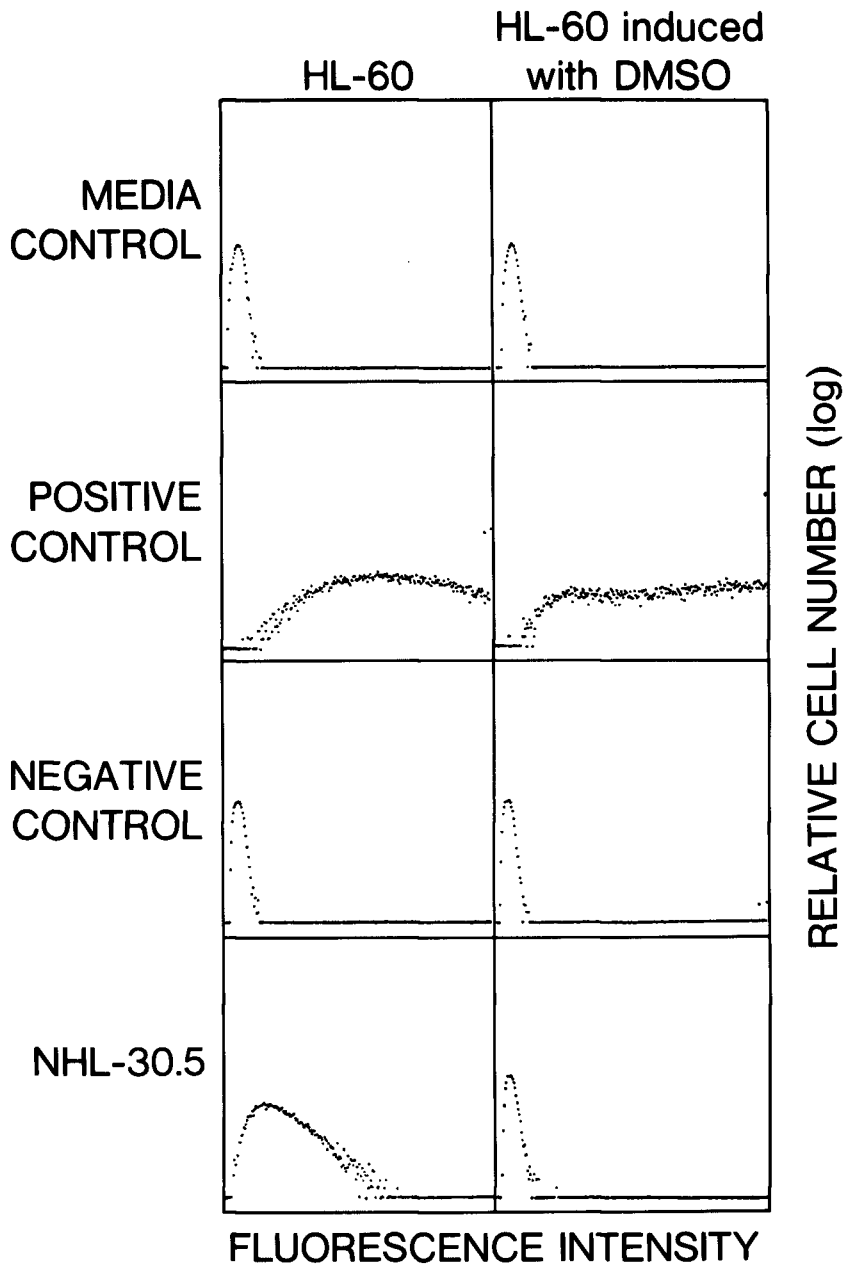

FIGURE VI FACS analysis of binding of NHL-30.5 to HL-60 cells before and after induction of differentiation with DMSO. Cells were incubated in the presence of 1.25% DMSO for 5 days and analyzed as described in Figure III.

TABLE VIII

Reactivity of NHL-30.5 with hemopoietic cells from patients in the acute phase of CML

PATIENT	PHILADELPHIA CHROMOSOME	BLAST CRISIS*	% BLASTS		FACS ANALYSIS % POSITIVE	
			PB	BM	PB	BM
1 CML	+	myeloid (M4)	40	41	15	34
3§ MPD	NA		53	NA	66	NT
4 CML	+	biphenotypic¶	48	69	10	15
5 CML	+	lymphoid	19	34	1	NT
6 CML	+	lymphoid	25	90	2	<1
7 CML	+	lymphoid	44	84	1	<1

* based on morphological, cytochemical, and cell surface phenotyping criteria.

§ All patients were considered to have typical Ph^1-positive CML before they entered blast crisis with the exception of patient #2 (MPD, unspecified myeloproliferative disorder). The blast population in this patient was unusual in that it expressed HLA-DR and CALLA but was TdT-negative.

NA = information not available
NT = not tested

¶ Biphenotypic in this context indicates the presence of both a myeloid and a lymphoid blast population.

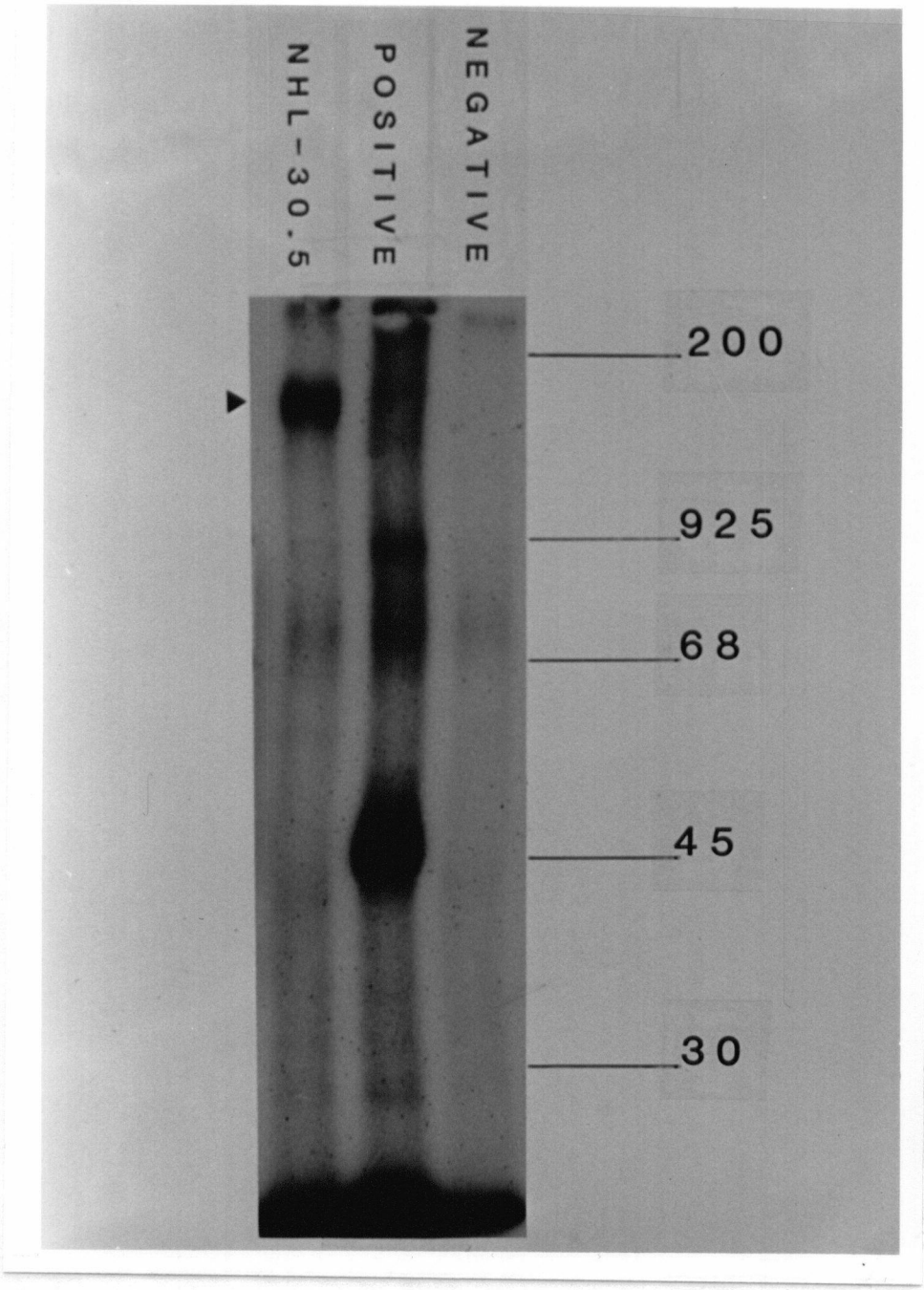

FIGURE VII Analysis of the NHL-30.5 antigen by immunoprecipitation. HL-60 cells were surface labeled with ^{125}I, a cell lysate was prepared, and the antigen was immunoprecipitated with NHL-30.5 monoclonal antibody. Analysis was carried out on SDS-PAGE (10%) under reducing conditions. Two controls were used: a positive control (described in Figure III), and a negative control (no monoclonal antibody added).

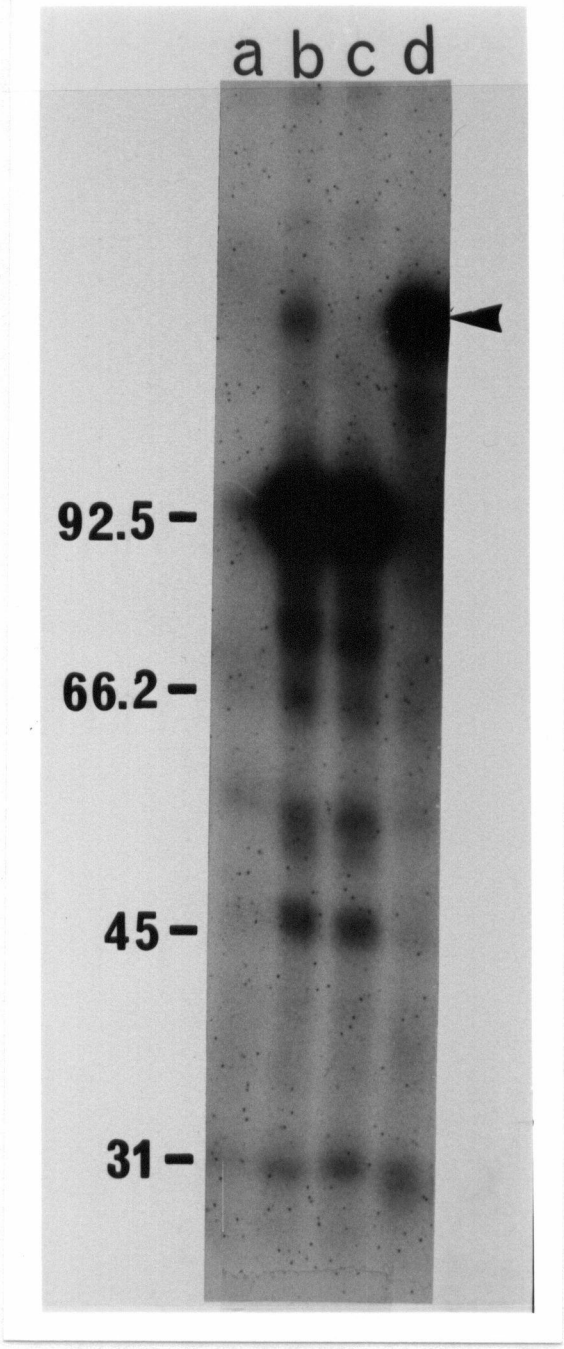

FIGURE VIII Immunoprecipitation of the NHL-30.5 antigen from the peripheral blood cells of a patient with AML (patient #37, Table IV). The sample contained 77% blast cells (M4 classification). The lysate from the iodinated cells was immunoprecipitated with a control antibody described in Figure III (lane a), with anti-transferrin receptor-specific monoclonal antibodies (lanes b and c), and with NHL-30.5 (lane d, arrow).

Since this peripheral blood sample contained only the occasional blast cell and bone marrow cells were not tested, the negative result may have been due to the sensitivity limit of the FACS and binding assays. This might also explain the negative result obtained for one of the M2 patients. Six other AML samples with significant blast counts however, did not show detectable numbers of NHL-30.5 positive cells. Four of these were monocytic leukemias (M5) and two were myelomonocytic (M4). Since NHL-30.5 does not react with differentiated monocytes, it is possible that AML blasts expressing more differentiated characteristics, such as M5-AML, no longer express NHL-30.5. It should be noted that even amongst positive patients there was no correlation between the blast count in the sample and the % positive cells determined by FACS analysis.

Five patients with AML and related disorders were tested at least twice during the course of their disease (Table VI). Patient #1 (AML) did not react upon presentation (possibly due to the low blast count in the specimen analyzed), nor did he react when tested following treatment, and later when he was in remission. Upon relapse the patient was strongly positive. Patient #2 (AML) was positive at presentation, and negative in remission, and patient #3 (AML) was positive both at presentation and relapse (remission not tested). Patient #4 was initially diagnosed as a childhood myeloproliferative disease with cells monosomic for chromosome #7. Monosomy 7 is the diagnostic criterion for one of the more common myeloproliferative states in childhood and it is known to carry a high risk of progression to AML (10,11). This patient ultimately developed AML and on the two occasions that he was tested (2.5 months apart), reactivity with the NHL-30.5 monoclonal antibody increased with the increased blast count. The fifth patient, initially diagnosed as an unspecified myeloproliferative disease, entered an acute phase characterized

by the presence of HLA-DR and CALLA-positive blast cells that were TdT-negative (typical CALLA-positive ALL is also TdT-positive). This might be interpreted as the anomalous expression of both myeloid and lymphoid markers since the blast cells can not be clearly designated as either myeloid or lymphoid. Nevertheless, the reactivity of these cells with NHL-30.5 was shown to increase when the blast count increased. Although only five patients were analyzed sequentially, the results obtained with these patients suggest that expression of NHL-30.5 correlates with the active phase of their disease.

NHL-30.5, in addition to reacting with cells from patients with a diagnosis of AML, bound to cells from a number of other patients not considered to have AML at the time of study. The first of these was a patient with MF who had 19% blasts in his peripheral blood and whose blood and marrow cells produced numerous abnormal colonies in methylcellulose assays. Eight months later, this patient developed overt AML. The second 'non-AML' but NHL-30.5-positive patient was diagnosed as a CMML with 28% blasts in his bone marrow. This patient also produced abnormal colonies in methylcellulose cultures, but remained in stable condition eight months after testing. Since CMML is considered to be a preleukemic disorder, the demonstration of some phenotypic similarities with AML may not be surprising. The third patient was an adult ALL with 92% blasts. At present we have no explanation for this finding since 14 other ALL patients with significant blast populations were all clearly negative. The expression of the antigen on the positive ALL sample might be interpreted as a form of lineage infidelity (8). Alternatively, the NHL-30.5 antigen may be a differentiation antigen expressed on certain primitive lymphoid as well as myeloid cells.

A fourth patient appeared to be an ALL on initial examination, but cytochemical studies and cell surface phenotyping demonstrated a myeloid, as

well as a lymphoid, blast population. Subsequently the patient was shown to be Ph^1-positive suggesting early blast crisis in a patient whose primary disorder was CML. Another patient with an unspecified myeloproliferative disease also entered a biphenotypic blast crisis and cells from this individual strongly reacted with NHL-30.5. Four other patients with CML in blast crisis were tested and one of them was shown to contain NHL-30.5-positive cells. This particular patient was in myeloid blast crisis. The three patients with NHL-30.5-negative blast crisis had the lymphoid variant. Reactivity with ficoll-hypaque-separated hemopoietic cells from patients in the chronic phase of CML appeared to be less frequent. Of 26 CML's studied only seven of them possessed greater than 10% (but no more than 20%) NHL-30.5-positive cells. If buffy coat preparations of CML cells were used, very few NHL-30.5-positive cells could be detected (<2%). Since ficoll-hypaque treatment of CML suspensions (which removes mature granulocytes) enriches for NHL-30.5-positive cells, the negative result observed with buffy coat preparations might reflect a dilution effect mediated by the excess of mature granulocytes predominating in this disorder.

The results presented in this Chapter have led to the tentative designation of the NHL-30.5 antigen as a leukemia-associated marker. Such a designation is based primarily on the analysis of its cellular distribution on hemopoietic cells from normal and leukemic individuals. The molecule is expressed predominantly on leukemic populations from patients with AML and to a lesser extent other hematologic disorders characterized by the presence of immature myeloid blast cells. Normal mature hemopoietic cells are clearly negative, as are the vast majority of the differentiating cells found in normal bone marrow. Since the NHL-30.5 antigen is also found on undifferentiated HL-60 cells but not following DMSO-induced HL-60

differentiation, it is possible that this antigen is a normal differentiation antigen, present on a minor population of primitive myeloid cells that is subsequently lost during the differentiation process. If this hypothesis is correct, then one would not expect a normal bone marrow to show reactivity with the antibody (Table VII) because of inherent limitations in the sensitivity of the assays used. Expression of the antigen on myeloid leukemic cells could then be interpreted as indicative of a type of differentiation block leading to the selective amplification of a clone of cells expressing the NHL-30.5 marker. A second possibility is that, at least within the context of the hemopoietic system the NHL-30.5 antigen is a novel molecule associated with aberrant gene expression in the leukemic cells. Whichever mechanism is postulated, the reactivity of the NHL-30.5 monoclonal antibody with cells from patients with AML, and its lack of reactivity with the majority of normal hemopoietic cells, suggests a potential use for this reagent in the immunophenotyping of myeloid leukemias. Approaches designed to determine whether NHL-30.5 is leukemia-specific or differentiation-specific are presented in Chapters IV and V.

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C H A P T E R IV

DIFFERENTIATION-LINKED EXPRESSION OF AN AML-ASSOCIATED ANTIGEN ON MYELOID LEUKEMIA CELL LINES

1) INTRODUCTION

The concept that AML cells are unable to differentiate or mature normally *in vivo* is an old one. The imposition of maturation arrest upon the malignant cells appears to abrogate normal differentiation yet at the same time allows the maintenance of the proliferative ability that characterizes normal immature cells at a corresponding level of differentiation. Current evidence suggests that leukemogenesis results in an uncoupling of proliferation from differentiation rather than the loss of genes that regulate the control of normal growth and differentiation (1). The morphological, cytochemical, and cell surface phenotype of leukemic cells is thus generally believed to reflect that of a corresponding normal cell lineage and stage of maturation (2). It is therefore of considerable importance to determine to what extent AML cells remain subject to the regulatory mechanisms that control the growth and development of normal myeloid precursors. In one study the introduction of myeloid leukemia cells into the mouse embryo at an appropriate stage of development produced healthy mice whose granulocytes contained a marker derived from the leukemic clone (3). The implication of this result is that the leukemic cells were able to participate in normal myeloid differentiation programs, presumably due to the influence of an appropriate combination of signals derived from the developing fetus. This phenotypic reversion of malignancy has been confirmed

in vitro by inducing the normal sequence of myeloid differentiation in clones of leukemic cells using a physiological inducer of differentiation termed 'macrophage and granulocyte inducer' (MGI) (1).

The analysis of the control of gene expression and functional activity during human myeloid differentiation has been greatly facilitated with the establishment of cell lines derived from patients with myeloid leukemias. Although these lines appear to be blocked at specific stages of differentiation, a number of studies have shown that in some cases the cells may be released from maturation arrest and induced to terminally differentiate in the presence of a variety of inducing agents (4). Table IX summarizes the differentiation potential of these cell lines as defined by such inducing agents.

Induction of Granulocytic Differentiation

The acute promyelocytic leukemia cell line HL-60 is unique among the myeloid cell lines in that it is capable of undergoing differentiation along either the granulocyte or macrophage lineages (5-7). Other myeloid lines demonstrate at best only partial granulocytic differentiation potential (4). Numerous physiological and nonphysiological agents are capable of inducing this pathway of differentiation in HL-60 cells, including: dimethylsulfoxide (DMSO), dimethylformamide, hexamethylene bisacetamide, butyric acid, hypoxanthine, actinomycin D, methotrexate, 5-azacytidine, vitamins A and D and their metabolites, cyclic nucleotides, proteolytic enzymes, and a factor derived from various conditioned media believed to be a member of the CSF family (4,8,9). Unfortunately the wide variety of compounds that trigger differentiation in these cells makes it difficult to identify common features that may play a role in the initiation of the granulocytic differentiation program.

TABLE IX

The differentiation potential of established human myeloid leukemia cell lines. Adapted from ref (4).

CELL LINE	LEVEL OF DIFFERENTIATION BLOCK	DIFFERENTIATION POTENTIAL	INDUCING AGENTS
KG-1a	Early myeloblast	-	-
KG-1	Myeloblast	Macrophages	Phorbol esters
HL-60	Promyelocyte	Granulocytes or macrophages	Numerous
ML-1&3	Myelomonoblast	Macrophages	Phorbol esters
U937	monocytoid	Macrophages	Phorbol esters
K562	Early blast/ erythroblast	Erythroblasts	Hemin/butyrate
HEL	Erythroblast	Erythroblasts or macrophages	Hemin Phorbol esters

Commitment of HL-60 to granulocytic differentiation occurs very rapidly, within 8-18 hours (10), and the lineage into which the cells will differentiate can be switched by passaging the cells at pH 7.2 to become neutrophilic granulocytes, and pH 7.6 to become eosinophilic granulocytes (11). The induced HL-60 cells display phenotypic and functional characteristics of mature granulocytes and these are summarized in Table X. It appears that the capacity for terminal differentiation is partially defective however since at least two well known markers of mature granulocytes, namely lactoferrin and leukocyte alkaline phosphatase, are not expressed in differentiated HL-60 cells and there also appears to be incomplete expression of some cell surface antigens (12).

Induction of Monocytic Differentiation

The tumour-promoting phorbol diesters, the prototype of which is 12-O-tetradecanoylphorbol 13-acetate (TPA), can induce the KG-1, ML-3, HL-60, U937, and HEL myeloid cell lines to differentiate into cells possessing markers of monocytic differentiation (Table XI) (4,13). The erythroleukemia cell line HEL is particularly interesting in that it can be induced to express either erythroid-specific markers when cultured in the presence of hemin, or monocyte-specific markers in the presence of TPA (14). Current evidence suggests that the phorbol esters may trigger differentiation through an interaction with protein kinase C, and that this interaction leads to a cascade of cellular events that are involved in the monocyte differentiation program (15).

TPA has also been shown to induce the differentiation of the majority of fresh AML blasts into macrophage-like cells (16,17). Most AML cells do not appear to retain granulocytic potential however, since the culture conditions known to induce HL-60 cells into granulocytes trigger no or minimal granulocytic differentiation in fresh AML blasts. Nevertheless, the

TABLE X

Characteristics of HL-60 leukemic promyelocytes before and after induction of differentiation along the granulocyte lineage. Adapted from refs (4) and (8).

CHARACTERISTIC	UNINDUCED	INDUCED
Morphology	promyelocytes	differentiating granulocytes
DNA synthesis	+	-
Proliferation	+	-
Colony formation (soft agar)	+	-
Tumorigenicity	+	-
Chemotaxis	-	+
Phagocytosis (bacteria)	-	+
NBT reduction*	-	+
Bactericidal activity	-	+
Fc receptors	+	+
Complement receptors	-	+
Alkaline phosphatase	-	-
Lactoferrin	-	-
Expression of differentiation antigens consistent with granulocytic differentiation.		
Alterations in oncogene expression with differentiation.		

* Nitro-blue tetrazolium (NBT) is a histochemical stain that identifies a functional phagocytic system in mature granulocytes.

TABLE XI

Characteristics of HL-60 leukemic promyelocytes before and after induction of differentiation along the monocytic lineage. Adapted from refs (4) and (8).

CHARACTERISTIC	UNINDUCED	INDUCED
Morphology	promyelocytes	adherent monocytes
DNA synthesis	+	-
Colony formation	+	-
Phagocytosis (bacteria)	-	-
Phagocytosis (latex beads)	-	+
Non-specific esterase	-	+
NADase	-	+
Cytotoxicity for tumour cells	-	+
Expression of differentiation antigens consistent with monocytic differentiation.		
Alterations in oncogene expression with differentiation.		

therapeutic implication of these biological modifiers of myeloid differentiation is stimulating a systematic search for agents that could possibly induce terminal differentiation of myeloid leukemia cells in vivo (18-21).

Oncogenes and Differentiation

The identification of specific genes that play a role in normal myeloid differentiation is crucial to an understanding of how the gross chromosomal abnormalities observed in leukemic cells can influence the pathogenesis of myeloid leukemia. Several authors have suggested that a number of cellular oncogenes might encode products that control cell growth and differentiation. In support of this view are reports of alterations in the transcriptional expression of a number of these genes in myeloid leukemia cell lines following induction of differentiation (Table XII). Although induction of differentiation results in the cessation of proliferation, the decreased expression of at least the c-myc gene appears to be directly related to the differentiation process rather than to a cell cycle-related phenomenon (22).

Cell Surface Antigens and Differentiation

A number of studies have shown that the morphological and functional maturation of myeloid leukemia cell lines, as induced by various agents, involves a coordinated series of alterations in cell surface antigen expression that reflects the normal myeloid differentiation scheme. The HL-60 cell line for example is known to express a promyelocyte antigen termed Pro-Im1, but does not express the monocyte antigen OKM1. Induction of monocytic differentiation in these cells results in a loss of the Pro-Im1 antigen, and the subsequent expression of the OKM1 molecule (23). Induction of granulocyte differentiation on the other hand results in the induction of C3 receptors, the increased expression of a number of granulocytic markers,

TABLE XII

Alterations in the transcriptional expression of a number of cellular oncogenes in HL-60 cells following induction of differentiation. Adapted from refs (4) and (29).

	STAGE OF DIFFERENTIATION		
Oncogene	Promyelocytes	Granulocytes	macrophages
myc	+	↓	↓
myb	+	↓	↓
fes	+	+	↓
abl	+	+	+
ras^H	+	+	+
fos	-	-	↑

+ = transcriptionally active
↓ = marked decrease in transcription
↑ = marked increase in transcription

and the diminished expression of HLA-ABC and β_2-microglobulin determinants, as well as a variety of other myeloid differentiation antigens (6,12,24-28). Although the pattern of expression of these cell surface antigens is generally consistent with that of a normal myeloid differentiation pathway, partial defects in antigen expression have been reported, and the differentiation-linked changes observed in a particular cell line is not necessarily reproduced in another (12). It has been suggested that heterogeneity in antigen expression observed between the different myeloid lines when induced to differentiate might reflect heterogeneity in the differentiation block of each cell line, and that individual phenotypic characteristics are responsible for the extent of differentiation obtained with a given inducer (12). Nevertheless, these molecular changes on the cell surface may have important roles in the sequential stages of the myelopoietic differentiation program, and they offer a powerful approach to the study of myeloid differentiation at the molecular level.

In the preceding chapter the cellular distribution of the NHL-30.5 antigen was shown to be restricted to leukemic specimens containing immature myeloid blast cells. In this section the expression of the NHL-30.5 antigen was analyzed in detail on a series of myeloid leukemia cell lines induced to differentiate in vitro.

2) RESULTS

Reactivity of NHL-30.5 with Leukemic Cell Lines

A number of hemopoietic cell lines were tested for reactivity with the NHL-30.5 monoclonal antibody. None of the lymphoid cell lines tested (SU-DHL 1, 4, 6, 8, and 10, and Jurkat) showed any fluorescence above background. A number of the myeloid lines did react however, and these are listed in Table XIII. Since the HL-60 cell line has been the most extensively characterized,

these cells were initially chosen to study the effect of differentiation on the expression of the NHL-30.5 antigen.

Induction of Granulocytic Differentiation

Approximately 70-90% of HL-60 cells showed reactivity with NHL-30.5 under normal conditions and the binding of ^{125}I-labelled purified NHL-30.5 antibody gave an estimation of 4,000 molecules/cell. When the line was induced to differentiate along the granulocyte lineage by incubating in the presence of DMSO (Figure IX) or retinoic acid (Figure X), the number of cells expressing the antigen decreased. This decrease began on the first day following the addition of the inducing agent and continued to decline steadily until the fluorescence profiles of the induced cells became identical to those of the negative control. By the fifth day only 3% of the DMSO-induced cells and <1% of the retinoic acid-induced cells were positive by FACS analysis. Furthermore, only 250 NHL-30.5 molecules/cell could be detected on the induced cells using iodinated antibody. An additional peak was observed in the fluorescence profile of NHL-30.5-labelled cells from the retinoic acid-treated cultures on day five (Figure X). This was also present in the negative control however, and may reflect the declining health of the cultures associated with overgrowth (control cells) or differentiation (retinoic acid-treated cells). Following exposure to the differentiating agents >80% of the cells showed morphological evidence of differentiation beyond the promyelocyte stage.

NHL-62.14, a monoclonal antibody with apparent specificity for the transferrin receptor (produced in the same fusion as NHL-30.5) was used as a positive control. Greater than 95% of cultured HL-60 cells were positive for NHL-62.14, but if induced to differentiate only 60% of the cells were positive and fluorescence intensity was much weaker.

TABLE XIII

Reactivity of NHL-30.5 monoclonal antibody with various hemopoietic cell lines

CELL LINE	CHARACTERISTICS	FACS ANALYSIS % NHL-30.5-POSITIVE
HEL	erythroleukemia (AML)	>90%
HL-60	promyelocytic leukemia (AML)	70-90%
KG-1	myeloblastic leukemia (AML)	30-50%
KG-1a	immature variant of KG-1 (AML)	<10%
K562	early blast/erythroleukemia (CML)	<10%
U937	early monocytoid (histiocytic lymphoma)	<1%

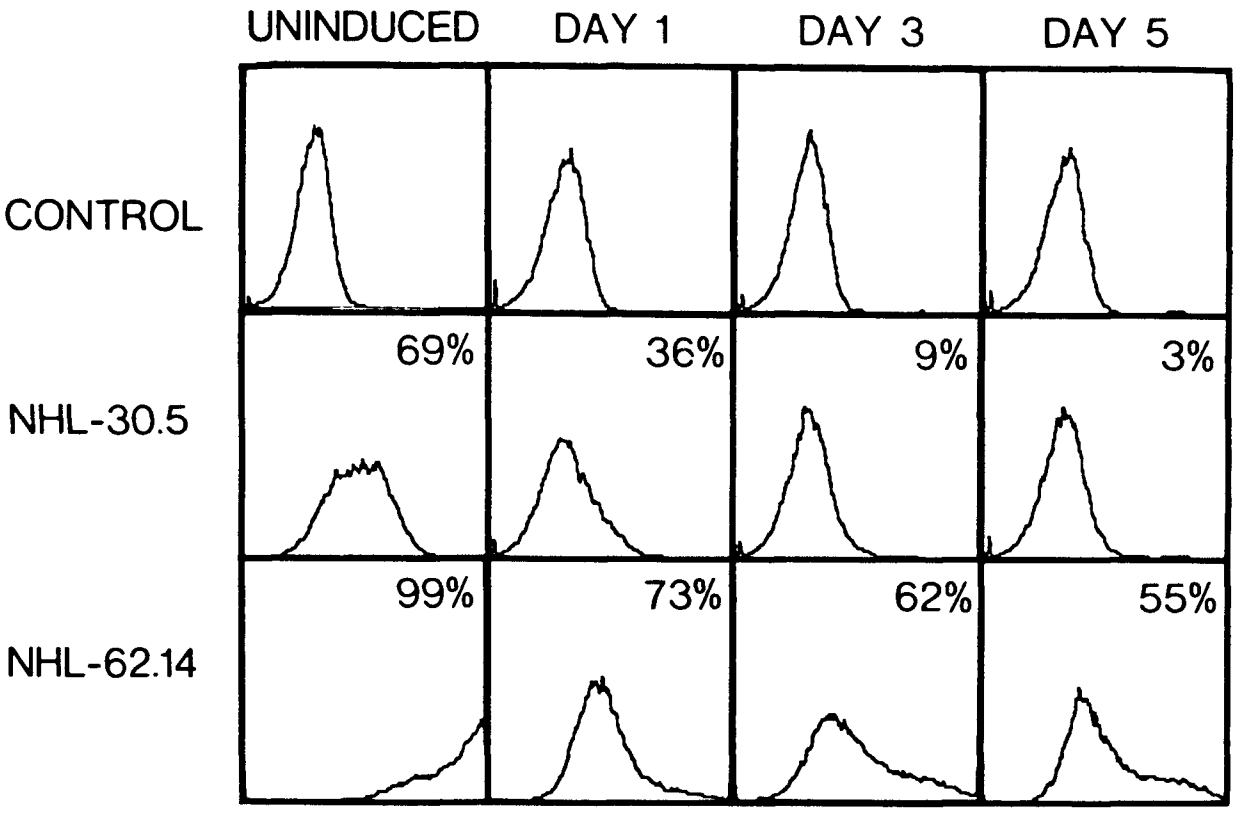


FIGURE IX FACS analysis of the reactivity of NHL-30.5 monoclonal antibody with HL-60 cells induced to differentiate with DMSO. Cells were cultured in the presence of 1.25% DMSO and analyzed for reactivity on days one, three, and five following the addition of DMSO. The control antibody is an unrelated monoclonal antibody raised against mouse lymphocytes and NHL-62.14 is an anti-transferrin receptor antibody. Percentages refer to the % positive cells. The horizontal axis represents fluorescence intensity (log) and the vertical axis represents cell number.

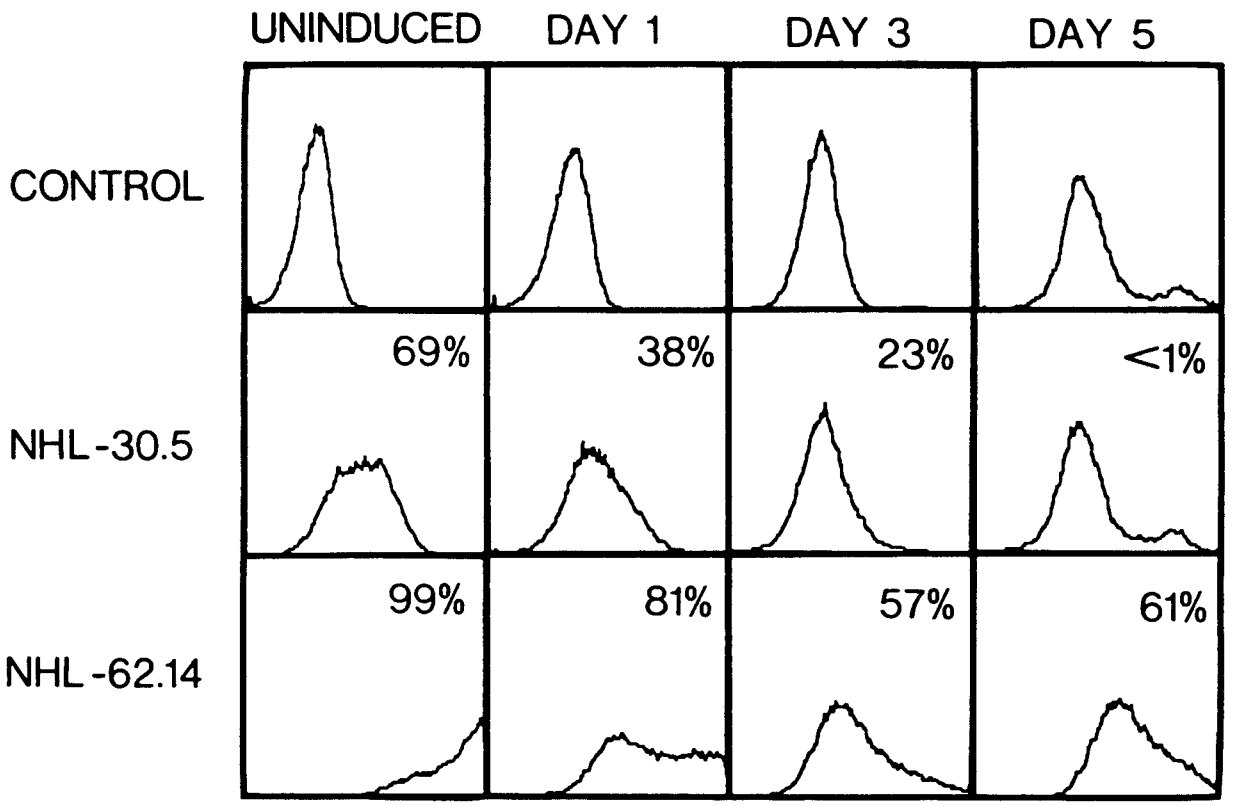


FIGURE X FACS analysis of the reactivity of NHL-30.5 monoclonal antibody with HL-60 cells induced to differentiate with retinoic acid. Cells were treated with 1 x 10^{-6}M retinoic acid and analyzed for reactivity on days one, three, and five following induction as in Figure IX

The NHL-30.5 and NHL-62.14 antigens were immunoprecipitated from the surface of iodinated HL-60 cells before and after induction with DMSO. Figure XI shows that NHL-30.5 could only be precipitated from the uninduced cells. The NHL-62.14 antigen was detectable before and after induction, although there was less precipitated from the induced cells. Immunoprecipitation of cells labelled with ^{35}S-Methionine or ^{3}H-leucine was unsuccessful. Metabolic labelling with ^{32}P failed to demonstrate phosphorylation of the NHL-30.5 antigen under conditions where the transferrin receptor was clearly labelled (Figure XII).

Induction of Monocytic Differentiation

The HL-60 cell line has previously been shown to acquire monocytoid characteristics following incubation in the presence of TPA (13). Figure XIII shows the fluorescence profiles of TPA-induced HL-60 cells stained with the NHL-30.5 and NHL-62.14 antibodies. Within one day following induction of differentiation, 85% of the cells were adherent and had to be removed with a rubber policeman. One and two days following addition of the TPA <1% of the cells (both adherent and nonadherent) expressed the NHL-30.5 antigen. Essentially all of the uninduced cells expressed high levels of the transferrin receptor but two days following induction only half of the cells were positive and fluorescence intensity was much weaker.

The KG-1 cell line was initially derived from a patient with myeloblastic leukemia and these cells retain the morphology of leukemic myeloblasts. Under normal culture conditions 30-50% of the KG-1 cells expressed the NHL-30.5 antigen and estimations of the number of molecules per cell varied between 500 and 2,300. Incubation of the KG-1 cells in the presence of DMSO had no effect on the expression of NHL-30.5. This confirms previous observations that KG-1 cells are resistant to agents known to induce granulocytic differentiation in

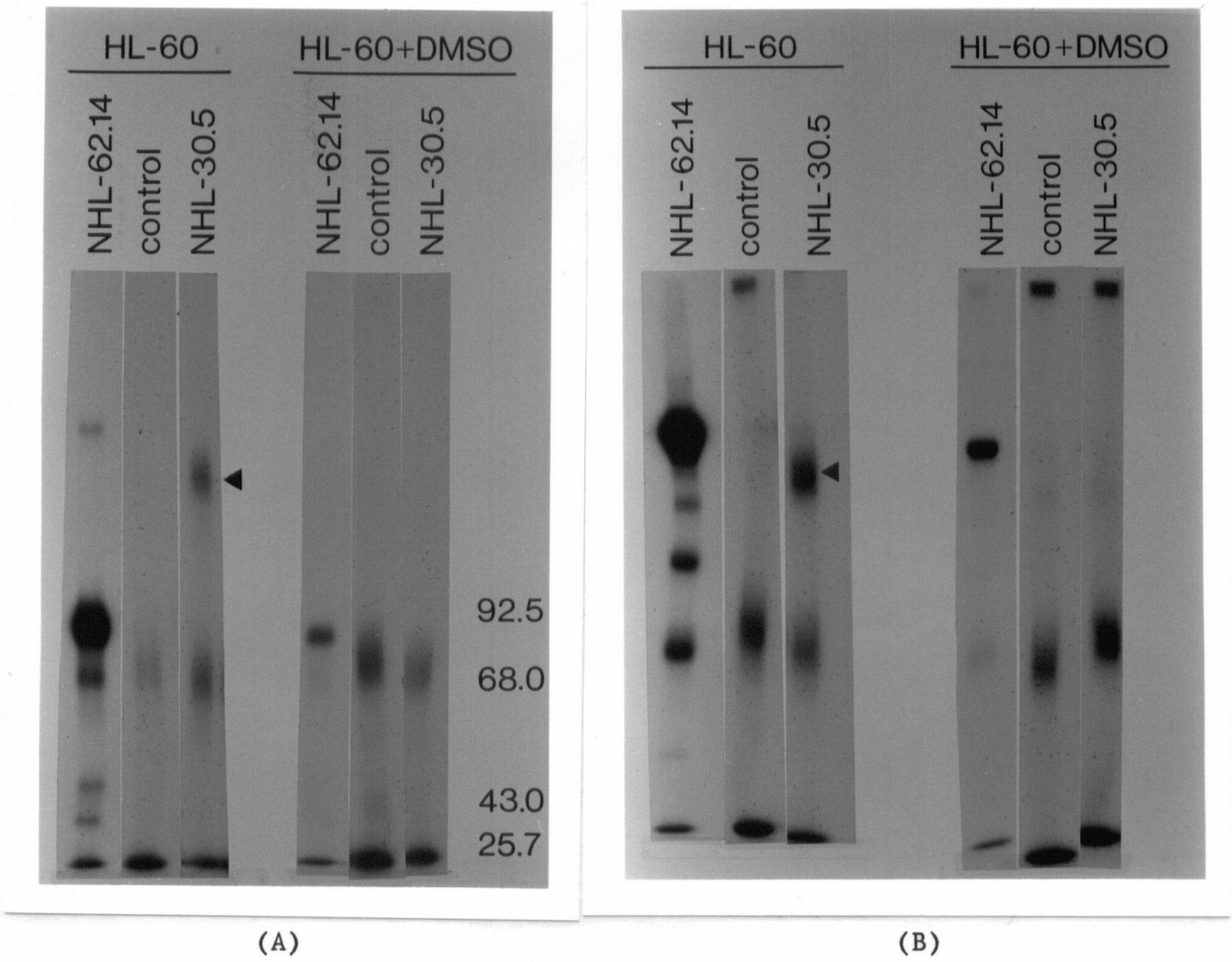


FIGURE XI Immunoprecipitation of NHL-30.5 and NHL-62.14 antigens on HL-60 cells before and after induction with DMSO (day 5). Target cells were labelled with ^{125}I and the antigens were immunoprecipitated from the cell lysates with the NHL-30.5 and NHL-62.14 monoclonal antibodies. The analysis was carried out on SDS-PAGE (5%) under both reducing (A) and nonreducing (B) conditions.

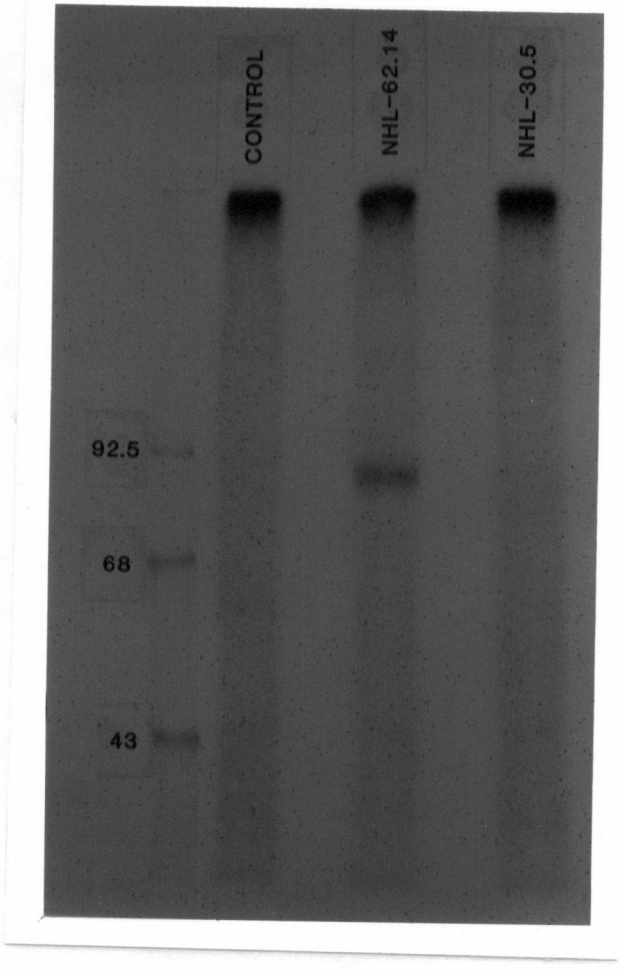


FIGURE XII Immunoprecipitation of NHL-30.5 and NHL-62.14 (transferrin receptor) antigens from HL-60 cells labelled with ^{32}P. Analysis was carried out on SDS-PAGE (7.5%) under reducing conditions.

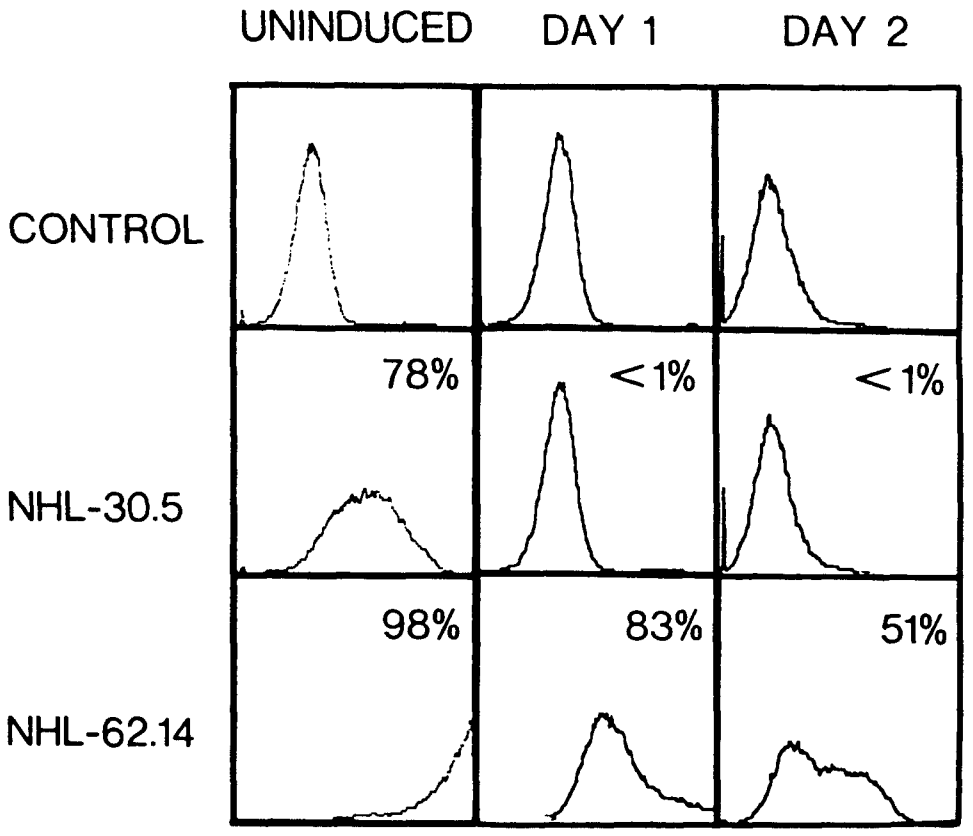


FIGURE XIII FACS analysis of the reactivity of NHL-30.5 monoclonal antibody with HL-60 cells induced to differentiate with TPA. Cells were harvested on days one and two following addition of the TPA and analysis was carried out as in Figure IX.

HL-60 cells (12). Agents that induce monocytic differentiation can induce KG-1 cells however. Within one day of treatment with TPA, 60% of the induced KG-1 cells became adherent although they were easily resuspended following a five minute incubation in saline containing 0.2% EDTA and 0.1% BSA. This is in contrast to the HL-60 cell line which became very strongly adherent in the presence of TPA and most cells had to be removed with a rubber policeman. Nevertheless, both the adherent and nonadherent cells from TPA-treated KG-1 cultures lose the NHL-30.5 antigen (Figure XIV).

The KG-1a cell line is a spontaneous variant of the KG-1 line and is considered to be more immature than its parental line. The cells are morphologically undifferentiated blast cells and are negative for all histochemical stains (12,30). No inducer has been described that is capable of stimulating a differentiation program in these cells. The NHL-30.5 antigen is weakly expressed on a small subpopulation of KG-1a cells (5%). This population appears to represent a distinct subset of NHL-30.5-positive cells (rather than background staining) since this proportion was increased to 15% by sorting the NHL-30.5-positive cells in the FACS followed by expansion of the cells in tissue culture.

The K562 cell line was established from a CML patient in blast crisis (31) and was initially felt to be blocked at a very early myeloid blast stage. The subsequent demonstration of the synthesis of the erythroid marker glycophorin (32) and the inducibility of globin gene expression in these cells suggested at least partial erythroid differentiation potential. Only 10% of the K562 cells express NHL-30.5.

The HEL cell line was initially derived from a patient with erythroleukemia and these cells strongly express the NHL-30.5 marker. Under normal culture conditions >90% are NHL-30.5-positive with an estimated 1.5 x

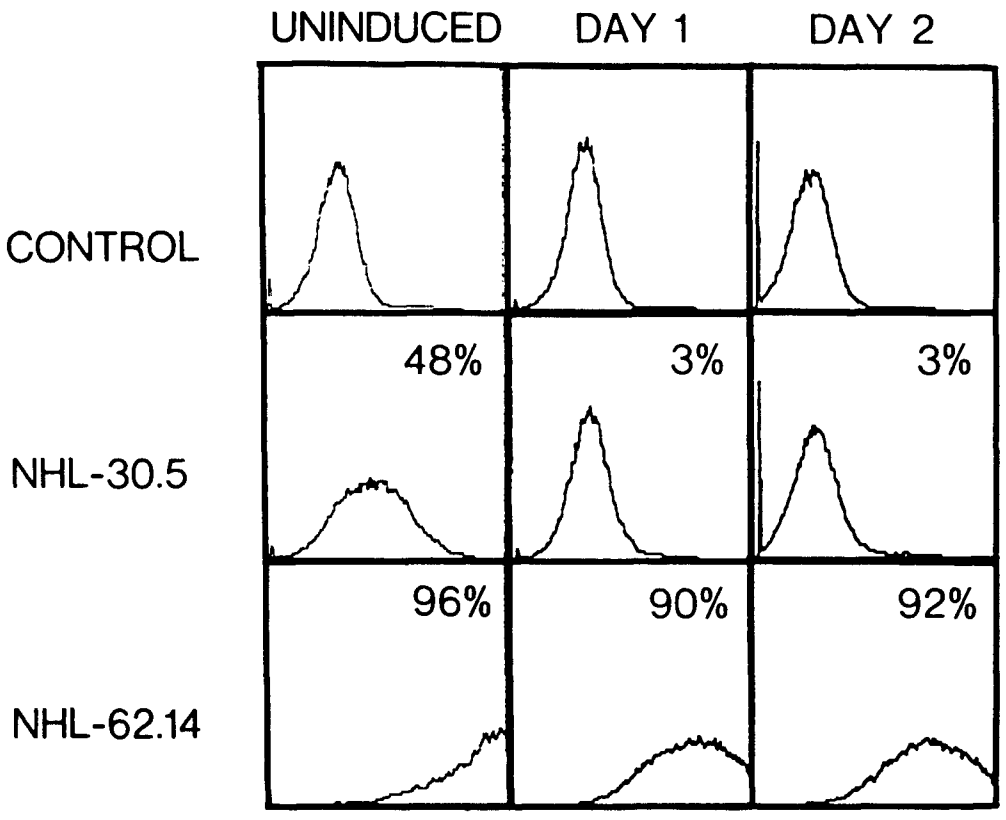


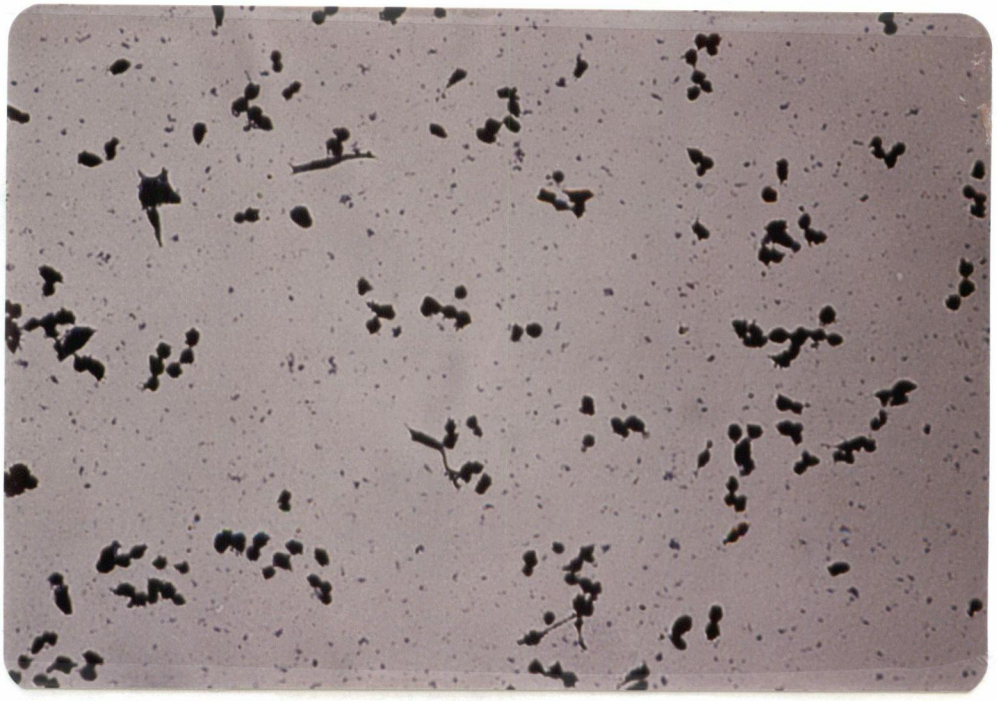
FIGURE XIV FACS analysis of the reactivity of NHL-30.5 monoclonal antibody with KG-1 cells induced to differentiate with TPA. Cells were harvested on days one and two following addition of the TPA and analysis was carried out as in Figure IX.

10^4 molecules/cell. The cell line generally grows in suspension although it is slightly adherent in tissue culture dishes. In the presence of TPA this adherence is greatly increased and the cells express markers of monocytic differentiation (33). Figure XV illustrates the morphology of HEL cultures prior to, and five days following, the addition of TPA into the medium. The fluorescence intensity of induced HEL cells stained with NHL-30.5 began to decrease within one day following induction and continued to decline until approximately 3,000 NHL-30.5 molecules/cell were remaining on day five. Likewise, the number of fluorescing cells progressively decreased to 40% and this paralleled the decreased expression of the transferrin receptor (Figure XVI). The nonadherent cells in the tissue culture dishes behaved in a similar manner (Figure XVII). When the nonadherent cells were removed and cultured in tissue culture dishes containing fresh medium they rapidly became adherent (within several hours) suggesting that a) their lack of adherence in the initial culture dishes was due to overcrowding on the dish and b) they were committed to become adherent since TPA was no longer required in the medium.

3) DISCUSSION

Expression of the NHL-30.5 antigen was examined on cell lines derived from patients with myeloid leukemias. Two of these lines, K562 and the undifferentiated variant of the KG-1 line (KG-1a) expressed the antigen weakly and on <10% of the cells. Further characterization of the NHL-30.5-positive and NHL-30.5-negative populations in these cell lines may reveal some important functional differences. Three AML-derived cell lines (HEL, HL-60, and KG-1) expressed the antigen on a significantly larger proportion of cells; HEL being the strongest, followed by HL-60 and then KG-1. These lines are phenotypically immature although they retain the capacity to acquire some of the differentiated characteristics of the myeloid lineage under the influence of an appropriate stimulus.

(A)



(B)

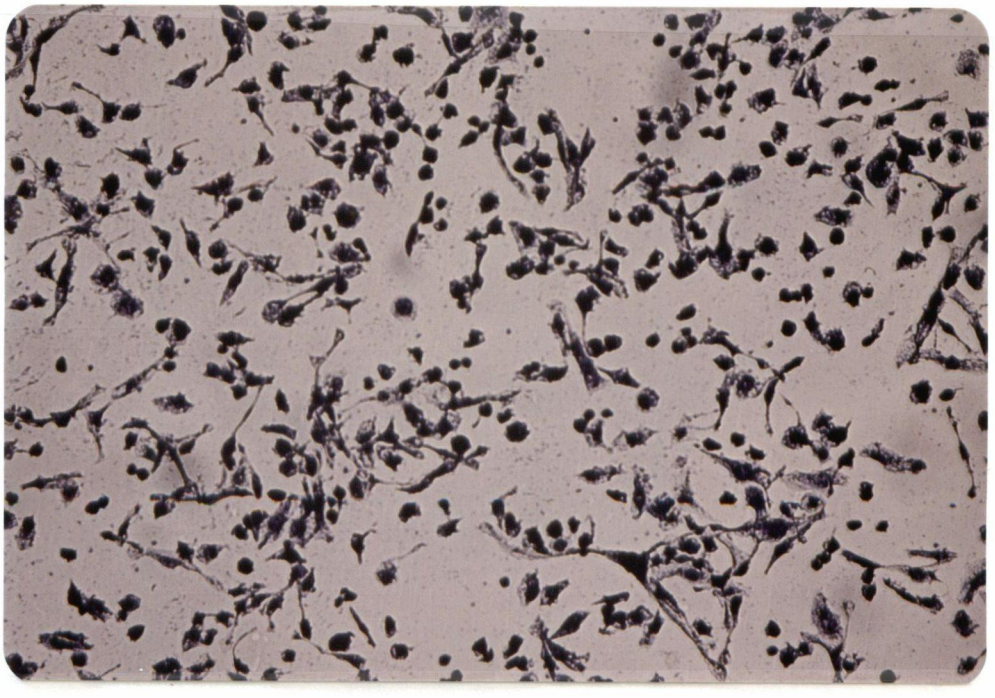


FIGURE XV Cultures of HEL cells before (A) and following (B) induction of differentiation with TPA. Cultures were stained with May-Grunwald-Giemsa.

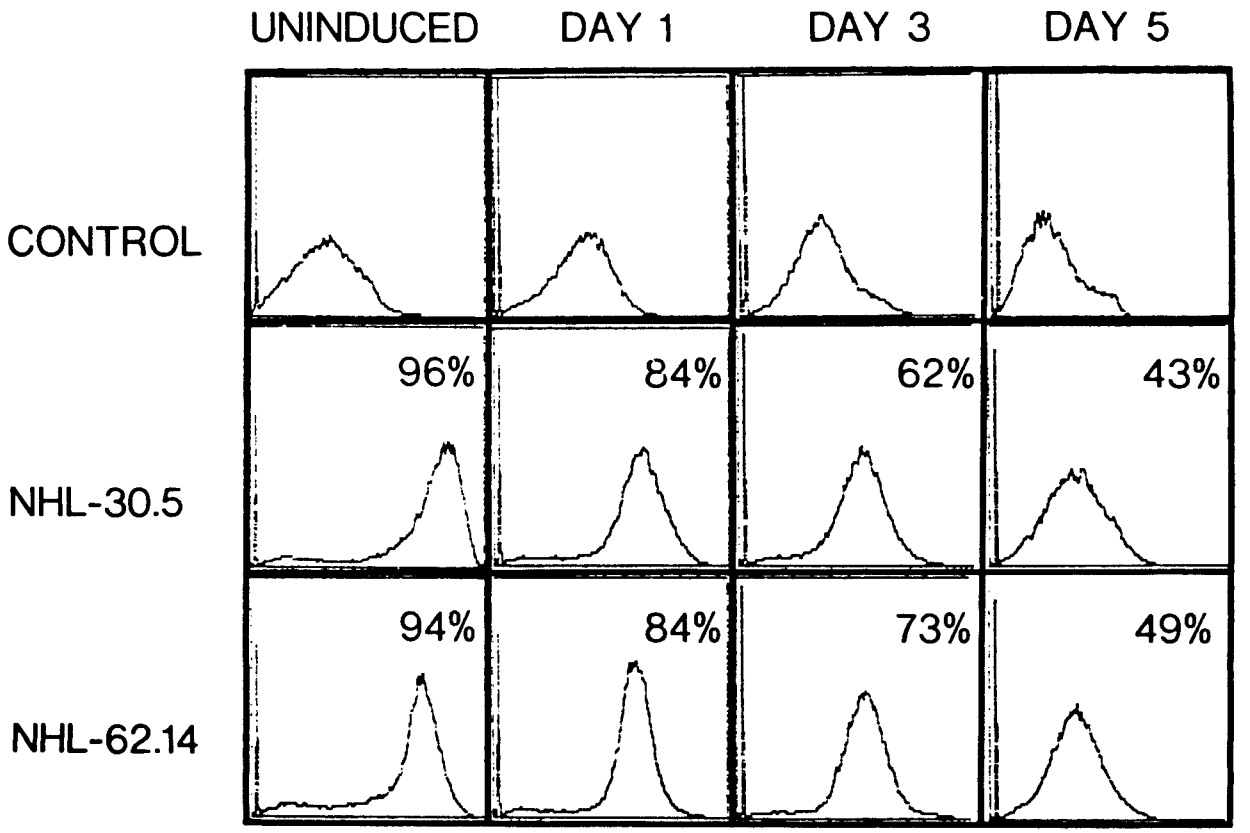


FIGURE XVI FACS analysis of the reactivity of the NHL-30.5 monoclonal antibody with HEL cells induced to differentiate with TPA. Adherent cells were harvested one, three, and five days following the addition of TPA and analysis carried out as in Figure IX.

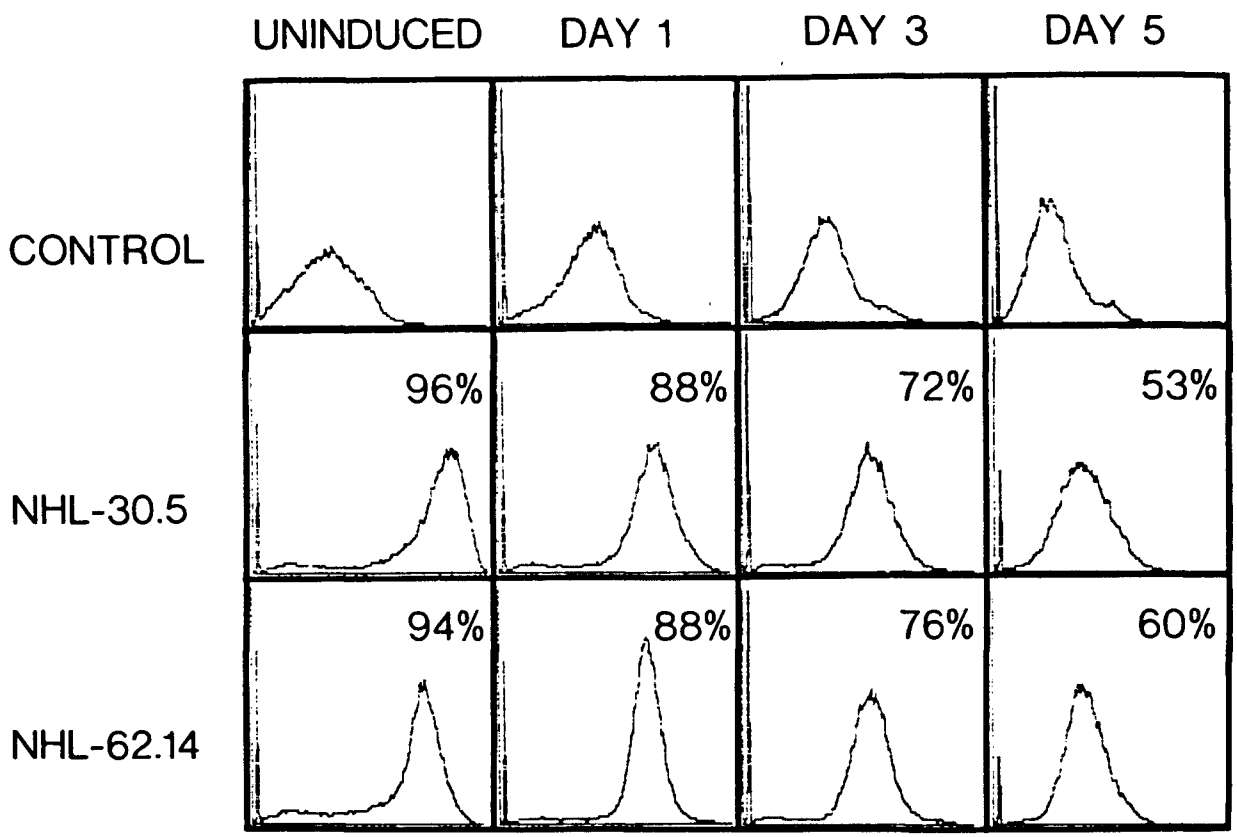


FIGURE XVII FACS analysis of the reactivity of the NHL-30.5 monoclonal antibody with the nonadherent cells from a TPA-induced culture of HEL cells. One, three, and five days following the addition of TPA the nonadherent cells were harvested and analyzed as in Figure IX.

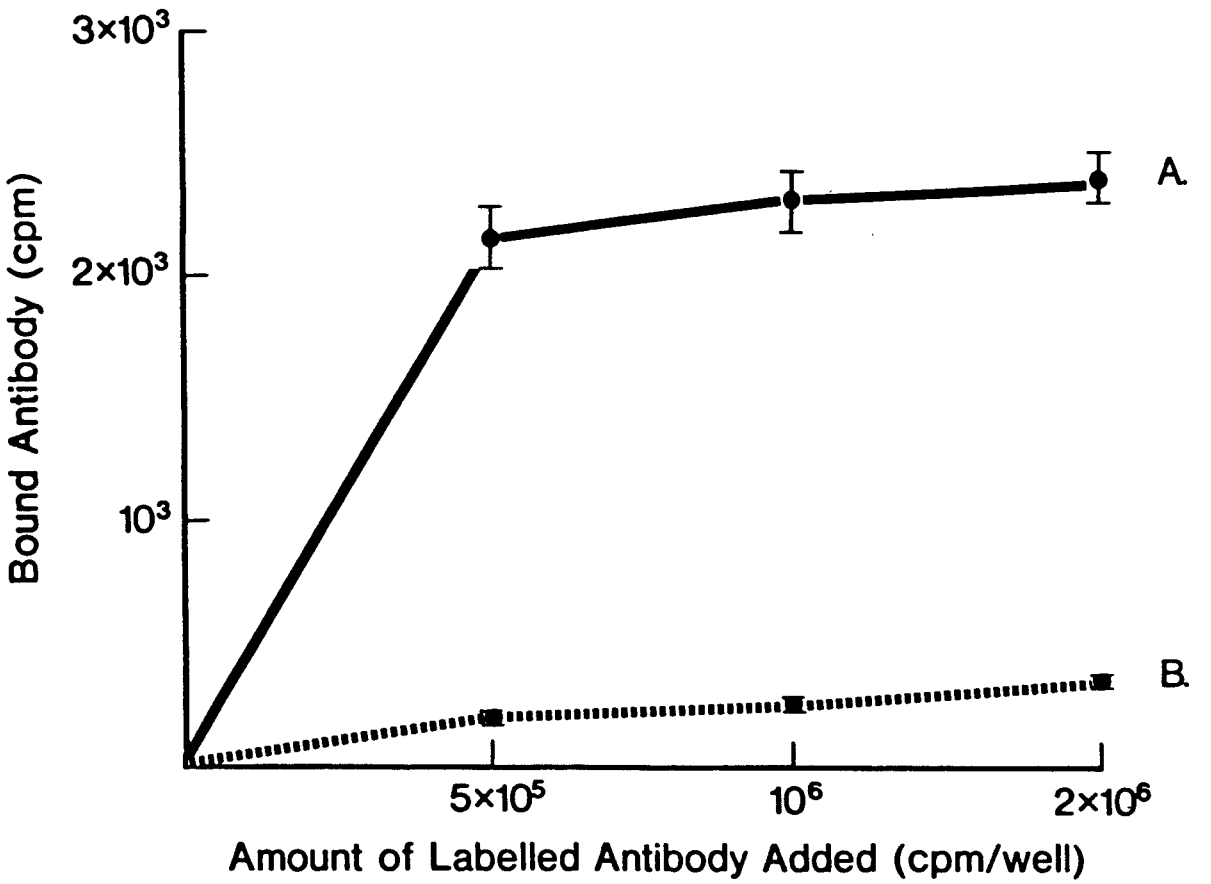


FIGURE XVIII Saturation curve for the binding of ^{125}I-labelled NHL-30.5 monoclonal antibody to HL-60 cells. An estimate of antigen density was obtained by substracting the amount of radioactivity bound to the cells in the presence of an excess of cold antibody (B) from the amount bound at saturation in the absence of cold antibody (A).

If the HL-60 cell line is induced to differentiate using DMSO or retinoic acid, the cells acquire properties of mature granulocytic cells. This process takes five days and, as demonstrated by fluorescent staining (Figures IX and X) and immunoprecipitation (Figure XI), involves the loss of the NHL-30.5 molecule beginning on the first day. The HL-60 cells can also be induced to differentiate into cells with markers of mature monocytic cells by incubating in the presence of TPA. Cells treated in this manner are also negative for NHL-30.5 antigen expression. The KG-1 cell line on the other hand is incapable of granulocytic differentiation but can be induced with TPA to become adherent and express monocytoid properties. The NHL-30.5 antigen is undetectable on both the adherent and nonadherent cells from TPA treated KG-1 cultures. In contrast to the HL-60 and KG-1 cell lines, induction of monocytic differentiation in the HEL cells does not reduce NHL-30.5 expression to undetectable levels. Approximately 1.5×10^4 molecules/cell are present on the uninduced cells and this is reduced five fold following induction of differentiation.

The functional significance of the differentiation-associated decrease in NHL-30.5 antigen expression is not known, although it lends support to the idea that NHL-30.5 is an early myeloid differentiation antigen that is progressively lost during the maturation process. This hypothesis would provide an explanation for the expansion of NHL-30.5-positive cells seen in AML and other hematologic disorders that are characterized by the accumulation of immature myeloid precursors. These observations lead into the following Chapter which will analyze the expression of this putative differentiation antigen on normal and leukemic myelopoietic progenitor populations.

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C H A P T E R V

RESTRICTED EXPRESSION OF AN ACUTE MYELOGENOUS LEUKEMIA-ASSOCIATED ANTIGEN (NHL-30.5) ON NORMAL HEMOPOIETIC PROGENITOR CELLS

1) INTRODUCTION

A variety of circulating blood cell types are now recognized by their unique morphologic, antigenic, and functional properties. Current evidence indicates that the production of these cells begins with the activation in primitive pluripotent progenitors of a unique program of gene expression that may take many cell generations to complete (1). Execution of such programs leads finally to the acquisition of specialized properties: although preliminary changes, including changes in responsiveness to various extrinsic growth factors, are also thought to occur. Some changes have been correlated with the loss of differentiative and proliferative potential that distinguish different populations of committed and pluripotent hemopoietic progenitors in clonogenic assays (2). Nevertheless, relatively little is known about the changes that distinguish these cells from more primitive hemopoietic progenitors or from their progeny in which evidence of terminal maturation can first be detected.

A widely adopted approach to identify gene products associated with early stages of hemopoietic cell differentiation has been to prepare monoclonal antibodies against antigens found on leukemic cell populations (3-9). Such an approach is based on the assumption that a morphologically recognized leukemic blast phenotype may be associated with the continued

expression of surface antigens normally restricted to primitive hemopoietic cell types. In the preceding Chapters the identification and partial characterization of a cell surface antigen expressed on hemopoietic cells from patients with AML was presented (9,10) (Chapters III and IV). The monoclonal antibody defining this molecule was shown to react with a substantial proportion of blood and marrow cells from over 80% of patients with newly diagnosed or relapsing AML. In contrast, normal mature blood cells including granulocytes, lymphocytes, monocytes and platelets were all found to be negative. Similarly, by FACS analysis, a detectable (>2%) positive population could not initially be demonstrated in buffy coat preparations of marrow aspirates from either normal or CML patients. Subsequent studies with light density peripheral blood preparations from patients with CML revealed a small but clearly detectable NHL-30.5 positive population to be present in some cases (7/26) (Table VII, Chapter III). A preliminary study with more highly purified populations of CML clonogenic granulopoietic progenitor cells also indicated that these reacted with NHL-30.5 (11).

In Chapter IV the expression of NHL-30.5 was analyzed on myeloid leukemia cell lines induced to differentiate, and was shown to have a maturation-linked pattern of expression. Together with the clinical data presented in Chapter III, these observations suggest that the NHL-30.5 marker is an early myeloid differentiation antigen, and that its increased expression on AML marrow or blood reflects the expanded population of immature myeloid cells characteristic of AML. If this hypothesis is correct then one might predict that the small (<5%) NHL-30.5-positive population in normal marrow would include myeloid progenitor cells. In this Chapter, the expression of NHL-30.5 on erythropoietic (CFU-E/BFU-E) and granulopoietic

(CFU-C) cells from both normal and leukemic individuals was evaluated using FACS sorting procedures and in vitro assays for clonogenic myeloid progenitors.

2) RESULTS

FACS Analysis/Cell Sorting

Since the peripheral blood of patients with CML typically contains elevated numbers of all progenitor classes (12-14), CML blood was first used to investigate the expression of the NHL-30.5 antigen on these primitive cell types. Cells from four patients were studied. Reactivity of NHL-30.5 with CML peripheral blood was variable depending on the patient (Table XIV). In cases where less than 5% of the cells were positive the sort gates were adjusted so that cells with the highest fluorescence intensity, comprising 5% of the total population, were sorted into the positive fraction and cells with the lowest fluorescence (the remaining 95%) were sorted into the negative fraction. The sorted fractions were then plated in standard methylcellulose assays to determine the distribution of the various myeloid progenitors within each sorted population. Table XIV illustrates the number of CFU-C, BFU-E, and CFU-E colonies per 10^5 cells from the four CML patients sorted in this manner. In the positive fractions granulocyte/macrophage progenitors were enriched an average of four fold while BFU-E and CFU-E were enriched six and seven fold respectively. Both the enrichment of progenitors in the positive fraction, and their corresponding depletion from the negative fraction was significant ($p < 0.05$, Table XIV). Sorted cells from two additional CML patients were plated in the absence of erythropoietin and a ten fold enrichment of CFU-C was observed in the NHL-30.5-positive fraction (data not shown).

TABLE XIV

The number of progenitors per 10^5 cells in sorted fractions from CML peripheral blood

		PATIENT NO.				
		1 (4.4%)§	2 (1.2%)	3 (6.8%)	4 (10.9%)	Mean of 4 experiments*
CFUC	Control	207	121	184	1535	290 (164-512)
	(+) Fraction	1092	414	1120	3750	1174 (748-1843)
	(-) Fraction	17	14	34	205	36 (20-67)
CFU-E	Control	262	443	46	1055	275 (142-530)
	(+) Fraction	2530	3200	36	5610	2012 (1107-3654)
	(-) Fraction	83	132	19	173	78 (48-127)
BFU-E	Control	80	167	39	1780	175 (76-401)
	(+) Fraction	1050	979	290	3120	982 (605-1596)
	(-) Fraction	30	79	20	263	60 (34-106)
CFU-GEMM	Control	1.7	5.3	0	0	
	(+) Fraction	164.4	6.5	2.5	0.5	
	(-) Fraction 0	1.2	0	0	0	

§ Figures in parentheses indicate % NHL-30.5-positive

* Geometric mean (Range defined by ± 1 S.E.M.)

Enrichment of all progenitors in the positive fraction and corresponding depletion from the negative fraction was significant when compared to the control in a one-tailed paired-sample t test ($p<0.05$).

Ficoll-hypaque separated normal bone marrow and peripheral blood cells were also stained, sorted, and assayed in a similar fashion. Although a distinct NHL-30.5-positive population was not observed in normal marrow, the sort gates were adjusted in each case so that cells with the highest fluorescence intensity, again comprising 5% of the total population, were sorted into the positive fraction. Table XV shows the frequency of myeloid progenitors in sorted fractions from six different normal marrow specimens. CFU-C were consistently, and significantly ($p < 0.05$), enriched in the NHL-30.5-positive fraction (on average, approximately 17 fold). Significant ($p < 0.05$) enrichment of BFU-E and CFU-E was also demonstrated, with corresponding depletion of these progenitors from the negative fractions. Although the anomalous behaviour of erythroid progenitors from bone marrow #3 (Table XV) suggests the existence of NHL-30.5-negative erythroid progenitors in this patient, the possibility that this result was an artefact can not be ruled out.

Table XVI shows the results of the same sorting experiments on ficoll-hypaque separated normal peripheral blood. The positive fraction again showed significant ($p < 0.05$) enrichment of all progenitor types (ten fold for CFU-C, five fold for CFU-E, and five fold for BFU-E). Cells plated from the negative fraction were also usually reduced in their content of these progenitors. Small numbers of CFU-GEMM (progenitors of mixed granulocyte-erythroid-macrophage colonies) were observed in some of the above experiments where the general trend suggested a similar staining behaviour to that of other clonogenic progenitors.

A summary of the distribution of the various progenitor classes in sorted fractions from CML peripheral blood, normal peripheral blood, and normal bone marrow is graphically illustrated in Figures XIX-XXI.

TABLE XV

The number of progenitors per 10^5 cells in sorted fractions from normal bone marrow.

		PATIENT NO.						
		1 (3.4%)§	2 (3.8%)	3 (<1%)	4 (4.9%)	5 (2.6%)	6 (3.8%)	Mean of 6 experiments*
CFU-C	Control	103	93	102	247	127	197	135 (114-159)
	(+) Fraction	2267	2054	1630	2640	3308	2350	2320 (2105-2556)
	(-) Fraction	20	15	72	15	69	19	28 (20-37)
CFU-E	Control	221	182	225	278	190	67	178 (145-219)
	(+) Fraction	2856	3243	340	3460	3487	1220	1896 (1296-2772)
	(-) Fraction	144	21	229	28	268	17	67 (40-112)
BFU-E	Control	55	65	93	171	27	197	82 (61-111)
	(+) Fraction	250	946	75	1530	450	2350	554 (330-930)
	(-) Fraction	34	13	105	42	64	19	37 (27-50)
CFU-GEMM	Control	1	1.5	0.5	0.4	0	0	
	(+) Fraction	0	13.5	0	0	0	0	
	(-) Fraction	0	0.4	0	0	0	0	

§ Figures in parentheses indicate % NHL-30.5-positive

* Geometric mean (Range defined by ± 1 S.E.M.)

There was significant enrichment of all progenitors in the positive fraction when compared to the control fraciton in a one-tailed paired-sample t test ($p<0.05$). Depletion from the negative fraction was significant for both CFU-C and CFU-E but was not significant for BFU-E (experiments 3 and 5 were not depleted of BFU-E in the negative fraction).

TABLE XVI

The number of progenitors per 10^5 cells in sorted fractions from normal peripheral blood.

		DONOR NO.				
		1 (<1%)§	2 (<1%)	3 (<1%)	4 (1.5%)	Mean of 4 experiments*
CFU-C	Control	6	11	4	11	7 (6-9)
	(+)Fraction	75	114	33	71	67 (52-87)
	(-) Fraction	3	3	1	1	2 (1-3)
CFU-E	Control	4	23	6	7	8 (5-12)
	(+) Fraction	54	105	12	48	43 (27-67)
	(-) Fraction	2	7	<1	1	2 (1-3)
BFU-E	Control	8	40	16	30	20 (14-28)
	(+) Fraction	94	176	66	127	109 (88-134)
	(-) Fraction	14	18	3	6	9 (6-13)
CFU-GEMM	Control	0.3	0.9	0.8	1.3	
	(+) Fraction	4.5	0.8	22.0	1.2	
	(-) Fraction	0.8	0.1	0.4	0.1	

§ Figures in parentheses indicate % NHL-30.5-positive

* Geometric mean (Range defined by ± 1 S.E.M.)

There was significant enrichment of all progenitors in the positive fraction when compared to the control fraction in a one-tailed paired-sample t test ($p<0.05$). Depletion from the negative fraction was significant for both CFU-C and CFU-E but was not sigificant for BFU-E (patient #1 showed no depletion of BFU-E from the negative fraction).

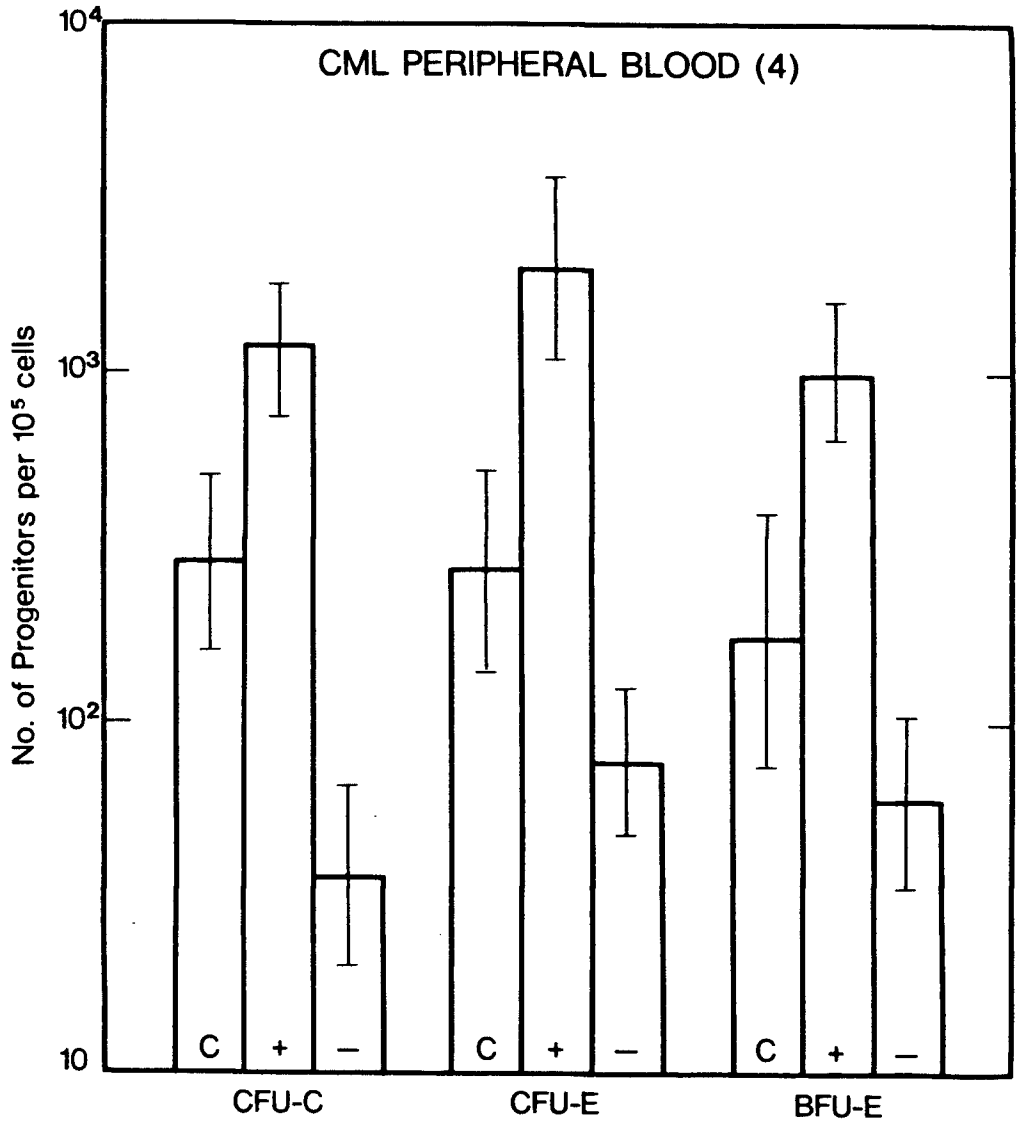

FIGURE XVIII Graphical representation of the frequency of myeloid progenitors in sorted fractions from CML peripheral blood. (C) control (unsorted) fraction, (+) NHL-30.5-positive fraction, (-) NHL-30.5-negative fraction.

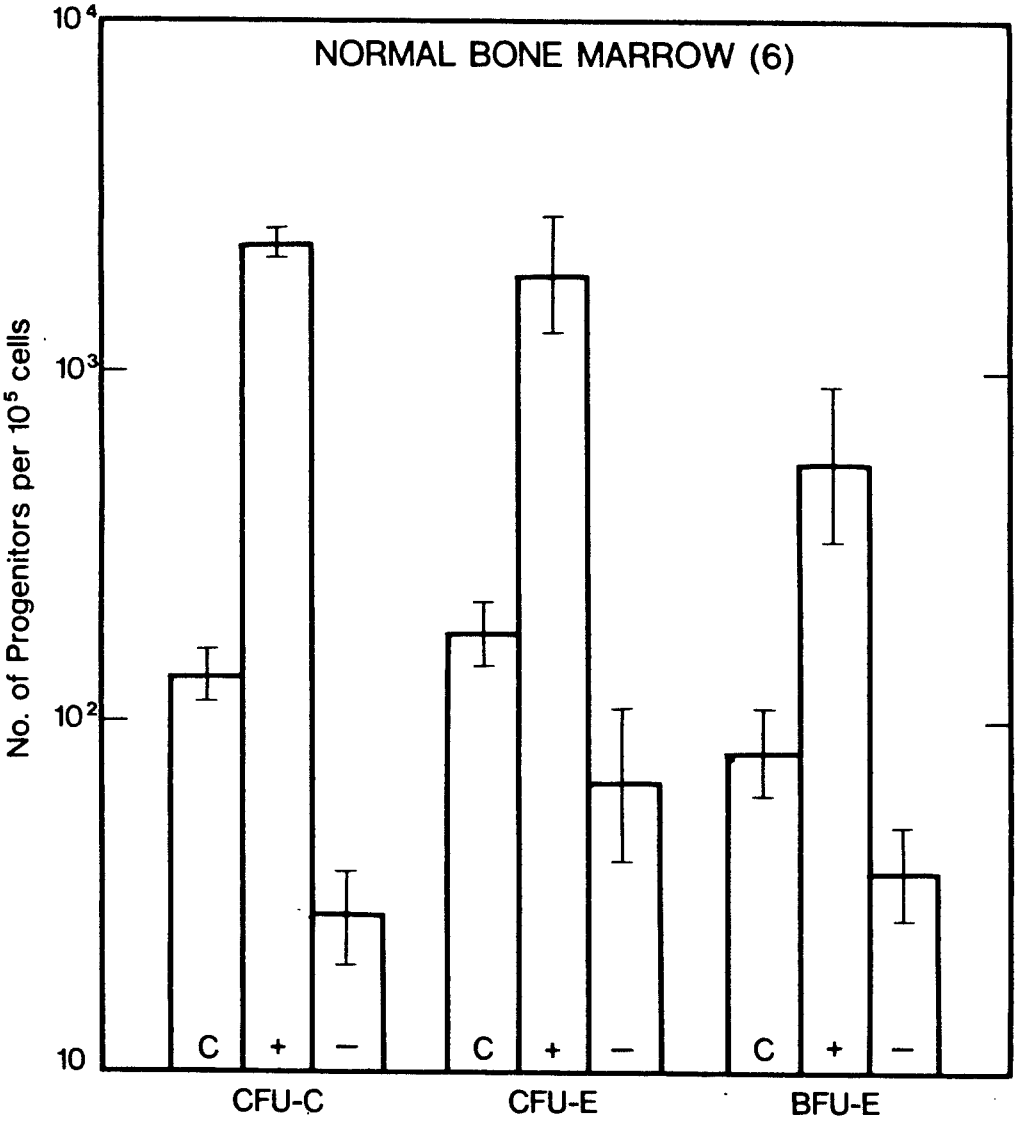

FIGURE XIX Graphical representation of the frequency of myeloid progenitors in sorted fractions from normal bone marrow. (C) control (unsorted) fraction, (+) NHL-30.5-positive fraction, (-) NHL-30.5-negative fraction.

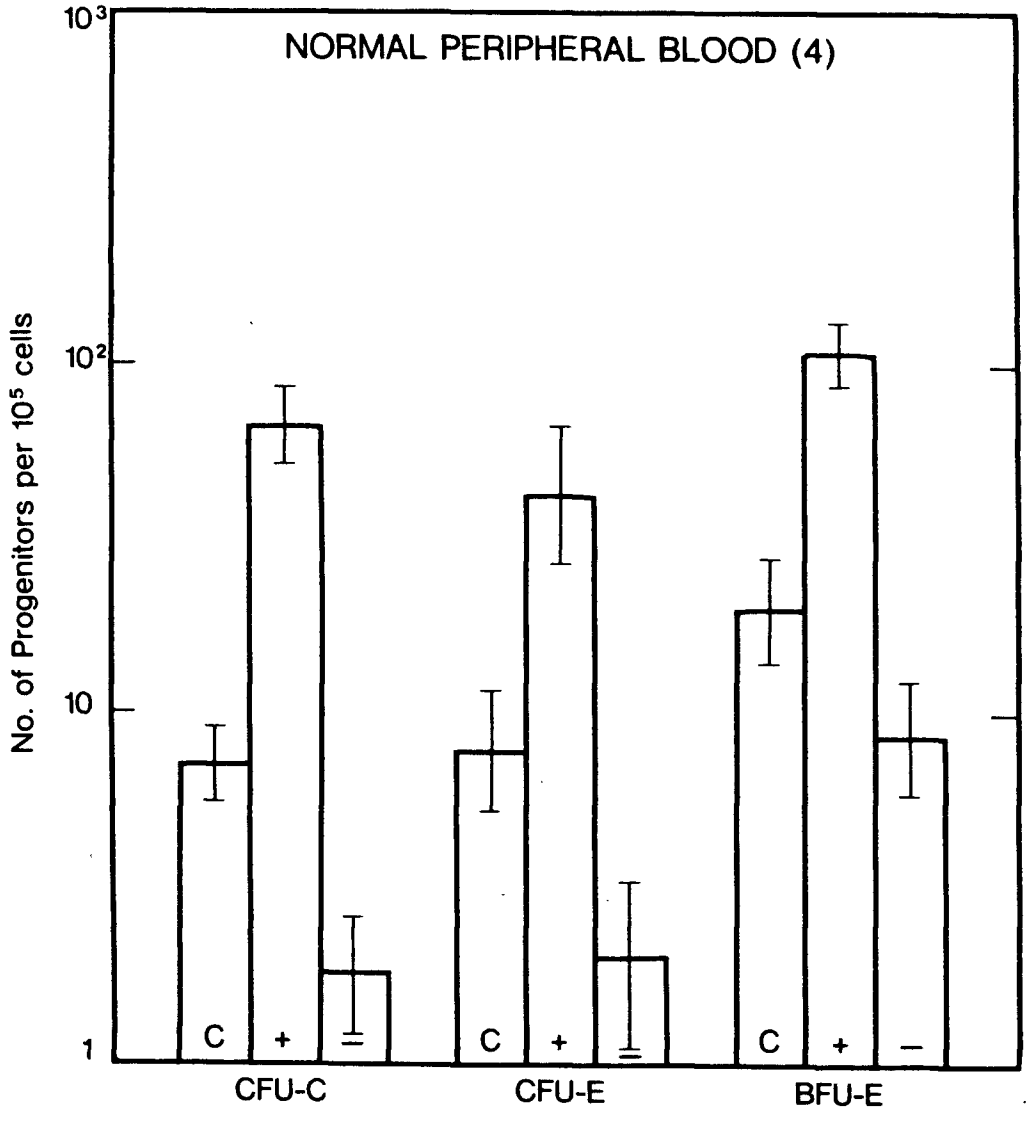

FIGURE XX Graphical representation of the frequency of myeloid progenitors in sorted fractions from normal peripheral blood. (C) control (unsorted) fraction, (+) NHL-30.5-positive fraction, (-) NHL-30.5-negative fraction.

In control experiments, some of these specimens were stained with media or an unrelated monoclonal antibody specific for phycoerythrin (IgG1) and the top 5% again sorted and assayed for progenitors. In such experiments, no enrichment of any progenitor type was obtained, providing direct evidence that the progenitor enrichment observed with NHL-30.5 staining was due to their selective reactivity with this antibody.

To test whether marrow fibroblasts and other minor adherent cell components of normal marrow aspirates express NHL-30.5, marrow adherent layers were established in 20% FCS in alpha medium by seeding 1-2 x 10^7 cells into 60 mm tissue culture dishes and maintaining them at 37°C with weekly feeding. After one or two subcultures the cells were allowed to reach confluence and then collagenased (15) prior to staining for FACS analysis. Such cell suspensions contained no detectable (<1%) NHL-30.5-positive cells (three separate marrows tested) whereas concurrently analyzed, and collagenase-treated, HL-60 cells were 40% positive.

In order to determine whether AML blast cell progenitors express NHL-30.5, the same sorting experiments were performed on peripheral blood and bone marrow cells from a patient with AML. Figure XXII illustrates the fluorescence profiles of the reactivity of the NHL-30.5 monoclonal antibody with cells from this patient. Three distinct populations were observed: a negative population (comprising ~40% of the total population), a positive population (~50%), and a strongly positive population (~10%). Cells were sorted at gate 'a' (top 60% positive) or gate 'b' (top 10%) and plated in standard methylcellulose assays. The patient produced numerous abnormal blast-like colonies in culture and the distribution of these colonies in the various sorted fractions is illustrated in Table XVII. The vast majority of the colony-forming cells sorted into the NHL-30.5-positive population, comprising the top 60% fluorescent cells,

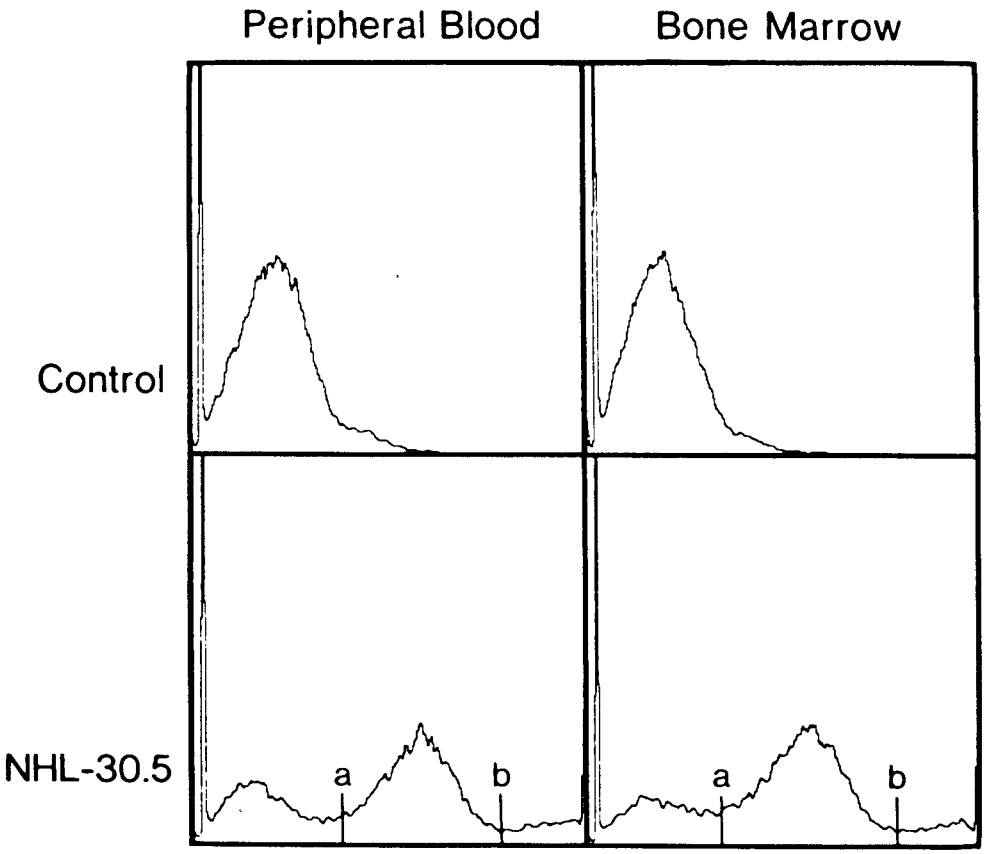

FIGURE XXII Fluorescence profiles of the reactivity of the NHL-30.5 monoclonal antibody with peripheral blood and bone marrow cells from a patient with AML. Cells were sorted at gate 'a' (60% NHL-30.5-positive cells) or gate 'b' (10% NHL-30.5-positive cells) and plated in methylcellulose assays. Colony data is presented in Table XVII.

TABLE XVII

The number of blast colony progenitors per 10^5 cells in sorted fractions from the blood and marrow of a patient with AML. Cells were sorted at gate 'a' (top 60% NHL-30.5-positive), or gate 'b' (top 10% NHL-30.5-positive). See Figure XXI.

Fraction		peripheral blood	bone marrow
Unsorted		141	79
Gate 'a'	positive (60%)	130	183
	negative (40%)	0	5
Gate 'b'	positive (10%)	53	75
	negative (90%)	98	122

although a few were present in the highly fluorescent population (top 10%).

Functional Studies of NHL-30.5

Additional experiments were undertaken to investigate whether the NHL-30.5 antigen might be involved in progenitor proliferation or differentiation responses. To test these possibilities purified NHL-30.5 monoclonal antibody was incorporated into the assay medium (up to 10 μg/ml). This, however, had no detectable inhibitory or stimulatory effect on colony formation by normal bone marrow (Table XVIII) or peripheral blood (Table XIX) progenitors in comparison to control cultures plated both with and without stimulators (erythropoietin and leukocyte conditioned medium). Addition of an equivalent concentration of mouse Ig (Sigma) was also without effect.

Immunoprecipitation

Since the pattern of reactivity of NHL-30.5 is most reminiscent of that observed with the My-10 monoclonal antibody (7), both antigens were compared directly by immunoprecipitation of surface components of KG-1 cells. The KG-1 line was selected for immunoprecipitation purposes since the My-10 antigen is known to be absent from HL-60 cells. The proteins immunoprecipitated with these two antibodies were clearly different in their mobility in SDS-PAGE (Figure XXIII). The NHL-30.5 antigen was shown to have a MW of 180,000 under both reducing and nonreducing conditions as previously demonstrated for HL-60 cells (9), whereas the My-10 antigen had an approximate MW of 115,000.

The NHL-30.5 antigen was further compared with My-10 by the reactivity of the respective antibodies with two AML cell lines in an indirect binding assay (Table XX). The NHL-30.5 monoclonal antibody reacted with both HL-60 and KG-1 cells, while My-10 reacted with KG-1 cells only. The My-10 monoclonal antibody was also unable to inhibit the binding of ^{125}I-labelled NHL-30.5 antibody to HL-60 cells in a blocking assay (Table XXI).

TABLE XVIII

The number of progenitors per 10^5 bone marrow cells plated in the presence of purified NHL-30.5 monoclonal antibody.

	PROGENITORS / 10^5			
ANTIBODY	CFU-E	BFU-E	CFU-C	MIXED
EXPERIMENT #1				
control	ND	ND	ND	ND
1 µg/ml anti-LFA-1	41	40	141	0
10 µg/ml NHL-30.5	38.5	19	197	0
1 µg/ml NHL-30.5	38.5	29	116	0
NHL-62.14.4*	13.5	5.5	126	0
NB-2*	0	9	101	0
EXPERIMENT #2				
control	200	100.5	59	1
20 µg/ml IgG	169	64.5	62.5	0.5
10 µg/ml IgG	183	59	61	2.5
1 µg/ml IgG	25	59	61	2.5
10 µg/ml NHL-30.5	161	68.5	48	2
1 µg/ml NHL-30.5	110	61.5	46.5	0.5
EXPERIMENT #3				
control	87	128	39	3
20 µg/ml IgG	90	118	36.5	1
10 µg/ml IgG	89.5	109	38	0
1 µg/ml IgG	70.5	128	51	0.5
10 µg/ml NHL-30.5	50.5	93	55	0
1 µg/ml NHL-30.5	45	89.5	35	0

* NHL-62.14 and NB-2 are two monoclonal antibodies produced in this laboratory with specificity for the transferrin receptor (unpublished).

TABLE XIX

The number of progenitors per 4 x 10^5 peripheral blood cells plated in the presence of purified NHL-30.5 monoclonal antibody.

	PROGENITORS / 4 X 10^5			
ANTIBODY	CFU-E	BFU-E	CFU-C	MIXED
EXPERIMENT #1				
control	25.5	40.3	42	1
20 µg/ml IgG	42	59.5	39.5	3
10 µg/ml NHL-30.5	41	56	37.5	3.5
EXPERIMENT #2				
control	11.5	28.5	5	1
20 µg/ml IgG	10	34	6.5	0
10 µg/ml IgG	12	35.5	5.5	0.5
1 µg/ml IgG	7.5	32.5	6	1.5
10 µg/ml NHL-30.5	6.5	22.5	6	0.5
1 µg/ml NHL-30.5	13	25.5	10	1
EXPERIMENT #3				
control	4	25.5	7.5	1
20 µg/ml IgG	4.5	21.5	6.5	1
10 µg/ml IgG	5	23	9	1
1 µg/ml IgG	3.5	21	5.5	0
10 µg/ml NHL-30.5	4	13.5	3.5	1
1 µg/ml NHL-30.5	5	18.8	6	0

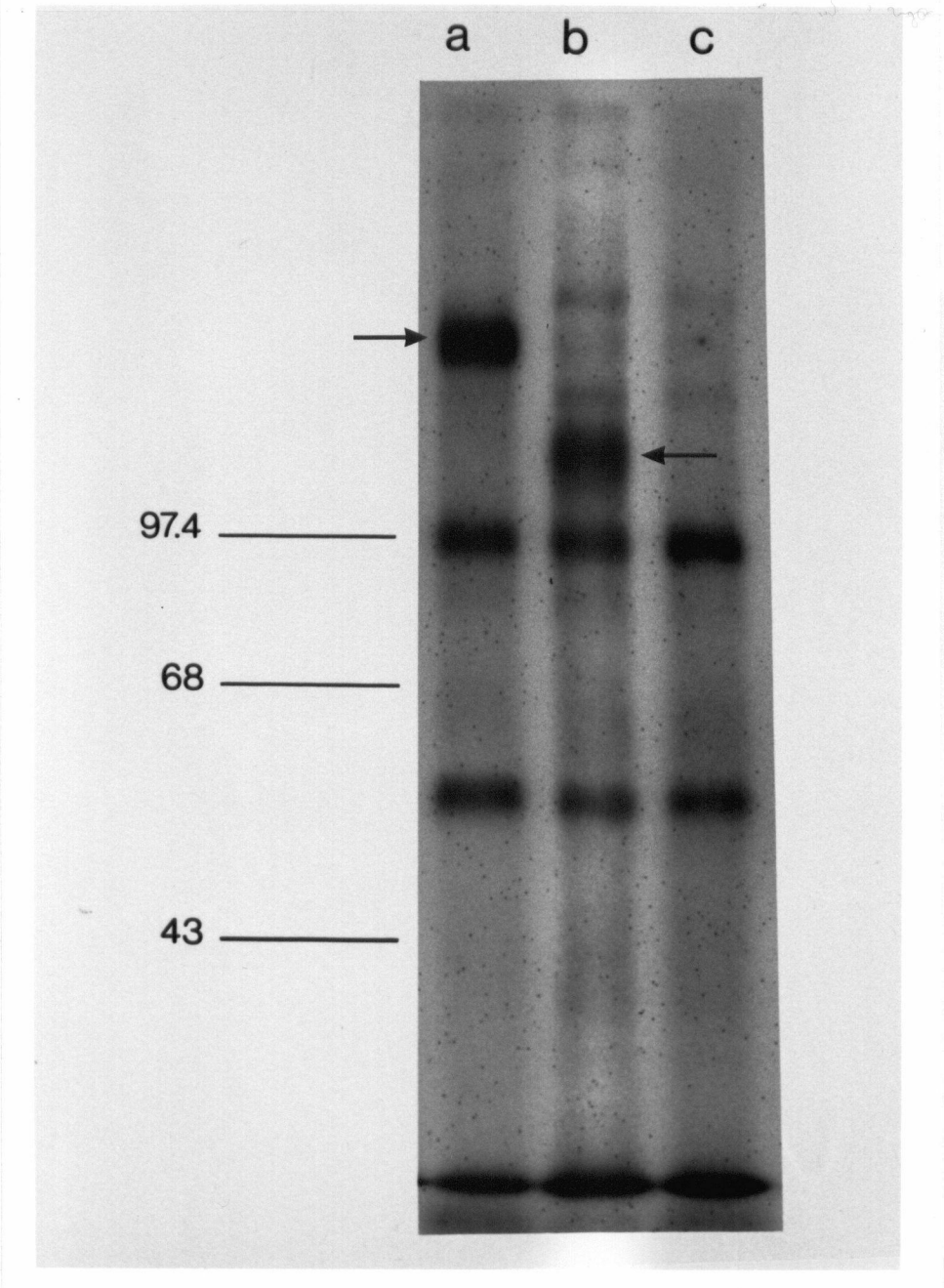

FIGURE XXIII A comparison of the NHL-30.5 and My-10 antigens by immunoprecipitation. KG-1 cell surfaces were labelled with ^{125}I and the antigens immunoprecipitated from the cell lysates using the NHL-30.5 and My-10 hybridoma supernatants. The analysis was carried out on SDS-PAGE (7.5%) under reducing conditions. Lane a: NHL-30.5, Lane b: My-10, and Lane c: control antibody raised against mouse lymphocytes (does not react with KG-1 cells).

TABLE XX

A comparison of the reactivity of the NHL-30.5 monoclonal antibody and My-10 monoclonal antibody on two AML cell lines using an indirect binding assay.

Antibody	HL-60 binding (cpm)	KG-1 binding (cpm)
media	213	261
anti-transferrin receptor (NHL-62.14)	13,760	13,488
negative control*	482	291
My-10	320	9,616
NHL-30.5	2837	855

* an unrelated antibody raised against mouse lymphocytes

TABLE XXI

Ability of the My-10 monoclonal antibody to block the binding of ^{125}I-NHL-30.5 monoclonal antibody to HL-60 cells in a direct binding assay.

First incubation	Second incubation	Binding (cpm)
media	^{125}I-NHL-30.5	2805
NHL-30.5	^{125}I-NHL-30.5	210
My-10	^{125}I-NHL-30.5	2543
negative control	^{125}I-NHL-30.5	2650

3) DISCUSSION

The purpose of the experiments described in this Chapter was to investigate the possible distribution of NHL-30.5 on rare but normal marrow elements whose presence would have escaped detection in standard binding assays or FACS analyses (Chapter III). Previous studies (Chapter III) had shown that purified populations of lymphocytes, monocytes, granulocytes, erythrocytes, and platelets from normal individuals did not react with this antibody and that normal bone marrow buffy coat preparations contained less than 2% NHL-30.5-positive cells. In the studies described in this Chapter, the light density fraction of all specimens was obtained by centrifugation of ficoll-hypaque (density = 1.077g/cc). This appeared to increase the number of NHL-30.5-positive cells by a few percent. As a result a small but distinct (>5%) positive population could sometimes, although not always, be demonstrated in the peripheral blood of CML patients in chronic phase. However, this was not the case for any normal marrow or peripheral blood sample, where NHL-30.5-positive cells failed to reach the 5% level. Nevertheless, when cells with the highest fluorescence intensity (comprising 5% of the total population) were sorted and assayed, a significant and selective enrichment of clonogenic progenitors including CFU-E and BFU-E as well as CFU-C, was obtained. These findings suggest that expression of NHL-30.5 is part of an early stage of hemopoietic cell differentiation that persists even after lineage restriction. Consistent with these results is the observation that, at least in the one patient studied, the majority of the blast colony-forming progenitors in AML (that presumably help to maintain the blast population in vivo) are also NHL-30.5-positive (Figure XXI and Table XVII). In contrast to the positive results obtained with clonogenic progenitors, assessment of adherent marrow cell populations that are capable

of regulating progenitor turnover in vitro (16) showed these to be NHL-30.5-negative.

Few monoclonal antibody reagents are currently available that identify antigens restricted in their expression to early stages of myeloid cell differentiation (3,7-9). The My-10 monoclonal antibody (7) has a pattern of reactivity that shows some similarity to that of NHL-30.5 in that both react with immature myeloid populations and some leukemic blasts, but not with differentiated myeloid cells. The present study establishes that the antigen detected by My-10 is electrophoretically distinct from that detected by NHL-30.5. The former has an approximate molecular weight of 115,000 in contrast to 180,000, the molecular weight of the NHL-30.5 antigen. The inability of the My-10 monoclonal antibody to block the binding of iodinated NHL-30.5 antibody to KG-1 cells supports the idea that these antigens are distinct molecules. Also consistent with this difference is the finding that NHL-30.5 reacts with cells from most (80%) patients with AML (Chapter III), whereas My-10 appears to react with cells only from a minority (28%) of AML patients (7).

The stage of normal hemopoietic cell differentiation at which NHL-30.5 first appears remains to be determined. Since the KG-1 cell line (myeloblastic) expresses both the NHL-30.5 and My-10 antigens, while the HL-60 cell line (promyelocytic) expresses only NHL-30.5 (which it loses if induced to differentiate), it is possible that myeloid progenitor cells (NHL-30.5-positive and My-10-positive) become My-10-negative somewhere after the myeloblast stage but remain NHL-30.5-positive until sometime following the promyelocyte stage. Our limited observations to date suggest that clonogenic pluripotent progenitors (CFU-GEMM) like their various lineage-restricted but clonogenic progeny are NHL-30.5-positive. However, even CFU-GEMM are not believed to represent stem cells with long-term repopulating potential. In

standard short-term assays most CFU-GEMM display limited self-renewal capacity (17-19) and thus differ from high self-renewal pluripotent cells that have recently been shown to be initially refractory to growth stimuli in vitro (20,21). Recently it was reported that the lineage-restricted progenitors observed in four to six week old long-term marrow cultures may be derived from a population of cells that differ in HLA-DR expression from CFU-GEMM as well as CFU-C and BFU-E (22). Experiments are currently underway to determine whether such differences also extend to NHL-30.5 expression.

The normal functional role of NHL-30.5 is also unknown. Since most clonogenic progenitors in normal peripheral blood are not actively cycling, in contrast to those in CML blood or all but the most primitive classes present in normal bone marrow (23-27), it seems unlikely that regulation of NHL-30.5 expression is directly related to cell cycle status. Tests to evaluate whether NHL-30.5 binding could inhibit or stimulate normal progenitor proliferation and differentiation also showed no effect, although this might reflect the particular determinant recognized by the NHL-30.5 monoclonal antibody rather than the physiologic role of the antigen of which it is a part.

The data presented in this Chapter, together with the results outlined in Chapters III and IV, provide a strong basis for the designation of the NHL-30.5 marker as an early myeloid differentiation antigen. AML appears to represent the expansion of a neoplastic clone that continues to express NHL-30.5 because the cells are unable to differentiate to those stages characterized by the loss of the NHL-30.5 antigen. Progenitor-restricted antigens such as My-10 and NHL-30.5 may have important roles in modulating progenitor cell behaviour.

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C H A P T E R VI

SUMMARY AND CONCLUSIONS

Analysis of cellular differentiation relies upon the identification of phenotypic characteristics that are associated with specific levels of maturation. Since the vast majority of normal marrow cells are in the terminal stages of differentiation and are therefore recognizable as precursors of the granulocyte, monocytic, megakaryocytic, or erythroid lineages, the developmental sequence of these myeloid cells has been easily studied using morphological and cytochemical criteria. A more powerful approach is the immunological analysis of differentiation-associated cell membrane proteins using monoclonal antibodies. Such antibodies have proven useful in the study of the terminal myeloid differentiative events (Chapter I, 5(B)) but also offer a unique approach to study gene products expressed on the numerically infrequent stem cell population (1).

Regulation of proliferation and differentiation in these stem cells is thought to reside in cell surface receptors that are responsive to extrinsic growth factors or cellular interactions. Very little is known about the cell surface antigenic changes that distinguish stem cells from their differentiating progeny and consequently cells within the stem cell or progenitor cell compartment have remained rather elusive. The identification of stem cell-restricted surface antigens would provide an easy route to the purification of these cells and, more importantly, might identify cell surface molecules that mediate specific cellular functions.

In AML the neoplastic cells are unable to follow normal differentiation pathways, although the continued expression of some normal features of myeloid differentiation are usually evident. Current evidence indicates that the cell surface phenotype of the blast population can generally be viewed within the framework of normal myelopoietic differentiation, and this provides a useful basis for current immunological classification schemes (2).

As an approach to identify unique gene products associated with the early stages of myeloid cell differentiation, a number of monoclonal antibodies were raised against an AML-derived cell line (HL-60) considered to be blocked at an immature stage of myeloid maturation. One of these antibodies, NHL-30.5, identified a cell surface antigen with a cellular distribution reflecting that of a myeloid differentiation antigen. The identification of this antigen and analysis of its cellular distribution is discussed in this thesis.

Clinical Association of the NHL-30.5 Antigen with AML

The NHL-30.5 monoclonal antibody detected a cell surface antigen with a molecular weight of 180,000 on the HL-60 cell line and on fresh leukemic cells from a patient with the M4 classification of AML. The antibody reacted with hemopoietic cells from 40/48 patients with AML and two patients with preleukemic disorders characterized by the presence of myeloid blast cells (a chronic myelomonocytic leukemia (1/1) and a myelofibrosis (1/2)). In contrast it did not react with normal mature hemopoietic cells, including lymphocytes, monocytes, granulocytes, erythrocytes, platelets, and splenocytes. Lymphoid reactivity appeared to be a rare event since only one of 15 acute lymphoid leukemias demonstrated reactivity and all lymphoid cell lines were uniformly negative. Reactivity with cells in the chronic phase of CML was also rare (7/26) and, when positive, the number of NHL-30.5-positive

cells was low (1-20%). Cells from the acute phase of CML reacted in a manner analogous to AML if the blast crisis was of the myeloid variant (1/1), but were clearly negative in lymphoid blast crisis (3/3). An unusual case characterized by a CALLA$^+$, TdT$^-$, HLA-DR$^+$ blast population was also positive, as was a biphenotypic blast crisis containing both myeloid and lymphoid blasts. Notably the vast majority of normal differentiating bone marrow cells were negative (<5% positive), including bone marrow fibroblasts.

Differentiation-Linked Expression of the NHL-30.5 Marker

Three AML-derived cell lines, considered to be blocked at an early stage of maturation were shown to express the NHL-30.5 antigen. The HEL cell line (erythroleukemia) contained over 90% NHL-30.5-positive cells with an estimated 1.5×10^4 molecules/cell. The HL-60 (promyelocytic) cell line was 70-90% NHL-30.5-positive with ~4,000 molecules/cell and the KG-1 line (myeloblastic) was 30-50% positive and antigen estimations varied between 500 and 2,300 antigens per cell. Cell lines derived from lymphoid leukemias were clearly negative, as was the U937 (early monocytoid) line. K562 (early blast/erythroid) and KG-1a (variant of KG-1) were weakly positive (1-10%).

The three NHL-30.5-positive AML cell lines are known to possess myeloid differentiation potential. HL-60 cells are unique in that they respond to stimulation with DMSO or retinoic acid by initiating a granulocytic differentiation program, or to stimulation with TPA by entering a monocytic differentiation program. Induction of either pathway of differentiation was shown to result in the loss of the NHL-30.5 antigen, as detected by flow cytometry and immunoprecipitation.

The KG-1 and HEL cell lines are incapable of granulocytic differentiation but are inducible along the monocyte pathway with TPA. Differentiated KG-1 cultures were also shown to lack the NHL-30.5 molecule.

In contrast to HL-60 and KG-1, induction of monocytic differentiation in the HEL cell line did not reduce antigen expression to undetectable levels, although a five fold decrease in expression was observed.

Expression of the NHL-30.5 Marker on Normal Myeloid progenitors

In order to determine whether the NHL-30.5 antigen is a normal myeloid differentiation marker, its expression was studied on clonogenic erythropoietic and granulopoietic cells from both normal and leukemic individuals. Analysis of normal differentiating bone marrow cells and mature peripheral blood mononuclear cells stained indirectly with the NHL-30.5 monoclonal antibody and FITC-second antibodies did not reveal a distinctly positive population. However, the cells with the highest fluorescence intensities (comprising 5% of the total population) sorted on a fluorescence activated cell sorter were highly enriched in both erythropoietic (CFU-E/BFU-E) and granulopoietic (CFU-C) progenitors. Similar patterns of enrichment were observed in suspensions of cells from CML peripheral blood suggesting that the NHL-30.5 antigen is expressed on both normal and leukemic progenitors detectable as CFU-E, BFU-E, and CFU-C. Since the NHL-30.5 antigen has a cellular distribution similar to the My-10 antigen, both molecules were compared by immunoprecipitation and shown to have clearly different molecular weights.

Conclusion

The data presented in this thesis suggest that the NHL-30.5 antigen is a novel myeloid differentiation antigen restricted in its expression to the early stages of myelopoiesis, and that the loss of this molecule from a myeloid cell can be viewed as a stage in the normal myeloid differentiation program. The precise stage of maturation at which the NHL-30.5 antigen is lost is not clear, but the vast majority of terminally differentiating marrow

cells are clearly negative, as are the fully mature cells in the peripheral blood. The data support the concept that NHL-30.5-positive cells accumulate in AML patients due to a block in their capacity to differentiate into the stages characterized by loss of the NHL-30.5 molecule.

A number of questions regarding this antigen remain unanswered. With respect to its cellular distribution, it is not yet known whether all of the myeloid progenitors express NHL-30.5. Identification of a clearly NHL-30.5-negative population of progenitors might identify a unique functional subset of progenitors analogous to the immunologically defined T-cell subsets in the peripheral blood (3). Similarly, and of relevance to potential use of this antibody in the treatment of AML, is whether or not the precursors of these myeloid progenitors are NHL-30.5-positive. Preliminary data suggests that CFU-GEMM are NHL-30.5-positive, although it remains to be established whether stem cells with long-term repopulating potential are also positive. If the antigen is expressed on these high self-renewal pluripotent stem cells, then the NHL-30.5 mononclonal antibody can not be used as part of an 'antibody cocktail' to purge leukemic marrow for autologous bone marrow transplantation.

Nevertheless, the clinical data presented in Chapter III suggests that the NHL-30.5 antibody, in conjunction with other monoclonal antibodies, is potentially a useful reagent to monitor patients with preleukemic disorders for possible evolution into AML, and to distinguish myeloid leukemias from lymphoid leukemias (particularly in the acute phase of CML). Since the NHL-30.5 antigen is expressed at relatively low levels, techniques designed to increase the sensitivity of its detection, such as antibody bridging procedures (5), may be beneficial in this regard. The antibody is currently being used in this laboratory for the cell surface phenotyping of leukemias

to further evaluate its use in the diagnosis and classification of hematologic malignancy.

Finally, and perhaps the most interesting question, is the function of this molecule. The restricted expression of the antigen suggests that it may play an important role in modulating progenitor cell behaviour but this has yet to be proven. In Chapter V it was shown that the NHL-30.5 monoclonal antibody was unable to inhibit or stimulate colony formation in vitro. However, not all monoclonal antibodies raised against a cell surface receptor are capable of inhibiting its function (5), and so it will be important to raise monoclonal antibodies against different determinants on the purified antigen. Further characterization of this molecule, such as amino acid sequencing, determination of its orientation in the plasma membrane, analysis of associated tyrosine kinase activity, and ultimately the cloning of its gene would help determine if NHL-30.5 is a cell surface receptor for an as yet unspecified ligand.

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