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THE UNIVERSITY OF ALBERTA

K562 - A MODEL FOR LEUKEMIC PLURIPOTENTIAL STEM CELL DIFFERENTIATION

by

JUDY ANN SUTHERLAND

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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.....
Supervisor

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Date ..April..16..1984

To my Family

I'm walking out easy I'm walking out slow
I'm taking this old road to a place that I know
and I can't feel the wind & I don't see the snow
over my mountain

And it's cold gettin' colder but I'm feeling so warm
I'm flying so high I just can't see the storm
and it's a long time ago I was here on my own
over my mountain

So in sad isolation I'm feeling so good
I've drifted too far but I knew that I would
and it seems that I'm laughing and I knew that I could
over my mountain

Dougie Maclean

ABSTRACT

This study investigated the capacity of K562, a poorly differentiated human leukemic cell line, to differentiate in response to exogenous inducing agents. The results indicated that K562 is a pluripotential stem cell line, that, when stimulated with an appropriate agent, can differentiate along erythrocytic, granulocytic, monocytic and megakaryocytic lineages. Induction of differentiation of K562 cells suggests that treatment of poorly differentiated leukemias with inducing agents may be beneficial in these diseases as well as in chronic leukemias.

K562 cells synthesised and expressed Class I MHC antigens when induced with sodium butyrate, interferon, and phorbol diesters. These results are significant because of growing evidence of a relationship between expression of MHC antigens, malignancy and differentiation.

This study also provides additional evidence of the role of monoclonal antibodies in conjunction with conventional cytochemistry in classifying acute leukemias.

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Differentiation and Carcinogenesis

Cell differentiation is a complex, highly regulated process based on selective activation and repression of genetic material. Thus, cells with the same genome are able to perform unique and highly specialized functions. Differentiating cells are characterized by a progressive restriction in their repertoire of gene products. Although unique cell products are dramatic evidence of cell differentiation, most differences between cells are believed to be quantitative rather than qualitative (1). Cell differentiation is generally regarded to be a unidirectional and irreversible transformation which will ultimately lead to cell death. The cells progress through stages which are responsive to different stimuli and which have varying degrees of proliferative capacity.

In a 1978 Symposium on Fundamental Cancer Research, Clement Market stated in his keynote address that "Neoplasms are disorders of cell differentiation stemming from misprogramming of gene function. This misprogramming can have many causes, but all cancers must act by changing the function of regulatory DNA" (1). He goes on to suggest that the changes in the molecular mechanisms responsible for activating and silencing structural genes and regulating quantitative gene expression in normal differentiation can be used to explain the cancer phenotype. One of the major supporting pieces of evidence

for a lesion in regulatory as opposed to structural DNA is that neoplastic cells make no molecules that are not also found in normal adult or embryonic tissues.

In normal tissues, differentiation is intimately linked with proliferation so that at a specific stage of maturation the cell loses the ability to proliferate. However in neoplastic cells, these two processes often become autonomous (2), and the cancerous cells continue to proliferate. It has been suggested that neoplastic cells are blocked at a certain stage of differentiation (3). If differentiation stops before the cells lose their ability to proliferate, this may also account for the growth advantage that cancer cells have over normal tissues.

It has been expressed by several authors that the mechanisms of carcinogenesis and normal cell differentiation are similar if not identical (4,5) and that in both processes cytoplasmic factors are important (6).

Differentiation appears to be essentially a cytoplasmic event with no permanent changes to the cells genome (6). The only strong evidence in the hematopoietic system that violates this constancy hypothesis is observed in the differentiation of B lymphocytes. The generation of diversity in immunoglobulin specificity involves rearrangement of immunoglobulin gene segments (7).

Extracellular factors play an important role in both differentiation and neoplasia (6). Cell growth is controlled by substances such as hormones, growth factors and chalones. Carcinogenesis is often the result of

external factors such as viruses and radiation.

The ability to divide appears to be a prerequisiste for both carcinogenesis and differentiation (6). Pierce pointed out that tumours arise mainly in tissues capable of mitosis (8). For example, polymorphonuclear phagocytes, which are incapable of further division, cannot initiate leukemia.

One of the major reasons for interest in the hypothesis that cancers result from disordered cell differentiation is that the process might be reversible and that some cancers might be curable by spontaneous or induced differentiation (9). There is evidence in the literature that some tumors do occasionally differentiate spontaneously, for example neuroblastomas not uncommonly differentiate to form non-malignant ganglioneuromas (10). In experiments done with teratocarcinomas, transplantation of these tumors resulted in differentiation into many types of adult tissues which showed neither invasiveness nor persistant mitotic growth (11). There are hundreds of reports in the literature that describe spontaneous regression of cancer including carcinomas of the bronchus and the stomach (12).

One of the major controversies concerning the nature of cancer and its relationship to differentiation is whether the phenotype of cancer cells reflects that of normal cells arrested at a particular stage ir differentiation, or whether it reflects a diso

assembly of normal segments of differentiation programs (9). This question arises from the observation that tumour cells occasionally express functions or features that appear to be mutually exclusive in differentiated normal tissues.

Because of its relationship to neoplasia, the study of differentiation in normal and cancerous tissues will likely provide more insight into the initiation, progression and management of cancer.

Chapter II. Review of the Literature

One of the most widely used models for studying differentiation is the hematopoietic system. It is attractive becauses one has relatively easy access to human normal and malignant cells representing different degrees of differentiation. In addition, blood-forming tissue is one of the few which continuously renews itself, even in adults.

In this system pluripotent stem cells differentiate, probably by a multistep process, into mature cells of various cell lineages. Investigation of hematopoietic differentiation has practical importance because the pathogenesis of many hematopoietic disorders can be related to abnormalities in stem cell proliferation, in their differentiation or maturation processes, or in the cells which regulate these processes.

This literature review will describe the development of methodology that is used to measure differentiation and the contribution of these techniques to our overall understanding of hematopoiesis and leukemia today. It will also review two major models used to describe differentiation programs of hematopoietic pluripotential stem cells. It will then specifically discuss how human myelogenous leukemia cell lines can be used as models to study the biology of blood cell differentiation.

A. The Development of Methodology for Studying Hematopoietic Differentiation.

1) Morphology

The earliest theories of blood cell differentiation date back to the seventeenth century when erythrocytes were observed and described for the first time. Leukocytes were first seen in the eighteenth century, but platelets were not recognized until 1842 because of the limitations of microscopy available at that time. The first compound microscopes produced images of cells surrounded by haloes, the consequence of spherical aberration and diffraction errors of low aperture lenses. These observations gave rise to the "globular theory", or the belief that all the organs of the body were composed of agglomerated globular corpuscles (13). Antonj von Leeuwenhoek, who first described erythrocytes, thought that the red cell was derived from six smaller particles. Boerhaave extended the hypothesis, he believed that the red cell was formed by six yellow globules of heat coaguable serum, while the serum globules were formed from six smaller lymphatic juice globules which in turn were derived from "limpid humours". Hewson modified the theory stating that leukocytes were formed in the lymph nodes and thymus, entered the circulation via the thoracic duct and in the spleen were transformed into erythrocytes, the leukocyte forming the centre of the red cell and coaguable lymph forming the periphery. These illusory globular

observations collapsed with the introduction of the achromatic microscope, but the theory of cell generation from exogenous granules survived with Schwann's cytoblastoma hypothesis and persisted for a long time in the cell transformation theories of hematopoiesis. Remak's observation in 1841 of an embryonic blood cell undergoing mitosis initiated the general theory of cell multiplication by division.

Leukemia was described almost simultaneously by Craigie, Bennett and Virchow in 1845 (14). Their observations were made at the autopsy of patients who had been ill for one to two years with increasing weakness, severe nosebleeds, and abdominal swelling. They noted an enlargement of the spleen and changes in the colour and consistancy of the blood. Bennett believed that the changes were related to an inflammatory response, but Virchow recognized that the cells did not represent an infection of the blood. He proposed the name leukemia or "white blood". He suggested that the defect was in those organs that he believed produced the cells; the lymph nodes, liver and spleen. He could not attribute the changes he had seen to overgrowth of the blood forming tissues, and eventually the proliferation of the white cells was attributed to new growth or neoplasia rather than hyperplasia. Virchow subdivided leukemia into two types based on cell morphology and clinical presentation. Those patients who had enlarged spleens were described as having "splenic" or "lienal" leukemia, and those with

swollen lymph nodes as having "lymphatic" leukemia.

In 1864, after the discovery of platelets, there was a school of thought developed by Lionel Beale, that platelets were minute particles of germinal matter, that might grow into red cells or leukocytes (15). In a modification of this theory, Georges Hayem maintained that platelets, which he referred to as hematoblasts, are an early stage in the development of the erythrocyte (16).

In 1868 Guilio Bizzozero and Ernst Neumann almost simultaneously discovered the hematopoietic function of the bone marrow, and so resolved the mystery of the site of red cell production in adults (17). Neumann also suggested that blood formation is a continuous process in adults and not just a feature of embryogenesis. Both of these ideas were highly controversial.

In 1870 Neumann further suggested that in "splenic leukemia" the marrow rather than the spleen was the source of excess blood cell production. He named this condition "splenomedullary leukemia" which was later shortened to "myeloid" or marrow derived leukemia (14).

Once the marrow was established as the site of blood cell formation, investigators began to search for their precursor cells (18). When these cells were found in the marrow, investigators hypothesised that these cells originated there. Until 1925, it was believed that these cells were derived from the vascular wall within the bone marrow.

The development of radiobiologic methods and transplantation techniques refined these hypotheses. The marrow was described as a production center whose environment supports the differentiation and proliferation of blood cells. The pluripotent stem cell circulates through the blood, but when it reaches the favorable environment of the marrow, it begins to divide and differentiate.

The origin of platelets remained in dispute until 1906, when James Wright determined that they originate from megakaryoblasts in the bone marrow (19).

The introduction of phase contrast and electron microscopy has added another dimension to the morphological investigation of blood. Ultrastructural studies have been particularly useful for studies of differentiation in the megakaryocyte lineage (20).

2) Cytochemistry

Paul Ehrlich, a German bacteriologist and pathologist, introduced the use of aniline dyes in 1877 for staining granules in leukocytes. He used this staining procedure as the basis of the differential blood count technique.

Ehrlich described a primitive, large, basophilic mononuclear cell with a vesicular nucleus and few or no granules, in stained bone marrow smears. He called this cell a "myelozyt" or marrow cell and he believed this cell to be the precursor of granulocytes. Ehrlich postulated that lymphocytes had an entirely different line of development,

originating in the lymphoid tissue (17). This dualistic concept of hematopoiesis was the forerunner of the concept that cells could maintain their own numbers by cell division and yet produce cells that would eventually mature into blood cells.

Staining blood and marrow simplified the classification of leukemias. Ehrlich confirmed that granulocytes and their precursors were the predominant lineage in splenic or myeloid leukemias (14).

Jenner in 1889 found that the precipitate formed when eosin and methylene blue are mixed could be dissolved in methyl alcohol to form a useful stain combining certain properties of both parent dyes. Jenner's stain is very similar to the May Grunwald stain used today. Romanowsky in 1890, found that when old methylene blue was used, the resulting stain had a much wider range and could stain cell nucleii and platelet granules which Jenner's mixture failed to stain.

Ehrlich's work was followed by Artur Pappenheim's who used Romanowsky stains to study bone marrow cells. He observed that the transitional forms of all blood cells could be organised into a pattern that began in a relatively featureless mononuclear cell which he called a "lymphoidozyt". The debate between Ehrlich's dualist concept and Pappenheims monophyletic theory lasted over thirty years, each trying to describe the most primitive cells that could be functioning as stem cells.

Di Guglielmo described erythroleukemias in the early

1900's first as a mixed myeloblastic erythroblastic form, and later as a pure erythroleukemia which he called acute erythremic myelosis. These were the first descriptions of leukemias of erythroid precursors (21).

In 1938 Forkner hypothesised three possible causes of leukemia; infection, metabolic disorder, or a malignancy (14).

It was later shown that infection is a consequence rather than a cause of leukemia.

A defect in metabolism where there could be either excessive or deficient production of a factor involved in maturation of blood cells was supported by the discovery in the 1920's that pernicious anemia, a disease of ineffective red cell production characterized by unsynchronized nuclear and cytoplasmic maturation, could be controlled by a "liver factor" in the diet.

Today leukemias are considered to be malignant neoplasms, a cancer of blood forming cells, but there continues to be a lot of work done identifying and characterizing factors that regulate growth and maturation of blood cells.

Modern Romanowsky stains, which differ only in the method of ripening the methylene blue, are used today for routine cell differentials. This stain allows discrimination between three classes of granulocytes based on differential staining of cytoplasmic granules. It also allows us to measure the maturity of cells in the erythroid

lineage based on the relative amounts of methylene blue and eosin that bind to the cells.

Cytochemical demonstration of specifically reacting cell components at an intracellular level has provided information on the constitution of the cells supplementary to that acquired from morphological examination, and also contributes to our knowledge of cell function. Specific stains for the demonstration of iron, lipids, and leukocyte proteolytic enzymes were developed around 1900. Since 1930, there have been methods developed for staining carbohydrates, nucleoproteins, proteins and numerous specific enzymes. Today cytochemistry allows us to discriminate and classify some otherwise morphologically undifferentiated cells into particular developmental lines (22).

3) Colony Assays

The colony assays have been extremely useful in defining groups of progenitor cells that differ in their capacity to self renew, to undergo terminal differentiation, to proliferate, and to respond to specific growth factors. The conventional depiction of hemopoiesis, in the form of lineage diagrams is also based to a large extent on colony assay techniques.

In 1961 Till and McCulloch developed a spleen colony method for quantitating mouse pluripotent hematopoietic stem cells (23), confirming the monophyletic view of the totipotential stem cell which was first suggested by

Pappenheim in 1900. By 1965 semi-solid culture methods, an in vitro assay of progenitor cells were being used to investigate human stem cells. The progenitor cells measured by these colony asays provided the link between the pluripotent stem cell and the various hemic cell lineages. Techniques for assaying committed progenitors preceeded those for pluripotent stem cells. CFU-GM or granulocyte-macrophage colony forming units were described by Pluznic and Sachs (24) and Bradley and Metcalf (25) in 1965. The assay system for erythroid colony forming units CFU-E was developed by 1971 (26). Erythroid burst forming units BFU-E, which are more primitive than CFU-E, were cultured first in 1974 (27). Culture methods for megakaryocyte progenitors were developed by Metcalf et al. in 1975 (28). Progenitors assayable in these clonal cultures represent a continuum of stages of differentiation in each cell lineage. Clonal assays for multipotential progenitors, CFU-GEMM, that give rise to cells in granulocyte, macrophage, erythrocyte, and megakaryocytic lineages were developed by 1978 (29). There are good indicators based on the sensitivity of these cells to cycle specific agents and replating experiments that CFU-GEMM and CFU-S represent equivelent stem cell populations in man and mouse (30). Other observations suggest that CFU-S and CFU-GEMM represent relatively mature pluripotent progenitors and that the small numbers of truly primitive stem cells may produce microscopic spleen colonies produce colonies smaller than CFU-GEMM in culture (31).

The recent development of new culture methods enables stem cell growth to be maintained in vitro for months and provides valuable insight into the relationships between stem cells and their immediate neighboring cells (31).

It is now widely accepted that all the mature blood cells, and possibly lymphocytes, originate from the same stem cell. This pluripotent cell has few distinguishing features and is therefore difficult to isolate and characterize. Based on colony assay techniques, pluripotent stem cells have the capacity for self renewal, in that they are able to give rise to new hematopoietic progenitors and in so doing, maintain their own population. Pluripotent stem cells also have the property of commitment, in that they are able to divide and generate progenitors, which are programmed to differentiate toward a specific lineage. The latter cells are able to proliferate extensively, but this is not associated with self renewal. These committed progenitors respond to humoral regulators such as erythropoietin and are responsible for maintaining the population of mature cells.

A number of models based on mathematical and other analyses of colony assays, that have been proposed for the mechanism of stem cell self-renewal and commitment. These models can be classified as stochastic or deterministic. The stochastic model of Till et al. proposed that the decision of a stem cell to renew itself or to generate committed progenitors is governed by a probabilistic rule

(33). Control of the stem cell compartment may be achieved by changes in the distributional parameter, p, the probability of self-renewal of stem cells. Humoral factors may exert extrinsic influences on these distributional parameters. An extension of this model suggested that stem cell commitment is governed by a progressive and stochastic restriction in the differentiation potential of hematopoietic stem cells (34). This model is supported by documentation of oligopotent hematopoietic progenitors which reveal terminal differentiation in two or three cell lineages in varying combinations. Several bipotent hematopoietic progenitors have been documented in cultures of murine and human cells such as erythroid-megakaryocyte progenitors (35), and neutrophil-erythroid progenitors (36).

Deterministic models are based on external mechanisms of regulation that instruct stem cells either to undergo renewal or differentiation. These models include the hematopoietic inductive microenvironment (HIM) model suggested by Trenton in which the commitment of multipotent stem cells to unilineage progenitors is determined by the specific microenvironment surrounding the cells (37). Other models postulate that competition between growth factors determines the lineage of commitment of the stem cell (38). It is generally believed, however, that erythropoietin and colony stimulating factor (CSF) act on committed progenitors later in differentiation.

Yet another model suggests that erythropoiesis is an

obligatory step in stem cell differentiation. According to this hypothesis, differentiation potential is successively lost until only the erythroid lineage remains (39).

Committed progenitors give rise to differentiated cells, such as erythrocytes, platelets and polymorphonuclear granulocytes which have specific functions but have lost the ability to proliferate.

4) Surface Marker Analysis

Recent advances in immunology have led to important insights in hematopoietic differentiation and the cellular origin of leukemia.

There are now available large batteries of monoclonal antibodies that identify lineage specific cell surface antigens, which in some cases, can be directly correlated with cell differentiation. It has been demonstrated in a number of cell culture systems and in the study of normal bone marrow cells that differentiation involves the modulation of cell membrane antigens. This process involves the selective loss of antigens which are characteristic of undifferentiated blasts, and the synthesis, insertion, modification, and selective retention of membrane antigens that are characteristic of the mature cell (40,41,42,43,44,45,46).

Ideally, the application of monoclonal antibodies to cell differentiation studies requires a biochemical description of the antigenic determinants identified. Consequently, most of the energy in this field is now

focussed on analyzing the haptens recognized by these monoclonal antibodies and in identifying the functions that they may provide.

Exhaustive searches for antigens specific for leukemia cells using monoclonal antibodies have been unsuccessful. This has been unfortunate in terms of therapeutic applications of monoclonal antibodies. However, this has established the belief that phenotypes of most malignant cells are not unique but instead reflect the phenotypes of normal cells at some stage of differentiation.

Monoclonal antibodies and antisera to differentiation antigens have been useful clinically in conjunction with cytochemical staining to distinguish undifferentiated forms of AML and ALL (47). This is important because their optimal therapies are quite different.

In the future, monoclonal antibodies may provide ideal probes for isolating hematopoietic progenitor cell populations and for identifying surface structures involved in regulatory functions.

5) Flow Cytometry and Cell Sorting

Up to this point, progress in understanding hematopoietic differentiation has been largely in the area of descriptive biology where the development of mature blood cells can be characterized by the sequential appearance of morphological features, specific enzymes, cell surface antigens, etc. The correlation between these events, has been much more difficult to measure.

Differentiated tissues consist of a number of cell types which complicate measurements of differentiation markers. In addition, continuous differentiation means that immature cell types are often present in a tissue with terminally differentiated cells. Cell sorters that can select cells according to concurrent expression of several related functions has opened the study of hematopoietic differentiation to a new level.

Some aspects of differentiation that can be probed with flow cytometry and cell sorting include: nucleic acid metabolism, membrane antigens and receptor sites, structure and ultrastructure and intracellular enzymes.

The relationship of DNA synthesis and the cell cycle to differentiation has been one area that has been examined using flow cytometry. Results have varied from those indicating that differentiation occurs independently of the cell cycle to the view that these processes are closely related (48,49,50,51,52). Within the erythropoietic system, the appearance of globin specific mRNA has been correlated with a marked reduction of cycling cells (53).

Messenger RNA metabolism is also amenable to study using flow cytometry. Differentiated cells can generally be distinguished from other cells by synthesis of specific proteins and are therefore rich in the mRNAs that code for such proteins. Cells that are not differentiated along that same pathway do not contain detectable amounts of those mRNAs. For example, it has been

established that non-hematopoietic cells contain no more than 0.01% the amount of mRNA for globin found in the reticulocyte (54).

Monoclonal antibodies are ideal probes for membrane antigens in flow cytometry analysis because of their high titer, specificity and homogeneity. The basis for measurement is an indirect fluorescent antibody technique. Flow cytometry has been widely used for investigating membrane antigen changes in erythropoietic differentiation. As differentiation proceeds, the level of HLA class I antigens on the cell membrane decreases and erythroid-specific membrane proteins such as glycophorin and spectrin increase (55).

6) Leukemic Cell Lines and Inducers of Differentiation

The study of leukemias, hematopoietic malignancies that retain many of the features of normal hematopoiesis provide another model system to study differentiation. The availability of large, relatively homogeneous populations of cells that are apparantly frozen at some point in the differentiation programs is an attractive tool.

The development of continuous cell lines derived from leukemic clones has made it possible to study hematopoiesis in a more highly controlled environment. Their use has also removed the restriction inherent in working with fresh cells with their limited in vitro survival. Many of these cell lines are responsive to a variety of exogenous physiological and non-physiological inducers of

differentiation. This is important for a number of reasons. They provide unique models to study the differentiation of blast cells to mature blood cells. They may provide the opportunity to examine the genetic defects responsible for the maturation arrest of leukemic cells in vivo. Finally, results of studies with leukemic cell lines may provide a new approach to cancer therapy.

The most detailed analyses of inducers of leukemic differentiation has been done in the mouse erythroleukemic cell lines (MELC). These cell lines were developed from spleen cells of Friend virus infected mice. In 1971, Friend et al. reported that erythroleukemia cells in culture could be induced to differentiate into orthochromic erythroblasts, synthesize alpha and beta globin mRNA and hemoglobins of the type found in the adult DBA mouse (56). The cells expressed erythrocyte-specific membrane antigens, and they synthesise carbonic anhydrase and the enzymes required for heme biosynthesis. These changes were in response to exposure to 2% dimethylsulphoxide (DMSO) for four days. Friend's report was the first to clearly indicate that a simple chemical compound could induce differentiation of leukemic cells into terminally differentiated end stage-blood cells. Since that time, a wide variety of unrelated chemicals have shown variable abilities to induce erythroid differentiation in the MELC lines (57). Later it was demonstrated that the DMSO treated MELC had a decreased level of leukemogenicity when injected into DBA/2 mice (56).

There have been a number of cell lines established from patients with acute myelogenous leukemia (AML). These lines have usually been composed of poorly differentiated lymphoblastoid-appearing cells with Epstein Barr virus associated antigens and lymphocytic cell markers and probably arose from EB virus transformation of non neoplastic lymphocytes present in the original culture inoculum.

Since 1975 several human myeloid or erythroid cell lines have been established. Some of the most thoroughly investigated cell lines include: HL60, KG1, HEL, and K562.

In 1977, Collins and his co-workers established the human myelogenous cell line HL60 from the peripheral blood of a woman with acute promyelocytic leukemia. In logarithmically growing cultures, most of these cells appear to be arrested at the promyelocyte stage of differentiation. They are strongly positive with cytochemical stains specific for granulocytic cells including myeloperoxidase, ASD-chloracetate esterase, and Sudan Black B (58). HL60 can be induced by DMSO (59) and retinoic acid (60) to undergo terminal differentiation to mature functional granulocytes, and by a phorbol ester, phorbol myristate acetate to become macrophages (61,62).

The KG-1 cell line was established in 1978 from the bone marrow of a man with erythroleukemia. During logarithmic growth, most of the cells are at the stage of myeloblast or promyelocyte and so are slightly less

differentiated that HL60 cells. Most of the cells are strongly positive for ASD-chloracetate esterase but only 1-2% are myeloperoxidase positive. KG-1 is the only human myeloid cell line that remains responsive to colony stimulating factor (CSF) (63). KG-1 cells are relatively unresponsive to retinoic acid or DMSO; there are no morphological changes in the treated cells but there is increased reactivity with monoclonal antibodies with myelomonocytic specificities (41). Like HL60, KG-1 can be induced with phorbol esters and teleocidins, to become macrophage like (64).

HEL, a human erythroleukemia line, was derived from the peripheral blood of a patient with Hodgkins disease who later developed erythroleukemia. The cytochemical profile of the these erythroblastic cells are consistant with erythroleukemia: positive for periodic acid schiff (PAS), acid phosphatase and butyrase stains and negative for granulocyte-specific stains. HEL cells can be stimulated by hemin to produce hemoglobin Barts (Hb gamma 4) and trace amounts of other embryonic hemoglobin species (65). It has recently been shown that HEL cells respond to phorbol esters in a similar way to HL60 and KG1 to become macrophage like (66) and that DMSO may induce megakaryocytic differentiation (personal communication with J. Breton-Gorius).

K562 is a cell line established in 1970 from cells in the pleural fluid of a woman with terminal chronic myelogenous leukemia (CML) in blast crisis (67). Although

it was originally believed to be a granulocytic line, and later to be an erythroleukemia line, the evidence now suggests that K562 is a pluripotential stem cell line. K562 is the subject of this investigation and its properties will be described in detail in the following chapter.

The tumour-promoting phorbol diesters, including 12-O-tetradecanoyl-phorbol-13 acetate (TPA) and 4β-phorbol 12β-myristate 13α-acetate (PMA), induce human AML cell lines blocked at the myeloblast - promyelocyte stage of differentiation, HL60 and KG1, and erythroblasts, HEL, to differentiate to macrophage-like cells. TPA or PMA-treated cells become adherent, develop pseudopods, display macrophage like characteristics by light microscopy, phagocytize yeast, reduce nitroblue tetrazolium salts and kill bacteria. Lysozyme secretion and the activities of beta glucuronidase and acid phosphatase increase 2 - 20 fold during macrophage differentiation. The phorbol diesters probably trigger differentiation through an interaction with high affinity phorbol diester receptors on the cell membrane. However, the expression of these receptors does not assure responsiveness to the compound (68).

DMSO is a polar planar drug widely used as a cryopreservative. The mechanism of its action as an inducer of erythroid and granulocytic differentiation is unclear, but studies on the Friend erythroleukemia cells

have shown that DMSO has a profound influence on the fluidity of the plasma membrane. It is freely permeable throughout the cell and it has a free electron pair which could interact directly with hydrogen bonds within the chromatin (69).

Vitamin A, its metabolites and analogues (retinoids), may participate in normal hematopoiesis. In the 1920's, investigators reported that experimental animals developed marrow hypoplasia when their diet was deficient in vitamin A. Anemia is also produced in humans who are deficient in vitamin A (70). Retinoids appear to enhance hematopoietic proliferation by increasing the responsiveness of the stem cells to the action of CSF (71) and erythropoietin (72). It has been suggested that retinoids may alter the number or affinity of receptors on the cell membrane to these growth factors (69).

HL60 cells can be triggered to differentiate along the granulocytic line by retinoic acid. The mechanism of induction of differentiation is not clear, but the recent discovery of a specific retinoic acid binding protein c(RABP) in various tissues has led to the hypothesis that the biological effect of retinoic acid may be mediated through this receptor (69). However, investigations have been inconclusive about the presence of these receptors on HL60 (73).

The effect of interferons on leukemic and normal myeloid differentiation is not clear yet. Studies have shown that alpha and beta interferons enhance the

differentiation of HL60 cells triggered by TPA or retinoic acid, but are ineffective when used alone (74). Interferon has been shown to increase hemoglobin synthesis in K562 (75) and has recently been shown to induce monocytic differentiation in U937, a human histiocytic lymphoma derived cell line (74,76). Interferon has also been shown to block or promote DMSO stimulated hemoglobin production in Friend erythroleukemia cells (77,78). In human systems, the delay of maturation of monocytes into macrophages by interferon (79), and enhancement of pokeweed mitogen induced B cell differentiation at low concentrations and inhibition at high concentrations of interferon have also been reported (80). The mechanisms of interferon induced differentiation and the enhancing effects of interferon on other inducing agents are unclear.

The addition of hemin to Friend erythroleukemia cells initiates an erythroid development program, with accumulation of globin mRNA and hemoglobin. Hemin has also been shown to trigger hemoglobin production and increase the expression of glycophorin in K562. It is believed that hemin affects the activity of protein initiation factors and may control the efficiency of translation of globin mRNA. Hemin may also have a direct effect on the transcription or stabilization of globin mRNA. Hemin has been shown to have a concentration dependent ability to reduce DNA synthesis and inhibit the proliferative capacity of erythroid cells (81).

Sodium butyrate has been shown to be an effective differentiating agent in the murine erythroleukemia system and in K562. The treated cells undergo morphological and functional changes consistant with erythroid differentiation. Sodium butyrate has also recently been shown to decrease in vitro tumorigenicity, elevate alkaline phosphatase activity and increase chorioembryonic antigen (CEA) content in human rectal and colonic tumor cell lines. The amount of CEA synthesised by tumours is believed to be directly correlated with the degree of differentiation in the cancer cells (82).

Sodium butyrate has been shown to have many effects on other mammalian cells, including the induction of the biosynthesis of new proteins (83), modification of the relative synthetic rates of specific proteins (84), augmentation or inhibition of various enzyme activities (84), induction of morphological changes in cultured cells (85), and inhibition of protein synthesis and cell proliferation (86). In addition, sodium butyrate induces massive hyperacetylation of histones in cultured mammalian cells (87,88) via inhibition of histone deacetylase (89).

Table 1. The Response of Human Myeloid Leukemia Cell Lines to Inducers of Differentiation

Cell Line	Stage of Differentiation	Inducers of Differentiation	Response to Inducing Agent	References
KG-1	myeloblast	phorbol diesters teleocidins	macrophages	64
HL60	promyelocyte	DMSO, retinoic acid phorbol diesters	granulocytes macrophages	59,60 61,62
U937	monocyte-like	phorbol diesters interferon	macrophages	76 74
HEL	early blast and/or erythroblast	hemin phorbol diesters DMSO	early erythroblast macrophages promegakaryoblast	65 66 (personal communication)
K562	early blast and/or erythroblast	hemin, butyrate phorbol diesters, butyrate interferon	early erythroblast promegakaryoblast increased hemoglobin	114,115 120,121 75

B. Differentiation Programs in Normal and Leukemic Hematopoiesis

Chronic myelocytic leukemia (CML) is a clonal hemopathy (21,90). The lesion is believed to be at the pluripotential stem cell level because the Philadelphia chromosome, a marker of this disease (91), is found in cells of the granulocytic, megakaryocytic and erythrocytic and the lymphocytic lineages (92,21).

The Philadelphia chromosome was described in 1960 by Nowell and Hungerford as an abnormally small chromosome in patients with CML (91). It was later determined that 80-90% of patients with typical CML have this defect. This marker was subsequently determined to be chromosome number 22 which had lost a portion of its long arm. In the majority of patients, the missing part of chromosome 22 is translocated to chromosome 9 (21).

The evidence that CML is derived from a single clone was obtained from the study of glucose-6-phosphate dehydrogenase isoenzymes in female heterozygotes with CML. Granulocytes, erythrocytes, platelets, monocytes and some lymphocytes display only a single G6PD isoenzyme in these patients rather than a mixture of the two isoenzymes (21,93).

Blast transformation occurs in all patients who do not die of other causes during the chronic phase of the disease. The mature leukemic cells are replaced by highly undifferentiated blast cells. Transformation is frequently

accompanied by additional chromosome abnormalities. The cell biology of transformed chronic granulocytic leukemia is quite variable. In about 30% of cases, the blast cells are highly undifferentiated and resemble those found in acute lymphoblastic leukemia (21). These blasts are believed to represent early hematopoietic stem cells not yet committed to either myeloid or lymphoid differentiation (94). They may express the common acute lymphocytic leukemia antigen (CALLA), and they may contain cytoplasmic immunoglobulin or have elevated levels of terminal deoxynucleotidyl transferase (TdT) which is correlated with acute lymphoblastic leukemia (21). Recent studies of lymphoid blast crises of CML, which examined DNA rearrangements of immunoglobulin heavy and light chain genes, have indicated that most cases of lymphoid blast crisis represent monoclonal expansions of cells at the B cell precursor stage of development (95). These patients frequently respond to vincristine and prednisone therapy which is effective in inducing remission in ALL.

The remaining 70% of cases of transformed CML involve myeloid committed stem cells. In most of these cases, the blast cells show features of granulocytic differentiation, while in a few cases, the transformed cells are monoblastic, erythroblastic or megakaryoblastic (47).

It seems unlikely that acute myelocytic leukemia (AML) begins in the same stem cell compartment as CML because shifts in AML - ALL patterns are very rare. Consequently

AML is tentatively assigned to the myeloid stem cell compartment. There is also some suggestive evidence that this disease may originate in a cell with even more restricted differentiation potential than a stem cell (21).

McCulloch believes that lineage diagrams based on colony assays provide an oversimplified and inflexible view of hematopoietic differentiation (92). He theorizes that the stages between the pluripotent stem cell and a functional end stage cell may be considered as components of differentiation programs (92,96). Each component represents gene activation or inactivation events modified by epigenetic or environmental influences. Diversity is increased at each division because the two daughter cells may follow different programs. He postulated considerable variation in the timing and sequence of the program components. Program segments with similar frequencies in timing would identify apparently discrete stages in differentiation. Certain program segments would have characteristics that are compatable with proliferation and differentiation in cell culture in the presence of selective conditions. Other program segments would be recognized by the appearance of macromolecules which would be considered as differentiation markers with or without lineage specificity. Normal programs of differentiation would terminate in functionally effective cells (92,97).

Determination based on lineage diagrams imply that once a cell has become committed to a specific hematopoietic pathway, its decendents will follow that pathway

faithfully. The concept of lineage fidelity, that cells only express markers of one lineage, appears soundly based in the post deterministic segments of differentiation programs (92).

However, there is uncertainty about earlier events in differentiation, but it has been postulated by McCulloch that only macromolecules that lack lineage specificity such as HLA-DR would appear on these pluripotential stem cells.

He has further suggested that the differentiation programs of blast populations are assembled abnormally from components that belong to normal programs and that the abnormal assemblage is a consequence of abnormal gene expression. Based on the premise that normal differentiation programs terminate in functional cells, he has stated that the programs of blast cells are abnormal because they terminate in nonfunctional cells (92,96).

The other piece of evidence that he offers in support of this theory is that of lineage infidelity (92,96,98,99) which is the expression of markers of different lineages in the same cell. This would violate the expected finding that commitment to one lineage excludes the expression of markers of other lineages. In a recent study of blast cells from twenty patients with acute leukemia, nine patients had blast cells showing lineage infidelity (98). Preliminary results indicate that these patients are less responsive to therapy. Normal marrow, marrow originating after transplantation, and multiple lineage colonies in

culture have been searched extensively and unsuccessfully for evidence of lineage infidelity.

Many other authors report that lineage fidelity is maintained in leukemic blast cells (100,101,102). Such lineage fidelity has been used to support models of leukemia that hypothesize either blocked differentiation or an uncoupling between self renewal and maturation (100,102,103). Therefore the termination of blast programs in non functional cells would represent an early termination of normal programs rather than evidence of abnormal programs. This appears to be more applicable to lymphoid rather than to myeloid leukemia.

An alternative model to McCulloch's has been proposed by Till (104). He has suggested that pluripotential stem cells may express a variety of markers, perhaps at very low levels, specific for each of the lineages within the stem cells repetoire for differentiation. This can be extrapolated perhaps to say that lineage infidelity is a normal feature of early differentiation. Determination and maturation would be associated with amplification of certain lineage specific features and repression of others. A test of this hypothesis awaits the availability of highly purified preparations of normal pluripotential stem cells to allow investigators to search for markers of multiple lineages on the same cell.

C. K562 - A Pluripotential Stem Cell Line.

The human leukemia cell line, K562, was originally established in 1970 in the laboratory of Lozzio and Lozzio at the University of Tennessee from a pleural effusion of a woman with CML in blast crisis (67). This cell line carries the Philadelphia chromosome, a marker of CML. This was the first permanent cell lined derived from a patient with CML that retained this marker for longer than a few months.

K562 cells are large, highly undifferentiated blasts. Their cytoplasm is basophilic, devoid of granules and contains a few small vacuoles. The nuclei are round and contain one to four prominent nucleoli. Numerous mitotic figures and binucleated cells can be seen. The cells grow as a single cell suspension in fluid tissue culture media with a mean doubling time of about twenty hours when grown in RPMI supplemented with 5-10% fetal calf sera, in 5% CO2.

K562 was originally believed to be a myeloid precursor arrested at an early stage of differentiation. This theory was based on the tissue of origin, failure to find detectable lymphoid surface markers (105,106), the presence of group specific granulocyte antigens (107), and the pattern of reactivity of anti K562 heteroantisera with bone marrow and leukemic cells (108).

Further evidence supporting a granulocytic phenotype include reactivity of K562 with anti MY-1, a monoclonal

antibody specific for a granulocytic differentiation antigen (99). In addition, K562 expresses two related antigens TG1 and TG3, defined by monoclonal antibodies, which are found only on myeloid cells in normal hematopoietic tissue. These antigens are lost when K562 is cultured with DMSO and sodium butyrate but not with hemin (109). Finally, there have been monoclonal antibodies raised against K562 that are able to inhibit myeloid colony formation (110). Another K562 monoclonal antibody, D5, is specific for maturing granulocytes beginning with the promyelocyte stage. Myeloblasts are negative, but the antibody cross reacts weakly with very immature erythroid and monocytic cells (111).

The majority of K562 cells have Fc receptors detected by sheep EA rosettes or binding of aggregated immunoglobulin. Changes were noted after culture with various inducing agents, but these were thought to reflect non specific membrane effects of the different agents (112).

Despite these myeloid features, K562 has been reported as unresponsive to phorbol esters (69), DMSO (112), and retinoic acid, and all attempts to induce granulocytic differentiation by these agents have been unsuccesful. Surface antigens characteristic of macrophages have not as yet been detected on these cells.

Further studies of K562 have provided evidence of erythroid differentiation. Andersson et al. demonstrated that K562 expresses glycophorin and spectrin, erythroid

specific membrane proteins (113). They later reported that sodium butyrate, a known inducer of differentiation in Friend erythroleukemia cells, induces K562 to form erythrocyte like particles, budding off the cytoplasm in a similar way to the formation of platelets (114). No other groups have been able to reproduce these observations. They also detected hemoglobin synthesis after sodium butyrate induction by the benzidine reaction and then confirmed by radioimmunoassay (114).

Biochemical proof of globin protein synthesis by K562 was provided by Rutherford, using hemin as an inducing agent (115). It was later demonstrated that K562 produces increased amounts of globin chains after hemin exposure because of increased production of specific globin messenger RNA (116). The pattern of hemoglobin production is of the embryonic type, with the presence of small amounts of fetal hemoglobin. Hemin triggers the synthesis of alpha chains and a subsequent increment of fetal hemoglobin (117). K562 does not synthesise adult hemoglobins either constitutively, or in response to inducing agents.

Heme synthesis is a pivitol event during erythroid differentiation. Sassa demonstrated changes in the concentrations and activities of heme biosynthetic enzymes during DMSO-induced differentiation of Friend erythroleukemia cell lines (118). It was subsequently shown in K562 that exogenous hemin stimulates an increased

activity of the enzymes from aminolevulinic acid dehydratase (ALAD) to ferrochetalase as demonstrated by the increased incorporation of radioactive aminolevulinic acid after hemin induction. Therefore endogenous intracellular heme biosynthesis accounts for at least a fraction of the heme incorporated into hemoglobin by K562 (116).

Tonkonow and Hoffman reported a dramatic change in the pattern of binding of an antiglycophorin to the membrane of K562 after hemin induction. There appeared to be a loss of a population of cells with a low level of reactivity to glycophorin A antisera, and an accumulation of a greater number of cells maximally reactive with antiglycophorin A (116). This is consistant with differentiation and maturation of normal human erythroid cells where glycophorins are present at the proerythroblast stage, before the onset of hemoglobin synthesis, but their amount greatly increases during maturation (119).

K562 lacks ABH, Rhesus, Lewis, Duffy, P, P1, and Pk antigens (112), but "i" antigen activity, a feature characteristic of fetal erythrocytes, is increased dramatically after hemin induction (116).

K562 has also been shown to have some features consistant with megakaryocytic differentiation. Breton-Gorius et al., using cytochemical ultrastructural studies, detected a weak platelet peroxidase activity (PPO), distinct from granulocytic peroxidases, in the nuclear envelope and rough endoplasmic reticulum in a small number of cells (120). By its location, the PPO resembled that of normal

and leukemic promegakaryoblasts. The addition of sodium butyrate or dimethyl formamide to K562 cultures increased the numbers of these cells, but did not modify the cytoplasmic maturation. Peroxidase activity is the first marker of differentiaton toward the megakaryocytic line in normal and leukemic promegakaryoblasts, and is detectable before any signs of cytoplasmic maturation. They were unable, however, to detect platelet glycoprotein I in K562 cells, using a monoclonal antibody. They have recently shown that K562 can be induced with PMA to express platelet glycoprotein IIIa and a glycoprotein common to the lymphoid lineage and platelets, as defined by the monoclonal antibodies C17 and J2 (121). Sodium butyrate also significantly increases the binding of monoclonal antibody J2. It is believed that platelet glycoprotein I appears in the maturation sequence later than platelet peroxidase or platelet glycoprotein IIIa.

Subsequently, Hoffman et al., using a rabbit antisera directed against purified platelet glycoproteins, found evidence for the constitutive expression of platelet glycoproteins on the surface of K562 cells (122). The glycoproteins were tentatively identified as IIa and III based on their migration in SDS gel electropheresis.

McCulloch et al. were the first group to publish direct evidence that K562 cells express markers from two different lineages simultaneously. They found that 10-30% of the cells had spectrin and My-1 on the membrane using a double

labelling immunofluorescent technique (99). K562 is the only human hematopoietic cell line which exhibits lineage infidelity. The concept of lineage infidelity appears to be central in the differing view that McCulloch and Till have on the nature of the pluripotential stem cell and its capacity for differentiation.

Membrane markers for T lymphocytes (sheep E rosettes), and B lymphocytes (surface and cytoplasmic immunoglobulin and C? receptors) are all negative. The major histocompatability antigens (HLA-DR, B2 microglobulin, and HLA-ABC) are not expressed on K562. None of these features changed after treatment with inducing agents (112' K562 is unusual because it lacks these MHC antigens. C ss I and II antigens are normally expressed on stem cells and on the majority of hematopoietic cells. There is some evidence now appearing that the MHC antigens may not only be involved in the immune recognition system, but also in the differentiation programs of the hematopoietic system.

There is some indication in the literature that some patients with CML can be treated with agents such as vitamin A to stimulate terminal differentiation of their leukemic cells (69). However, there has been little success in triggering maturation of less differentiated blast cells. K562 may provide a suitable model for developing treatment protocols based on induction of differentiation in patients with acute myeloid leukemias or transformed chronic myelocytic leukemias.

Chapter III. Materials and Methods.

This study investigated the capacity of the K562 cell line to differentiate toward the erythroid, granulocytic, monocytic and megakaryocytic lineages in response to exogenous inducing agents.

A panel of lineage-specific monoclonal antibodies, cytochemistry, functional assays and cell cycle analysis were employed to measure the differentiation capacity of these cells.

A. Cell Culture

1) Cells.

The K562 cell line was obtained from Dr. M. Longenecker (Dept. of Immunology, University of Alberta) in November 1981. Aliquots of the cells were frozen in liquid nitrogen at that time. The identity of the cell line was confirmed by indirect immunofluorescent staining of glycophorin on the cell membrane and by a karyotype analysis showing a single Philadelphia chromosome in all the metaphases studied.

2) Culture Conditions

K562 cells were grown in suspension culture in RPMI 1640 media (GIBCO Laboratories, Grand Island Biological Company, Grand Island, New York) containing 10% fetal calf serum (FCS)(Flow Laboratories - Flow General, Mclean, Virginia) and supplemented with 0.05 mg/ml of gentamicin

(Roussel Canada Inc., Montreal, Quebec). The cells were maintained at 37°C, in a humidified atmosphere, containing 5% CO_2. The cultures were diluted to low density (0.05 - 0.15 million cells/ml) three times weekly. Every four to six weeks aliquots of the cells were frozen in liquid nitrogen for retrospective analysis, if required, at a later date.

Cell numbers and cell volume distributions were determined using an automatic cell counter, equipped with a channelyzer (Coulter Counter, model ZBI, Coulter Electronics Inc., Hialeah, Florida).

Cell viabilities were assessed by trypan blue dye exclusion.

3) Cloning Procedure

The cell culture was diluted to a concentration of 10 cells/ml by serial dilutions. Aliquots (0.1 ml) of this suspension were distributed in individual microwells. Cells that proliferated and gave rise to cellular colonies after 8 to 14 days were picked off and subsequently grown in large amounts of media. Three such clones with different myeloid and erythroid surface marker phenotypes as measured by indirect immunofluorescence with monoclonal antibodies, were again cloned by the previous technique and subsequently studied. These clones were named K562-4, K562-11, and K562-17.

4) Freezing and Thawing K562 Cell Line.

Exponentially growing cells were concentrated by centrifugation to a final volume of 0.5 mls in RPMI 1640 containing 20% fetal calf serum. An equal volume of 20% dimethylsulphoxide (DMSO) in RPMI 1640 was added dropwise. The cells were cooled to -70°C at approximately 1°C per minute, and then stored in liquid nitrogen.

The frozen cell vials were thawed rapidly by immersion and gentle agitation in a 37°C waterbath. The contents of vials were transferred to a 12-ml conical centrifuge tube and diluted dropwise with 20% FCS in RPMI. The cells were centrifuged and then resuspended in 5 - 10 mls of RPMI 1640 supplemented with 20% FCS.

5) Mycoplasma Testing

The cell line was tested for mycoplasma contamination in April 1983. All three clones were found to be infected at that time. Subsequent investigation revealed that the original culture, obtained in 1981, was contaminated.

K562-4 was treated with Tylocine 250 ug/ml (GIBCO Laboratories), Kanamycin 1000 ug/ml (GIBCO Laboratories), Chlortetracycline HCl 100 ug/ml (GIBCO Laboratories), and Lincocin 1500 ug/ml GIBCO Laboratories) for two weeks. The dosages were determined for each antibiotic as the highest concentration the cells could tolerate without loss of viability. After two weeks of culture in standard media, the cells were retested for mycoplasma and were found to be free of contamination. The cells were tested again two

months later and were still free of contamination.

B. Induction Protocols

The inducing agents used in this study have been well described in the literature. These agents have been shown to induce erythroid, granulocytic, monocytic and megakaryocytic differentiation in human leukemic cell lines (Table 1). The optimal concentrations and duration of treatments for each inducing agent (Table 2) was obtained from the literature and by preliminary experimentation to find the dosage that stimulated the largest increment of membrane antigen changes without compromising cell viability as measured by trypan dye exclusion. A preliminary trial of the effects of each inducing agent on the three K562 clones showed no qualitative differences in the response of the cells. All subsequent investigations were done with the clone designated K562-4.

1) Sodium Butyrate

A 1 M stock solution of sodium butyrate (butyric acid - sodium salt, MW 110.1 gm/mole, JT Baker Chemical Co., Phillipsburgh, NJ) was prepared by adding 0.5505 grams of sodium butyrate to 5.0 mls of deionized water. This solution was sterilized by passing it through a Millex - GV 0.22 um filter unit (Millipore Corp., Bedford, MA). The stock solution was subsequently diluted with tissue culture medium to a final concentration of 1.5 mM. Exponentially growing K562 cells were seeded in duplicate at 5.0×10^4 cells/ml in the prepared media. The cultures were maintained for four days without replacing the media.

Duplicate untreated control cultures were prepared and maintained in parallel with the test cultures.

2) Hemin

A 4.0 mM stock solution of hemin (type III crystalline equine hemin, MW 652.0 gm/mole, Sigma Chemical Company) was prepared by dissolving 13.0 mg of hemin in 0.2 mls of 0.5 M NaOH, then buffered with 0.25 mls of 1 M Tris-HCl pH 8, diluted to 5.0 mls with deionized water, and then sterilized by filtration. The stock solution was subsequently diluted to 2.5×10^{-5} M with tissue culture media. Exponentially growing K562 cells were seeded in duplicate at 5.0×10^{4} cells/ml in the prepared media. Controls included duplicate untreated cultures and duplicate cultures supplemented with equivalent concentrations of NaOH and Tris-HCl buffer. The cultures were maintained for 4 days without replacing the media.

3) Retinoic Acid

A 1 mM stock solution of retinoic acid (all trans type XX crystalline retinoic acid MW 300.4 gm/mole, Sigma Chemical Company) was prepared by dissolving 0.003 grams of retinoic acid into 10.0 mls of ethanol. The stock solution was subsequently diluted to 1 uM with tissue culture media. Controls included duplicate untreated cultures and duplicate cultures supplemented with equivalent concentrations of ethanol. The retinoic acid stock solution and the untreated cultures were protected from

light degradation by wrapping the stock bottle and culture flasks in aluminum foil and working in subdued lighting as much as possible. Exponentially growing K562 cells were seeded in duplicate at 5.0 x 10^4 cells/ml in the prepared media. The cultures were maintained for four days without replacing the media.

4) Dimethylsulphoxide (DMSO)

A 0.175 M solution of DMSO was prepared by adding 0.625 mls of filtered DMSO (1.095 gms/ml, MW 78.13 gms/mole, Fischer Scientific Co., Chemical Manufacturing Divion, Fairlawn, New Jersey) to duplicate 50 ml portions of tissue culture media. Controls included duplicate untreated cultures prepared in parallel with the treated cultures. Exponentially growing K562 cells were seeded at 5 x 10^4 cells/ml and the cultures were maintained for four days without replacing the media.

5) 4β - Phorbol 12β-Myristate 13α - Acetate (PMA)

A 1.6 X 10^{-4} M solution of PMA was prepared by adding 0.001 grams of PMA (MW 616.8 gms/mole, Sigma Chemical Company) to 10 mls of DMSO. This solution was sterilized by filtration. The stock solution was subsequently diluted with tissue culture medium to a final concentration of 5 X 10^{-10} M. Exponentially growing K562 cells were seeded in duplicate at 2.5 X 10^5 cells/ml in the prepared media. Controls included duplicate untreated cultures and duplicate cultures treated with the concentration of DMSO equivelant to that of the test cultures. The cultures were

maintained for 20 - 24 hours.

6) Interferon

Alpha and cloned gamma interferons were obtained from Dr M. Fellous, Institut Pasteur, Paris and beta interferon was obtained from Dr. C. Tan, University of Calgary. Duplicate cultures seeded at 2.5×10^5 cells/ml were maintained for 20 - 24 hours and cultures seeded at 5×10^4 cells/ml were maintained for four days in culture media supplemented with 1000 U/ml of alpha, beta, or gamma interferon, without changing the medium.

Table 2. Summary of Induction Protocols for K562

Inducing Agent	Concentration	Duration of Exposure	Seeding Density
Sodium butyrate	1.5×10^{-3} M	4 days	5×10^4 cells/ml
Hemin	2.5×10^{-5} M	4 days	5×10^4 cells/ml
Retinoic acid	1.0×10^{-6} M	4 days	5×10^4 cells/ml
DMSO	1.75×10^{-1} M	4 days	5×10^4 cells/ml
PMA	5.0×10^{-10} M	20 hours	2.5×10^5 cells/ml
Interferon[a]	1000 units/ml	20 hours	2.5×10^5 cells/ml
Interferon[b]	1000 units/ml	4 days	5×10^4 cells/ml

[a]refers to alpha, beta and gamma interferons

[b]refers to alpha and beta interferons

C. Membrane Expression of Lineage Specific and Major Histocompatability Antigens

1) Flow Cytometry Analysis.

Flow cytometry analysis with an EPICS V cell sorter (Coulter Electronics, Hialeah, Florida) was used to measure the expression of cell surface antigens. Cell cultures were washed twice with phosphate buffered saline (PBS) with 0.2% bovine serum albumin (Pentex Bovine Albumin 30% solution, Miles Scientific, Naperville, Il.) and were then resuspended in PBS, 2% FCS, 0.02% sodium azide solution. Fifty ul aliquots containing 1 million cells were mixed with 50 ul of diluted monoclonal antibody (McAb) (table 3). After one hour at 4°C, the cells were washed three times with PBS, 0.2% BSA and then 100 ul of a 1/50 dilution of fluorescein conjugated F(AB')2 fragment rabbit anti-mouse IgG was added (Cappel laboratories, Cochranville, PA) The cells were incubated in the dark at 4°C for one hour, washed as described previously and then were resuspended in 0.5 mls of a 1% w/w formaldehyde solution (Formaldehyde solution, 37% w/w, Fischer Scientific Co.) in PBS. The samples were kept at 4°C in the dark until they were analyzed by a Coulter EPICS V flow cytometer using a 5 watt argon laser. Ten thousand cells of each sample were analyzed for green fluorescence using the multiple data aquisition display system (MDADS). The gates were set, based on 90°light scatter, to include both control and test samples, which may have changed in volume, and to exclude

Table 3. Characteristics of the Mouse Monoclonal Antibodies used to Measure the Expression of Lineage Specific and Major Histocompatability Membrane Antigens in K562.

Specificity	Antibody Designation	Reference
MHC ANTIGENS		
β2 Microglobulin	M18, M28	Fellous[a]
HLA-ABC Monomorphic	81H2, 83H1, 9H1	123,4,5
HLA-DR Monomorphic	7H3, 2206	" "
ERYTHROID LINEAGE		
glycophorin A	H85	Mannoni[a]
	B357	Salmon[a]
MYELOID LINEAGE		
Early Granulocytic	80H5,82H6,82H1	126,7,8,9
Late Granulocytic	80H3, 82H7	" "
Monocytic	82H3	Mannoni[a]
	MO2	130[b]
MEGAKARYOCYTE LINEAGE		
Factor VIII R Antigen		131[c]
Plt Glycoprotein IIb-IIIa	J15	132

[a]unpublished

[b]Coulter Clone

[c]Cappel

debris and agglutinated cells. Each test sample with its matched control was run consecutively to reduce error due to drift. Histograms showing the number of stained cells, as a function of the log fluorescence intensity were recorded. Controls analyzed included non-induced cells that were prepared in parallel with the treated cells, and non-stained cell suspensions to correct for background fluorescence.

The percentage of positive cells was obtained from the integral of cells that were more fluorescent than 95% of cells in the unstained cell preparations. In cases of weak fluorescence, and where a suitable match region existed, the IMMUNO Program (Coulter Electronics, Inc.) was used to determine the percentage of stained cells. This program reduces the high degree of error inherent in standard integration programs when the positive and negative immunofluorescent curves heavily overlap. IMMUNO is a least squares algorithm which extrapolates underneath the positives and subtracts out the negatives.

2) HLA Phenotype - Immune Fluorescence.

Immune fluorescence was performed as described earlier using cells induced with sodium butyrate and interferon, and on untreated cells using human sera containing monospecific HLA antibodies against A1, A2, B7 and B12 antigens. These sera were obtained from multiparous women and from multiply transfused individuals. The specificities of the antibodies were obtained previously by

testing with a panel of HLA phenotyped peripheral blood lymphocytes. The K562 cells were pretreated with 1% paraformaldehyde (Fisher Scientific Company, Fairlawn, New Jersey.) in PBS for five minutes to reduce non-specific binding of immunoglobulin to the Fc receptors on K562 (106).

3) HLA Phenotype - Cytotoxicity

Standard cytotoxicity HLA typing using eosin dye exclusion as the criteria of cell viability (133) was performed on K562 induced with sodium butyrate, interferons and on untreated cells. HLA - ABC prepared typing trays containing 95 different antisera were obtained from the Canadian Red Cross Blood Transfusion Service Reference Laboratory, Toronto, Canada. The McAb 81H2, 9H1 and 83H1 were used as cytotoxicity controls.

D. Investigation of the Mechanism of HLA Class I MHC Antigen Induction in K562

Immune precipitation of MHC antigens and analysis HLA class I mRNA from K562 induced with interferons and sodium butyrate and on control cells were performed by M. Fellous and Frederic Rosa, Institut Pasteur, Paris.

1) Analysis of HLA Class I mRNA in K562

Following induction with interferon and/or sodium butyrate, messenger RNA specific for HLA proteins was extracted from K562 cells, subjected to agarose gel electrophoresis, and then hybridized to pHLA [^{32} P] DNA as described previously (134).

2) Immune Precipitation of Class I MHC Antigens

After induction, the cells were labelled, washed, and then lysed as described previously (135,136). Using the detergent phase as the antigen source (137), immunoprecipitation was carried out as described by Ploegh (138). Immunoprecipitates were resolved and visualized as described previously (135).

E. Functional Assays

1) Hemoglobin Production

Qualitative estimation of hemoglobin production in K562 was determined by benzidine peroxide staining (139) of intact cells in suspension (140). Two hundred cells were scored after five minutes as positive (blue) or negative (yellow) using 40X magnification. The mean scores of duplicate preparations were recorded.

Quantitative analysis of hemoglobin production in K562 was carried out by a modification of spectrophotometric method described by Clarke (141). The non-carcinogenic benzidine derivative 3,3',5,5' tetramethyl-benzidine (Sigma Chemical Company, MW 240.4 gm/mole) was used instead of benzidine HCl. Duplicate aliquots of 100,000 K562 cells were washed with saline, lyzed with 40 ul of deionized water and frozen at -70°C until analyzed. Hemoglobin standards of 30, 100, 200, 300, and 400 ng/ml were prepared by diluting hemoglobin (human type IV 2X crystallized hemoglobin, Sigma Chemical Co.) in deionized water. This method was linear in the range of 30 ng to 400 ng of hemoglobin.

2) Nitro Blue Tetrazolium (NBT) Reduction.

The NBT test is used to investigate granulocyte and monocyte function and to measure terminal differentiation in granulocytic and monocytic cell lines. Except for chronic granulomatous disease, where the ability of neutrophils to chemically reduce NBT is impaired, the NBT

test is a measure of a cells ability to phagocytose complexed NBT, the dye acting as a visable marker in the process. NBT by itself is not readily phagocytosed and so it must be coupled with zymosan particles, Candida albican cells, or latex particles. Alternatively, a stimulant of phagocytosis, such as endotoxin or phorbol esters, may be added.

For NBT reduction studies, 2 million cells suspended in one ml of RPMI 1640 medium supplemented with 20% FCS were incubated for 20 minutes in the dark, in a 37°C waterbath, with an equal volume of 0.2% NBT (crystalline, grade III, MW 817 gm/mole, Sigma Chemical Company) dissolved in PBS with and without 200 ng of PMA. Preparations of fresh granulocytes were tested as a control for the method and reagents. The percent of cells containing intracellular reduced blue-black formazan deposits was determined on Wright-Giemsa stained Cytospin centrifuge (Shandon Southern Instrument Inc., Sewickley, PA) preparations of the incubated cells.

F. Morphological Examination and Cytochemist

A battery of cytochemical stains routinely used to evaluate the lineage of leukemic blasts (21) was used to measure the response of K562 to the inducing agents. The 5'-nucleotidase stain, which has recently been reported to be diagnostic in megakaryocytic leukemias (142), was also included.

1) Evaluation of Cell Morphology.

One hundred thousand to two hundred thousand cells suspended in tissue culture medium were centrifuged for five minutes at 600 RPM onto microscope slides in a Cytospin centrifuge. Air-dried preparations were fixed for five minutes in methyl alcohol and were then stained with May-Grunwald Giemsa stain. The slides were examined with light microscopy for morphological evidence of differentiation, such as reduction in cell size, loss of nucleoli, condensation of nuclear chromatin, cytoplasmic granules, and vacuolization.

2) Sudan Black B

Sudan Black B is a stain that reacts with a variety of lipids, including neutral fats, phospholipids and steroids. The distribution of sudanophilia in cells of the bone marrow and blood in normal and pathological states is now well established (22). Cells of the granulocytic series show increasing positivity with progressive maturity. Myeloblasts are negative or weakly positive. Lymphocytes

and their precursors are invariabley negative. Monocytes and their precursors may sometimes be n ative but frequently show variable numbers of fine or moderately course granules scattered discretely over the cell with little tendancy to concentrate in a paranuclear zone or in the cytoplasmic ring as in early myelocytes. Individual platelets are usually negative, as are megakaryocytes, although the latter may rarely show diffuse background staining with very fine sudanophilic granules scattered throughout the cytoplasm and over the nucleus. All cells of the erythroid lineage are negative. Results obtained with this stain correlate well with those of the myeloperoxidase stain, which has been used for may years as a specific marker of granules of cells of the myeloid series. The advantage, of sudan black stains over peroxidase is that there is no interference from the peroxidase activity of hemoglobin and fresh slides are not required.

Cytospin preparations of induced and non-induced K562 cells were stained by the method described by Miale et al. (143). Smears of normal bone marrow were used as a control for the method.

3) Non Specific Esterase - Alpha Napthyl Acetate Esterase

The biochemistry of esterases is complex and the nomenclature is confusing, but in cytochemistry the term esterase is generally restricted enzymes capable of hydrolizing the simpler esters of the N - free alcohols and

organic esters. Depending on the substrate used, a number of different reaction patterns can be identified in the granulocytic and monocytic lineages (22). The distribution of the enzyme reaction product when alpha - naphthyl acetate is used as a substrate is largely confined to monocytes while the use of naphthyl AS or AS-D acetate gives some degree of positivity in most haemic cells, but with particularly strong reactions in monocytes. Strong alpha naphthyl acetate esterase positivity is seen in the erythroblasts of Di Guglielmo's disease but normal erythroid precursors are negative. The reaction observed in leukemic erythroblasts is sensitive to fluoride inhibition like that observed in monocytes. In lymphocytic cells, B cells are negative, "null" cells show some fine granules and T lymphocytes show strong localized spots of positivity which are resistant to fluoride inhibition. Megakaryoblasts also show localized or granular positivity which is moderately sensitive to fluoride inhibition.

Cytospin preparation of induced and non-induced K562 cells were stained by the method described by Yam et al. (144). Smears of normal bone marrow were used as a control for the method.

3) Periodic Acid Schiff Stain (PAS)

The carbohydrate or more particularly the glycogen content of blood cells can be measured qualitatively by the the PAS stain. It is now well established that glycogen plays an important role in the primary function of

different types of hemic cells (22). The glycogen particle stores glucose and makes it available on demand. The presence of large stores of glycogen in cells such as neutrophils, transformed T lymphocytes in PHA cultures and platelets is related to their highly specialized functions which requires sudden bursts of energy.

The PAS stain has a complex pattern of reactivity which often changes markedly in pathological states (22). In normal hemic cells, the erythroid lineage does not normally show detectable amounts of glycogen at any stage of cellular development. The myeloid series is positive in all identifiable cells, with the strength of reaction correlated with the maturity of the cells. Lymphocytes have a much lower glycogen content than granulocytes, but PAS positive granules can often be demonstrated in the cytoplasm. From 10 - 40% of lymphocytes normally show positivity with one or two rings of perinuclear granules. More intense staining, with three or more concentric rings, or with heavy clumps of positive material is observed in 1 - 2% of lymphocytes. Monocytes contain small amounts of finely scattered granules. The PAS reaction shows diffuse and granular positivity in megakaryocytes. Platelets are strongly positive.

Myeloblasts and promyelocytes from most patients with acute leukemia are PAS negative, while lymphoblasts of acute leukemia show very strong PAS positivity in the form of concentric rings of course granules, or heavy blocks of glycogen against a negative background. Erythroid

precursors from erythroleukemic myelosis and erythroleukemias often possess concentric annular rings of moderately course PAS positive cytoplasmic granules in a variable proportion of cells. The intensity of the reaction and the percentage of erythroblasts showing positivity vary considerably in different patients and also in the same patient in the course of the disease. The strongest reactions are usually associated with bizarre cytological and mitotic abnormalities, but high PAS levels may also occur during relatively normal erythropoiesis. Cytospin preparations of induced and non-induced K562 cells were stained by the method described by Hayhoe et al. (145). Smears of normal bone marrow were used as a control for the method.

4) 5'-Nucleotidase Stain

The precise identification of human megakaryoblasts is difficult or even impossible with light microscopy alone. No diagnostic reactivity patterns can be demonstrated consistently with conventional cytochemistry in neoplastic megakaryocyte precursors, although patterns of course peripheral PAS positivity, combined with a positive alpha napthyl acetate esterase stain, is suggestive of megakaryoblastic leukemias (22). At the ultrastructural level, platelet peroxidase activity has been detected in the perinuclear space and endoplasmic reticulum of normal megakaryoblasts (20). Platelet peroxidase is now considered to be a specific marker of megakaryoblasts. However, there have been reports in the literature that

some leukemic megakaryoblasts may not show this peroxidase activity (146).

The function of 5'-nucleotidase is uncertain but a possible role for it in purine uptake has been suggested. It catalyzes the dephosphorylation of 5'-nucleotides to which the cells are generally impermeable. Cytochemical demonstration of 5'-nucleotidase has been confined mainly to the plasma membrane of lymphocytes (147). It has also been reported in lymphoid and myeloid blasts using an immunochemical assay. However, cytochemical demonstration of this enzyme appears to be restricted to leukemic megakaryoblasts. In a recent study of bone marrow smears from 36 patients with various types of acute leukemias, only the blast cells from the patient with acute megakaryoblastic leukemia were positive (142). The function of 5'-nucleotidase in megakaryoblasts is unclear but it may play a role in megakaryocytic maturation, because like platelet peroxidase, it seems to be synthesised early during megakaryocytic differentiation.

Cytospin preparations of induced and non-induced K562 cells were stained by the method described by Hayhoe et al. (126,131). Buffy coat smears from normal donors were used as a control for the method.

5) Alkaline Phosphatase

Alkaline phosphatases include a range of isoenzymes with the general ability to liberate phosphate from phosphomonoesters at pH levels above 7. These enzymes are

widely distributed in cells and tissues of the body. In hematopoietic tissues, alkaline phosphatase activity is confined almost exclusively to mature granulocytes, starting at the metamyelocyte stage (22). Reticuloendothelial cells of the marrow usually show strong enzyme activity, but other marrow cells including earlier granulocyte precursors, erythroblasts, megakaryoblasts, and almost all monocytes and lymphocytes are negative. Positive reactions have been observed in erythroblasts in conditions of erythroblastic hyperplasia and erythrocytosis.

Cytospin preparations of induced and non-induced K562 cells were stained by the method described by Hayhoe et al. (147). Buffy coat smears from normal donors were used as a control for the method.

G. Quantitative Staining of Cellular DNA - Flow Cytometric Analysis

According to the model developed by Howard and Pelc, the cell cycle consists of four phases designated; G1, S, G2 and M (148). A cell enters the G1 phase upon division and remains there until the onset of DNA synthesis. It is then considered to be in S phase until it has doubled its DNA content and entered the G2 phase. The mitotic or M phase follows and is marked by the condensation of chromatin into chromosomes and terminates when the cell divides into two G1 cells. The DNA distribution typical of an asynchronous homogeneous cell population is characterised by several distinctive landmarks. The largest peak, at unit relative DNA content is due to G1/G0 cells, the smaller peak at twice this DNA content is due to G2/M phase cells, and the continuum between them is due to S phase cells. There is some indication in the literature that there may be a relationship between DNA synthesis and cell cycle and the development of differentiated functions in hematopoietic cells (53).

Using the method described by Crissman (149), two million induced and control K562 cells were washed, then resuspended in 1.0 ml of cold saline. The cells were fixed by adding 5 mls of cold ethanol, dropwise with gentle agitation, to the cell suspension. At the time of analysis, the cells were treated with 200 ug/ml of ribonuclease (Ribonuclease A from bovine pancreas, 5X crystallized, type 1-A, Sigma Chemical Co.) to remove

double stranded DNA and 20 ug/ml of propidium iodide (Sigma Chemical Co.) to stain the DNA for twenty minutes. The cells were analyzed in a Coulter EPICS V flow cytometer. Fifty thousand cells of each sample were analyzed for red fluorescence using the Multiple Data Aquisition Display System (MDADS). Histograms showing the number of stained cells against DNA content were recorded. The percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle was determined using the INTEGRAL program (Coulter Electronics Inc.). Integrals were obtained from the channel that contained the maximum number of cells in G0/G1 and G2 phases.

A sample of cells from four separate experiments for each induction protocol were analyzed to determine the effects of the inducers on the distribution of K562 cells in the cell cycle.

Simultaneous analysis of DNA distribution and expression of lineage specific antigens was done to determine if there is a relationship between a cell's phenotype and its stage in the cell cycle. Two million cells were incubated with a particular monoclonal antibody and then stained with an FITC conjugated secondary antibody as described previously in this report. The cells were then resuspended in 1 ml of saline and fixed with 5 mls of 70% ethanol. At the time of analysis the cells were treated with ribonuclease and stained with propidium iodide as described above. A minimum of 50,000 cells were analyzed

for red and green fluorescence using the MDADS. Contour diagrams showing the number of cells expressing the membrane antigen on the Y axis and the DNA content on the X axis were recorded.

CHAPTER IV. RESULTS

A. SODIUM BUTYRATE

Sodium butyrate triggered profound changes in the morphology, expression of membrane antigens (Table 4) and cytochemistry of K562 cells.

Sodium butyrate at a concentration 1.5 mM is moderately inhibitory to cell growth (Figure 1), but it does not significantly affect cell viability as measured by trypan blue dye exclusion.

The induced cells were smaller than the control cells, they had increased vacuolization, decreased nuclear/cytoplasm ratio and a marked increase of the cell membrane perimeter accompanied by the development of pseudopods. The Golgi apparatus, represented by a clear area adjacent to the nucleus became very prominent.

The production of hemoglobin was slightly increased after induction. Control cells contained 0.17 +/- a SE of 0.012 pcg of hemoglobin per cell compared to 0.31 +/- 0.013 pcg of hemoglobin per induced cell after four days of exposure to sodium butyrate.

The membrane expression of glycophorin, defined by the binding of monoclonal antibody H85, was increased (Figure 2) after 48 hours exposure to sodium butyrate. However, the epitope which binds B357, also a glycophorin specific monoclonal antibody, was unaffected by sodium butyrate (Table 4).

Sodium butyrate triggered the synthesis of class I MHC antigens in K562 (Figure 3). This was confirmed by immuneprecipitation (PLATE I) and identification of specific messenger RNA (mRNA)(PLATE II). This response was seen within 24 hours of continuous exposure to sodium butyrate. HLA-DR antigens were not synthesised.

Exposure of normal human peripheral lymphocytes to 1.5 mM sodium butyrate for two days decreased the expression of MHC class I antigens on the cell membrane (Figure 4). In contrast, 1000 U/ml of alpha or beta interferon increased the expression of MHC class I antigens on the membrane of lymphocytes (Figure 4).

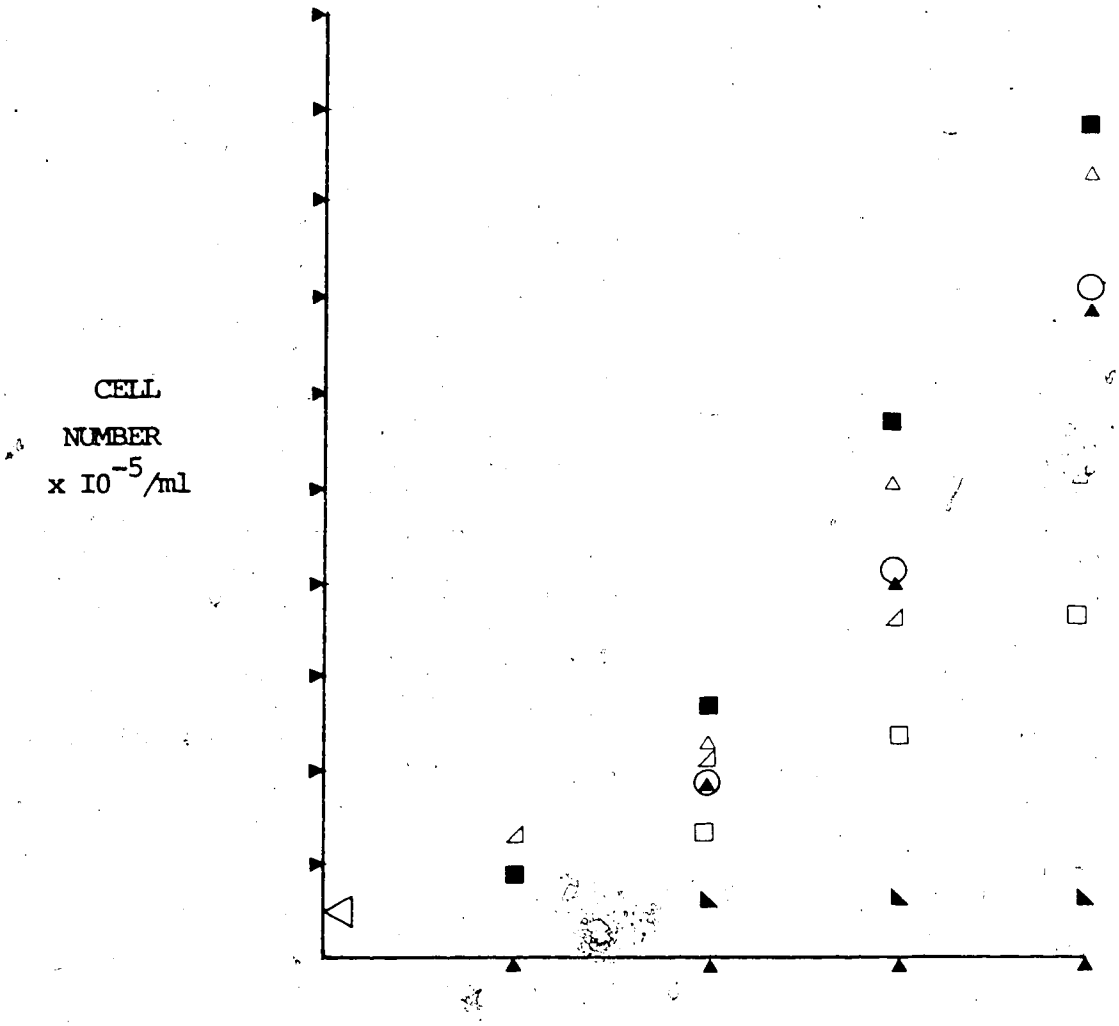
After exposure to sodium butyrate there was a marked reduction in the number of cells which bind 80H5 (Figure 5), 82H6 and 82H1 (Table 4), monoclonal antibodies which detect antigens that are expressed early in granulocyte differentiation. These changes were first observed after three days treatment with sodium butyrate, with the largest increment observed after four days. No changes were observed in the expression of monocyte or megakaryocyte antigens (Table 4).

After induction, 15% of the cells showed evidence of alkaline phosphatase activity (PLATE III), and 95% of the cells contained sudanophilic granules (PLATE IV). The control cells were negative for both stains. In addition, there was a marked increase of cytoplasmic glycogen after induction (PLATE V).

Analysis of DNA distributio revealed no significant

differences between the control and induced cells after four days of treatment. Fifty five percent +/- a SE of 3.37 of the untreated cells were in G1/G0, 28% +/- 3.79 were in S and 17% +/- 2.67 were in G2/M. Sodium butyrate treated cells were distributed 49.6% +/- 1.85 in G1/G0, 29.8% +/- 3.38 in S and 20.6% +/- 3.39 in G2/M.

Figure I. Growth Kinetics[a] of K562 Cells Treated with Various Inducers of Differentiation



[a]values represent the mean cell concentrations of four separate induction protocols

[b]refers to alpha and beta interferon

Control ■ ■ ■

Sodium Butyrate □ □ □

Hemin ◿ ◿ ◿

Retinoic Acid △ △ △

Interferon[b] ▲ ▲ ▲

DMSO ○ ○ ○

PMA ◣ ◣ ◣

Table 4. Membrane Expression of Lineage Specific and Major Histocompatability Antigens on K562 Cells after Induction with Sodium Butyrate

Membrane Antigens	McAb	Percentage of Positive Cells[a]	
		Control	Sodium Butyrate
MHC ANTIGENS			
β2 Microglobulin		1 ± .49	85[b] ± 1.48
HLA - ABC Monomorphic		2 ± 0.75	80[b] ± 1.50
HLA - DR Monomorphic		0	
ERYTHROID LINEAGE			
Glycophorin A	H85	55 ± 6.14	85[b] ± 2.30
	B357	75 ± 6.78	79 ± 4.23
MYELOID LINEAGE			
Early Granulocytic	80H5	79 ± 3.55	37[b] ± 4.62
	82H6	47 ± 7.26	9[b] ± 3.32
	82H1	17 ± 3.07	1[b] ± 0.69
Late Granulocytic		0	0
Monocytic	MO2	0	0
	82H3	68 ± 2.57	71 ± 3.57
MEGAKARYOCYTIC LINEAGE		0	0

[a] The percentage of positive cells was determined by flow cytometry analysis. Values represent the mean ± SE of five separate experiments.

[b] The differences between the means of induced and non-induced (control) cells are significant at the .05 level for Student's t test for paired data.

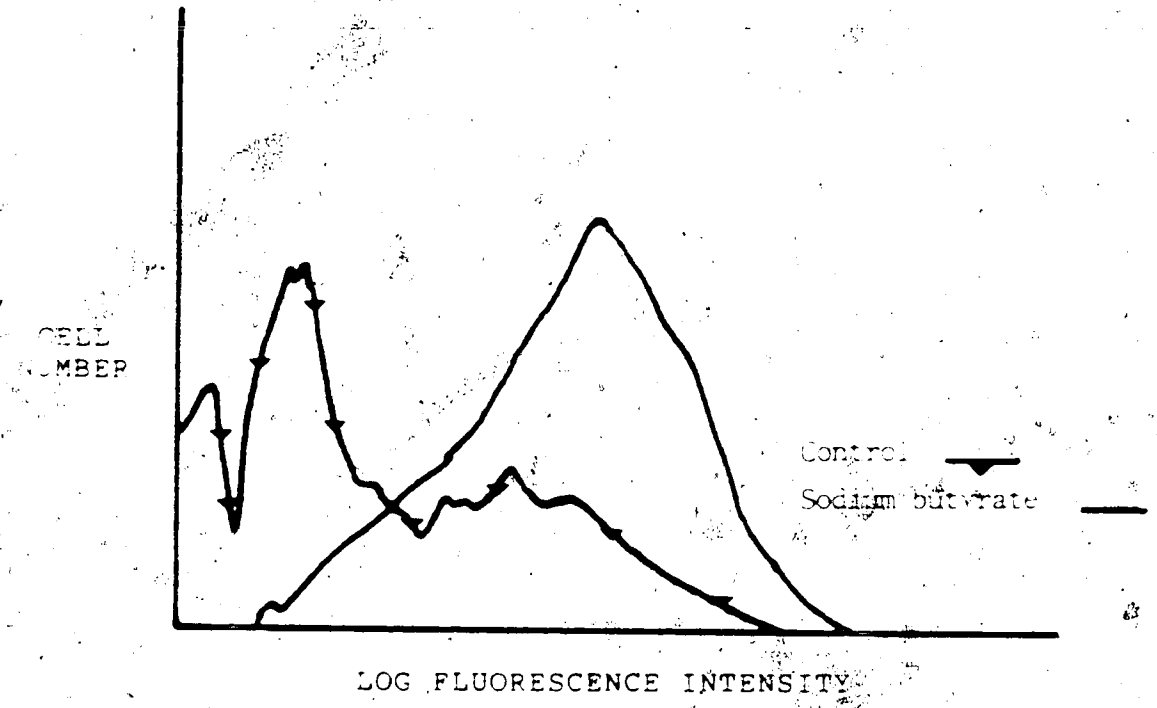


Figure 2. Reactivity of Monoclonal Antibody H85, anti Glycophorin, with K562 Cells Induced with 1.5 mM Sodium Butyrate for 4 Days.

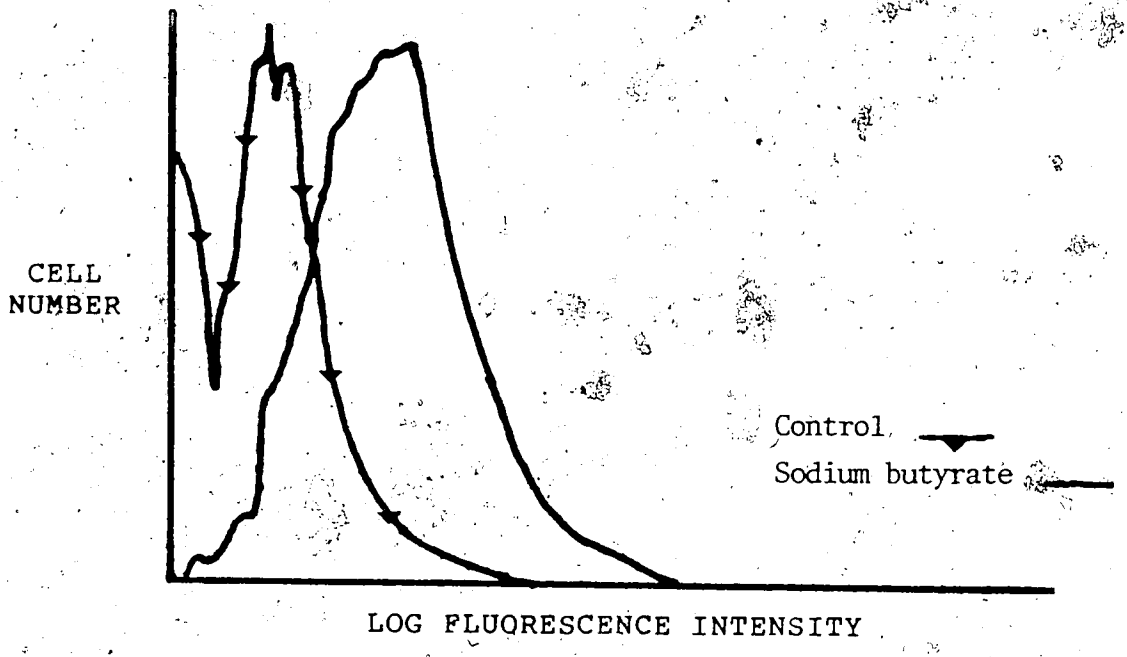


Figure 3. Reactivity of Monoclonal Antibody 81H2, anti HLA-ABC with K562 Cells Induced with 1.5 mM Sodium Butyrate for 4 Days.

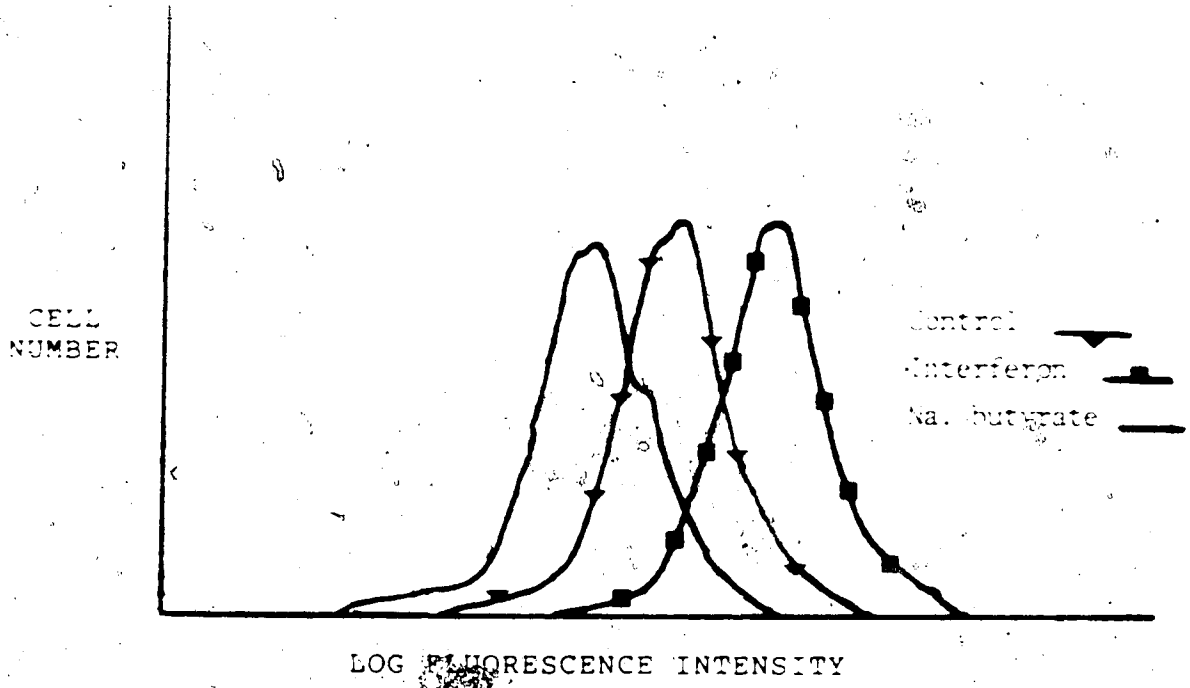


Figure 4. Reactivity of Monoclonal Antibody 83H1, anti HLA-ABC, with Normal Human Peripheral Lymphocytes Treated with 1.5 mM Sodium Butyrate or 1000 U/ml of Beta Interferon for 2 Days.

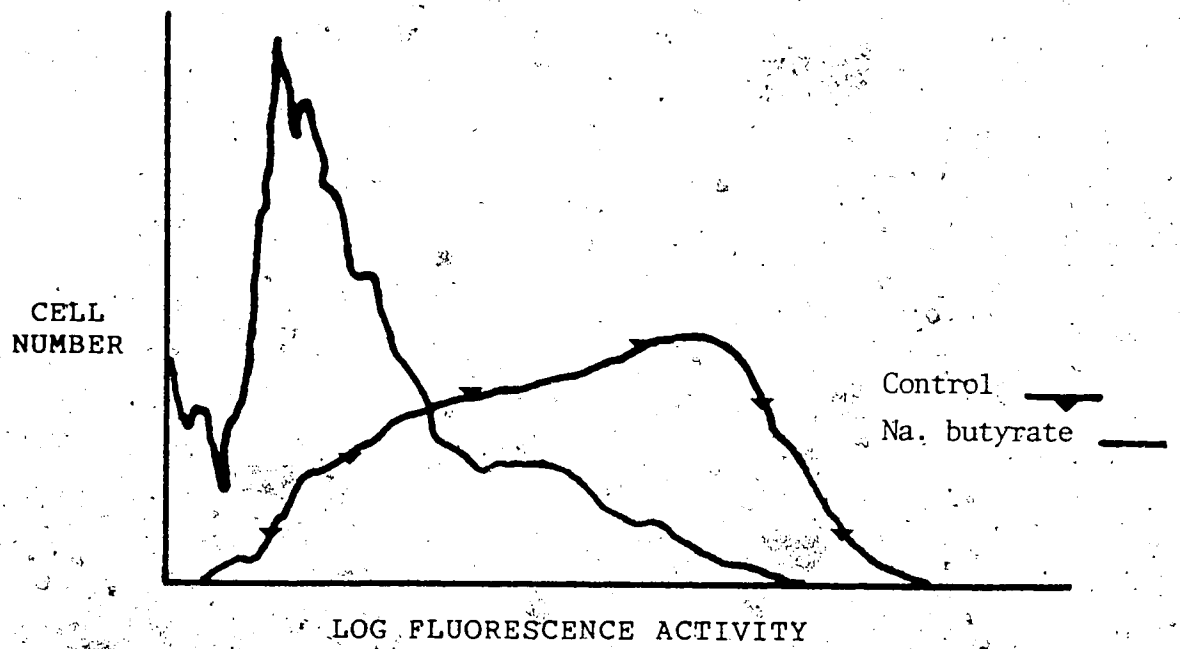
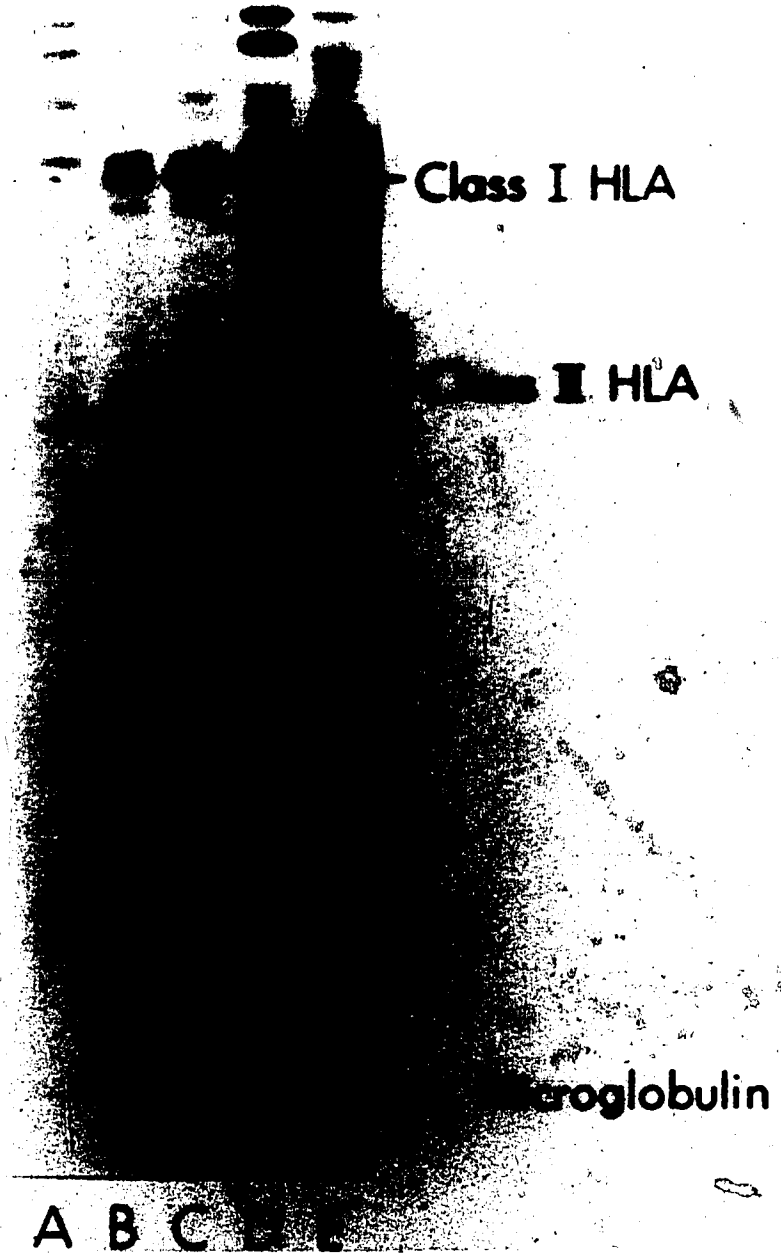


Figure 5. Reactivity of Monoclonal Antibody 80H5, anti Granulocyte, with K562 Cells Induced with 1.5 mM Sodium Butyrate for 4 Days.

PLATE I. SDS PAGE IMMUNEPRECIPITATION OF K562 CELLS INDUCED WITH SODIUM BUTYRATE AND INTERFERONS[a]

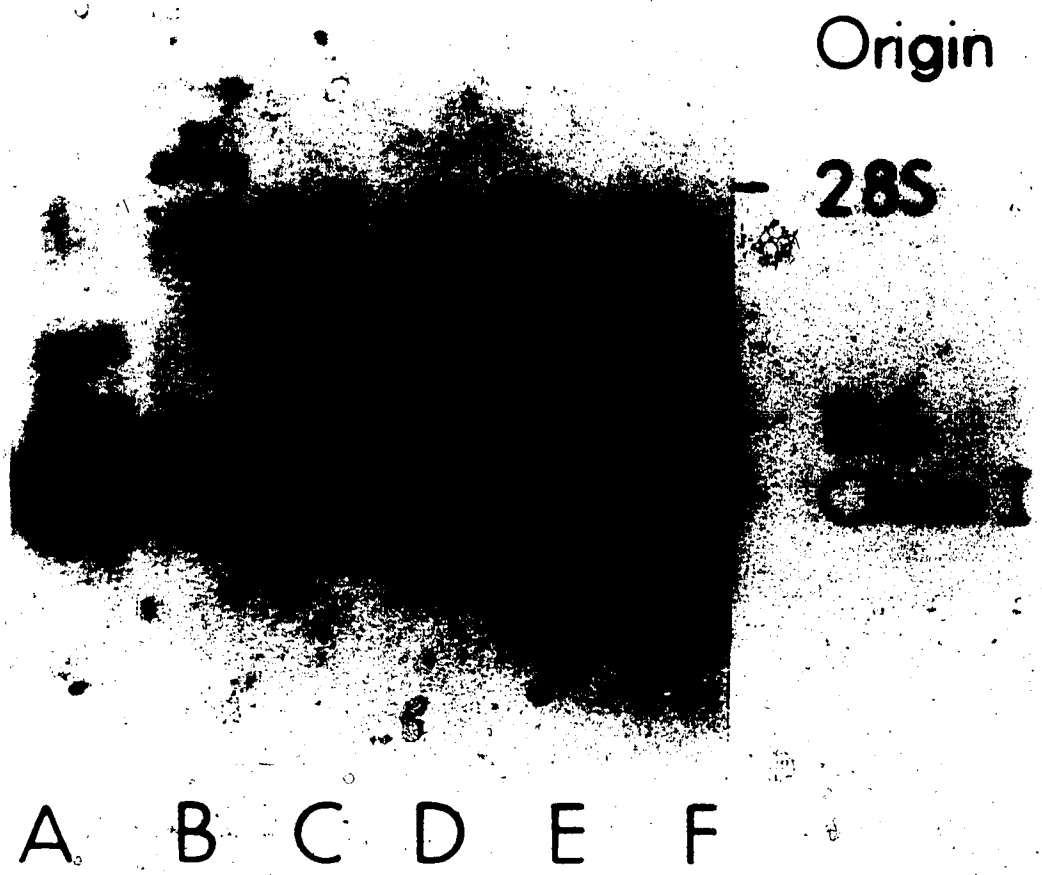


K562 induced with sodium butyrate or interferon synthesises class I MHC antigens which can be demonstrated by membrane fluorescence (Figures 3,6) and by immune precipitation of cytoplasmic and membrane antigens.

A. Untreated K562 Cells, B. Sodium Butyrate treated K562 Cells, C. Gamma Interferon treated K562 Cells, D. K562 cells treated with Sodium Butyrate and Gamma Interferon, E. Control - B Cell Line Ramos expressing MHC antigens.

[a]This work was performed by Dr. Fellous, Institut Pasteur

PLATE II. EFFECT OF INTERFERONS AND SODIUM BUTYRATE ON HLA - ABC mRNA IN K562 CELLS[a]

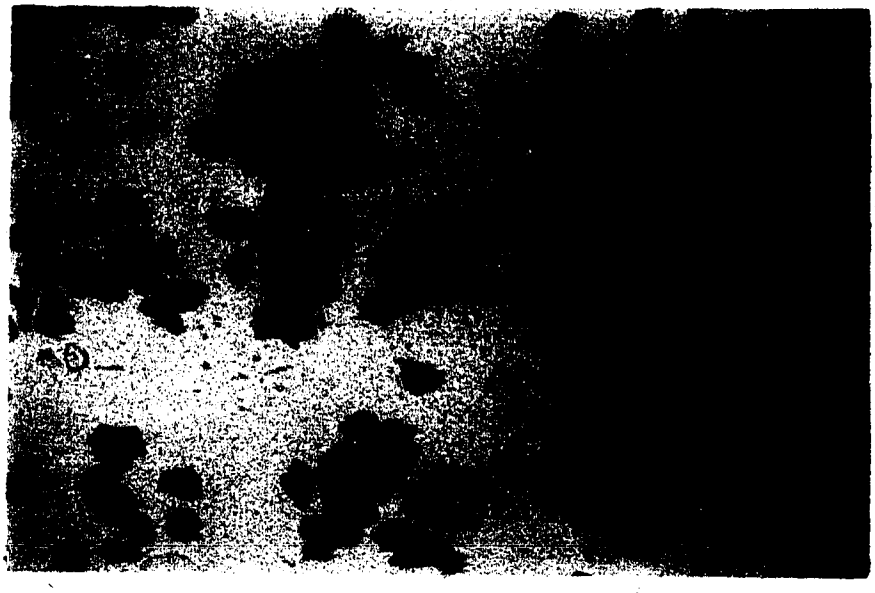


The increase of class I MHC antigens which can be detected on the cell membrane (Figure 3 and Figure 6) and in the cytoplasm (Plate I) of K562 induced with sodium butyrate and/or interferon can be attributed to an increase in class I MHC mRNA.

RNAs were extracted and analyzed with an HLA - ABC cDNA probe as described in Materials and Methods. A. K562 treated with Sodium Butyrate and Gamma Interferon, B. K562 treated with Sodium Butyrate, C. K562 treated with Gamma Interferon, D. Untreated K562, E. Untreated control culture Ramos, F. Ramos treated with Gamma Interferon

[a]This work was done by Dr. Marc Pellous, Institut Pasteur

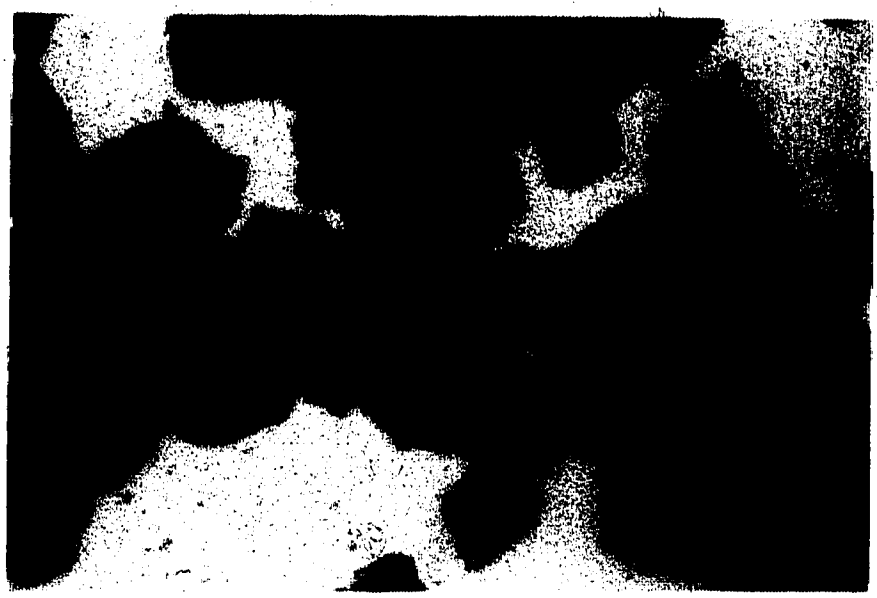
PLATE III. K562 CELLS SHOWING ALKALINE PHOSPHATASE ACTIVITY AFTER INDUCTION WITH SODIUM BUTYRATE



A) 40X Magnification of K562 Cells Induced with 1.5 mM Sodium Butyrate for 4 Days Showing Occasional Alkaline Phosphatase Positive Cells. Control Cells are Completely Negative.

B) 160X Magnification of a K562 Cell Showing Weak Alkaline Phosphatase Activity. This Culture was Induced with 1.5 mM Sodium Butyrate for 4 Days.

PLATE IV. SUDAN BLACK B STAINING OF K562 CELLS INDUCED WITH SODIUM BUTYRATE



A) 100X Magnification of Untreated K562 Cells stained with Sudan Black B

B) 100X Magnification of Sodium Butyrate Induced K562 Cells showing Positive Sudan Black B Staining. The Culture was Treated with 1.5 mM Sodium Butyrate for 4 Days

PLATE V. PERIODIC ACID SCHIFF STAINING OF K562 CELLS INDUCED WITH SODIUM BUTYRATE

A) 100X Magnification of Untreated K562 Stained with PAS

B) 100X Magnification of K562 Cells Induced for 4 Days with 1.5 mM Sodium Butyrate Showing Marked Increase of PAS Stained Glycogen.

B. INTERFERON

To further investigate the discovery that sodium butyrate could stimulate the synthesis of class I MHC antigens by K562 cells, the cells were treated with alpha, beta, and gamma interferons. All three types of interferon were able to stimulate the synthesis of HLA - ABC antigens and β2 microglobulin in K562 (Figure 6, PLATES I, II). This response could be seen within 15 hours of exposure of the cells to the interferons.

The increase of class I MHC antigens which can be detected on the cell membrane (Figures 3 and 6) and in the cytoplasm (Plate I) of K562 cells induced with sodium butyrate and/or interferon can be attributed to a specific increase in class I MHC mRNA (PLATE II).

When K562 was doubly induced with sodium butyrate and interferon, there appeared to be an additive response seen in the production of MHC Class I mRNA and in the concentration of cytoplasmic antigen, but which could not be detected on the cell membrane (data not shown). These results were particularly striking for gamma interferon.

The effect of sodium butyrate on the production of MHC class I antigens was not inhibitted by adding to the culture media an antibody to interferon (M. Fellous, personal communication).

Attempts to define the HLA phenotype of K562 induced by interferons or sodium butyrate, by standard cytotoxicity typing failed. No positive reactions were obtained with

any of the antisera. In addition, the lymphocytoxic McAbs 81H2, 83H1 and M18, which bind to K562 after sodium butyrate and interferon inductions were unable to kill the K562 cells (data not shown). However, when induced K562 cells were tested by immunefluorescence with a limited panel of human sera containing monospecific HLA antibodies, there were weak positive reactions observed with anti A2 and anti B12 (data not shown).

The interferons were slightly inhibitory to the growth of K562 (Figure 1), but they did not significantly affect the viability of the cells as measured by trypan blue dye exclusion. There were no changes in the morphology of the treated cells other than a slight decrease in cell volume.

Other than the changes described in the expression of class I antigens, alpha and beta interferon did not change the expression of any lineage specific membrane antigens during 4 days of treatment. HLA-DR antigens were not synthesised.

There was no change in hemoglobin production by K562 after 24 hours exposure to alpha or beta interferon, but after 4 days treatment, an enhancement of hemoglobinization was observed. Cultures treated with beta interferon contained 0.34 +/- a SE of 0.015 pcg of hemoglobin per cell compared to 0.18 +/- 0.012 pcg per cell in the control cultures. Alpha interferon had a similar effect on hemoglobin production with treated samples having a mean concentration of 0.32 +/- 0.014 pcg of hemoglobin per cell.

Because of extremely limited quantities of gamma

nterferon, its effects on hemoglobin production and the expression of lineage specific membrane antigens were not determined.

K562 cells treated with alpha or beta interferon stained the same as untreated cells with all the cytochemical stains.

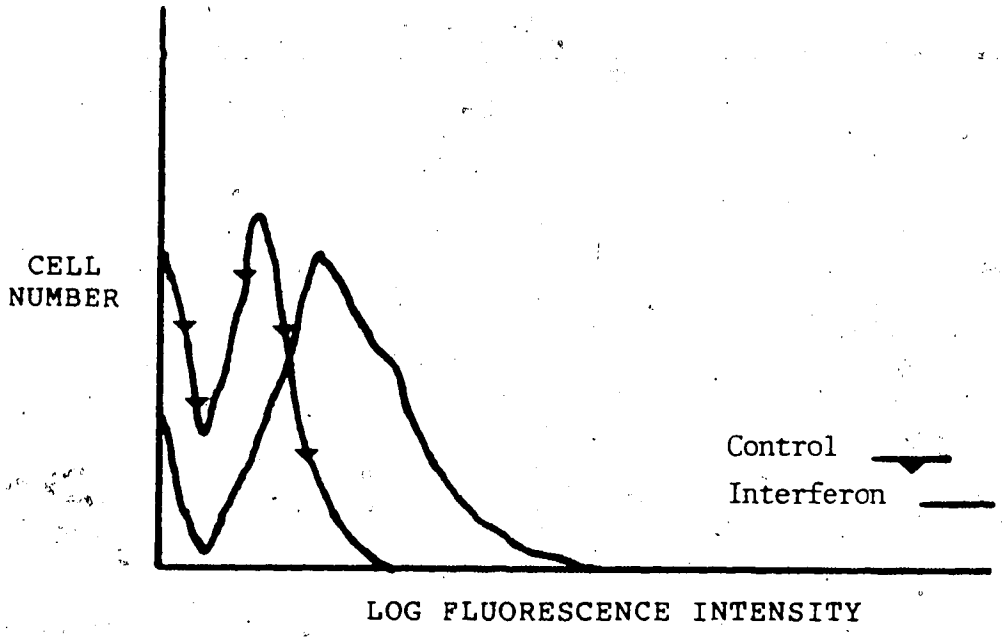


Figure 6. Reactivity of Monoclonal Antibody 83H1, anti HLA-ABC, with K562 Cells Treated with Beta Interferon for 24 Hours.

Equivelant results were observed with alpha interferon, gamma interferon produced a slightly larger increment.

C. Hemin

The exposure of K562 cells to hemin triggered significant changes in the phenotype of this cell line. As seen with sodium butyrate and interferon uM hemin was inhibitory to the cell proliferation (F but was not cytotoxic as measured by trypan blu clusion. The morphology of the induced cells resembled that of pronormoblasts with prominent nucleoli and a thin rim of deeply basophilic cytoplasm and induced cells had a more round and uniform appearance than control cells. The induced cells were slightly smaller than than the noninduced cells except for a minor population of enlarged cells which otherwise identical to the other hemin treated ce

Hemin sign cantly incr sed the production of hemoglobin by K562. After four days, treated cells contained a mean of 1.82 +/- a SE of 0.125 pcg of hemoglobin per cell compared to the control cells which contained 0.17 +/- 0.01 pcg of hemoglobin per cell. During four days of treatment, the benzidine staining of the cells reflected this response with a progressive increase of staining intensity and an increasing proportion of positive stained cells.

The induced cells remained negative with Sudan Black B stain, alkaline phosphatase and 5'-nucleotidase, and as expected, were unable to reduce NBT. The intensity of PAS staining of the cells was markedly reduced after induction

with hemin (PLATE VI).

There were significant changes in the expression of membrane antigens (Table 5). Within six hours, the expression of early myeloid markers represented by reactivity with 80H5 and 82H6 (Figure 7) began to increase. Membrane glycophorin was initially repressed and then began to increase after 24 hours of hemin exposure, reaching a maximum by 72 hours (Figure 8). Monocyte markers remained unchanged and the cells remained negative for MHC and megakaryocyte antigens (Table 5).

Induction with hemin resulted in a significant change in the number of cells in the G2/M phases of the cell cycle. After four days, 47.2% +/- a SE of 2.38 of control cells were in G1/G0, 38.5% +/- 2.26 in S, and 14.25% +/- 0.96 were in G2/M. Induced cells were distributed 43% +/- 1.87 in G1/G0, 32.75% +/- 0.89 in S and 24.2% +/- 2.02 in G2/M.

Table 5. Membrane Expression of Lineage Specific and Major Histocompatability Antigens on K562 Cells after Induction with Hemin

Membrane Antigens		Percentage of Positive Cells[a]	
		Control	Hemin
MHC ANTIGENS	McAb		
β2 Microglobulin		1 ± 0.44	1 ± 0.28
HLA - ABC Monomorphic		2 ± 0.63	2 0.75
HLA - DR Monomorphic		0	0
ERYTHROID LINEAGE			
Glycophorin A	H85	65 ± 4.91	86[b] ± 4.22
	B357	71 ± 7.27	83[b] ± 5.39
MYELOID LINEAGE			
Early Granulocytic	80H5	65 ± 4.13	86[b] ± 2.88
	82H6	41 ± 7.87	73[b] ± 8.15
	82H1	23 ± 2.56	42[b] ± 4.12
Late Granulocytic		0	0
Monocytic	MO2	0	0
	82H3	70 ± 5.70	75 ± 6.04
MEGAKARYOCYTIC LINEAGE		0	0

[a]The percentage of positive cells was determined by flow cytometry analysis. Values represent the mean ± SE of five separate experiments.

[b]The differences between the means of induced and non-induced (control) cells are significant at the .05 level for Students t test for paired data.

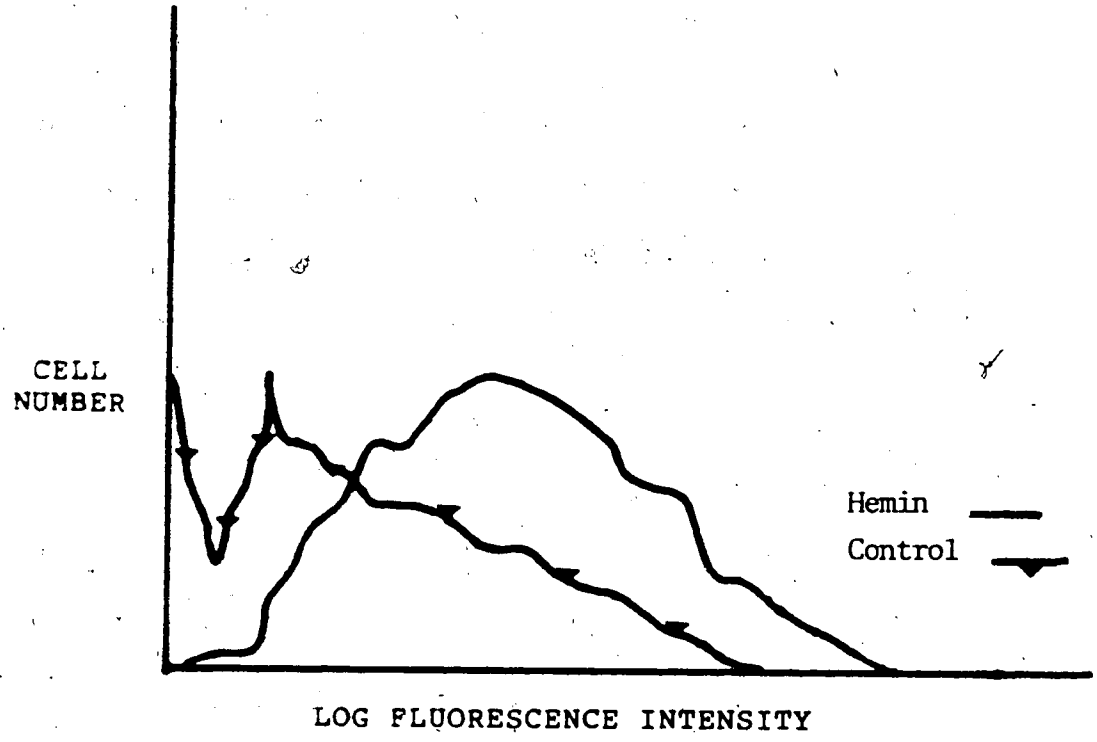


Figure 7. Reactivity of Monoclonal Antibody 82H6, anti-Granulocyte, with K562 Cells Induced with 25 uM Hemin for 4 Days.

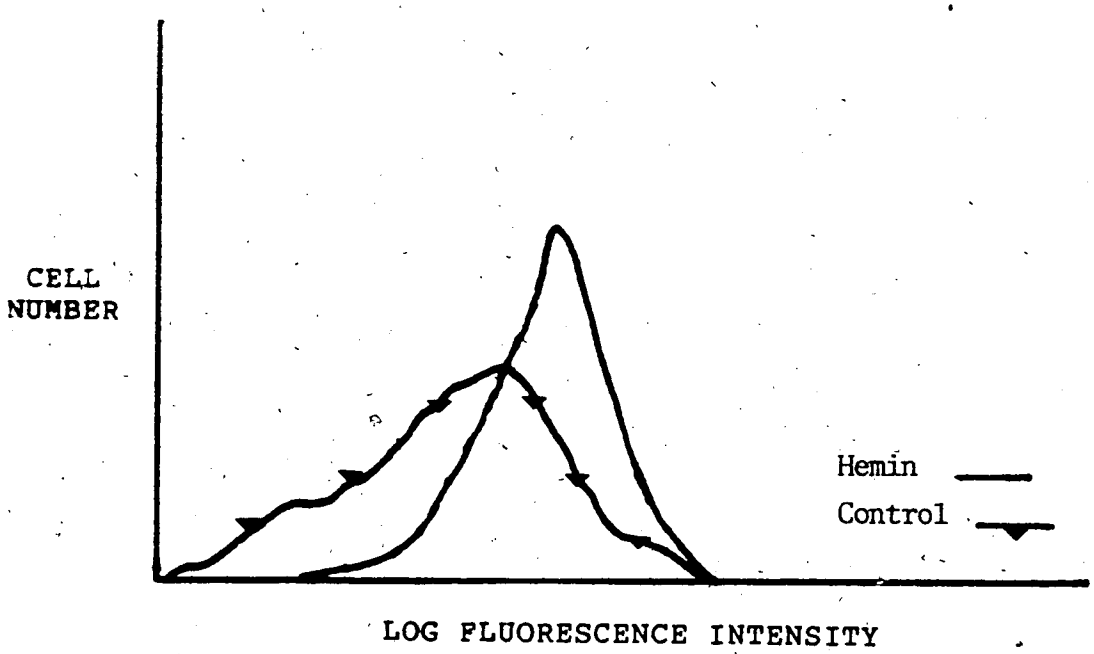
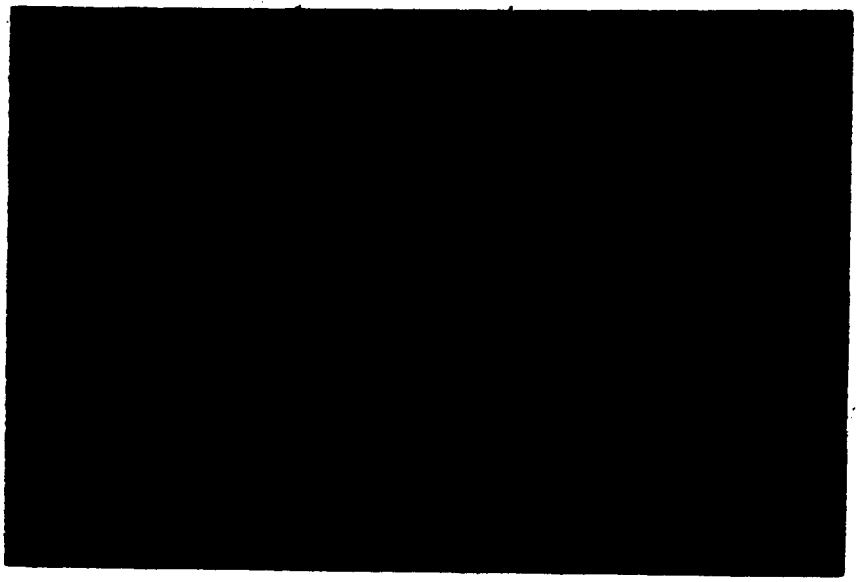
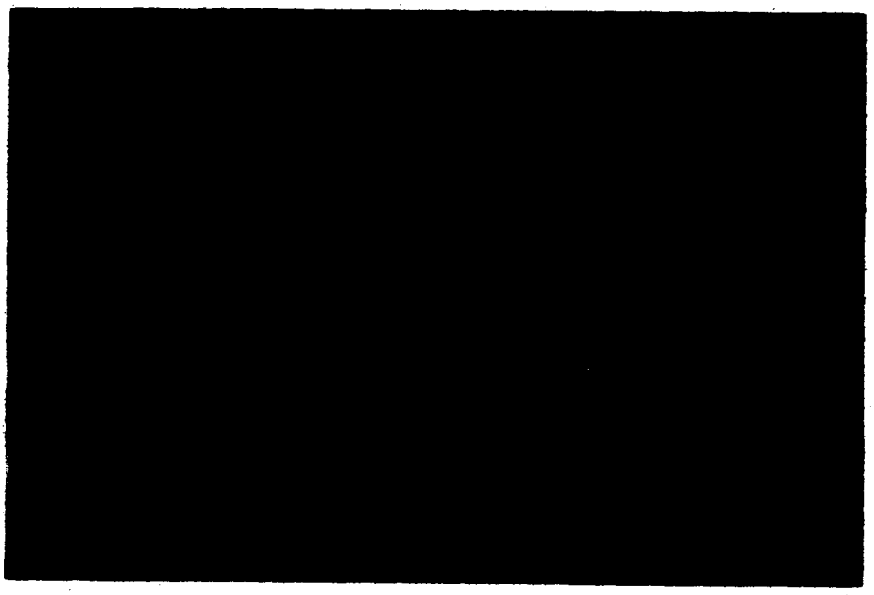


Figure 8. Reactivity of Monoclonal Antibody H85, anti-Glycophorin, with K562 Cells Induced with 25 uM Hemin for 4 Days.

PLATE VI. PERIODIC ACID SCHIFF STAINING OF K562 CELLS INDUCED WITH HEMIN



A) 100X Magnification of Untreated K562 Stained with PAS



B) 100X Magnification of K562 Cells Induced with 25 uM Hemin for 4 Days Showing a Marked Decrease of PAS Stained Material

D. Retinoic Acid.

Retinoic acid also triggered changes in the membrane expression of several lineage-specific antigens (Table 6) and in the production of hemoglobin by K562.

The erythroid features of the cell line, as measured by the membrane expression of glycophorin (Figure 9) and production of hemoglobin, were inhibited by retinoic acid, a known inducer of granulocytic differentiation in other cell lines (60). The cell hemoglobin content decreased from a mean of 0.17 +/- a SE of 0.013 pcg/cell to a mean of 0.07 +/- 0.009 pcg/cell after exposure to 1.0 uM retinoic acid for four days.

Antigens specific for early granulocyte differentiation decreased (Figure 10) but there was no change in the expression of the antigens 82H7 and 80H3 (Table 6) which are expressed later in granulocytic differentiation, or in the monocyte antigens, and the cells were unable to reduce NBT. The changes observed in glycophorin were first noted after 48 hours and reached a maximum after 96 hours, changes in 80H5 and 82H6 were not seen until 72 hours of exposure to retinoic acid.

There were no changes observed in the cytochemical profile, including those stains which are specific for granulocytic or monocytic diffentiation.

Retinoic acid, like sodium butyrate, hemin and interferon did not induce expression of megakaryocyte antigens.

The morphology of the cells was unchanged after induction except for a slight decrease in cell volume. Retinoic acid was slightly inhibitory to cell proliferation (Figure 1).

No significant changes in the distribution of cells in the cell cycle were noted after induction with retinoic acid. After four days, 51.8% +/- a SE of 2.27 of the control cells were in G1/GO, 31.7% +/- 1.16 were in S and 16.5% +/- 0.82 were in G2/M. Induced cells were distributed 48.4% +/- 1.08 in G1/GO, 33.8% +/- 2.11 in S and 17.9 +/- 1.78 in G2/M.

Table 6. Membrane Expression of Lineage Specific and Major Histocompatability Antigens on K562 Cells after Induction with Retinoic Acid

Membrane Antigens		Percentage of Positive Cells[a]	
		Control	Retinoic Acid
MHC ANTIGENS[a]	McAb		
β2 Microglobulin		2 ± 1.25	1 ± 0.66
HLA - ABC Monomorphic		2 ± 1.15	2 ± 1.25
HLA - DR Monomorphic		0	0
ERYTHROID LINEAGE			
Glycophorin A	H85	68 ± 3.43	59[b] ± 3.14
	B357	64 ± 3.62	40[b] ± 4.56
MYELOID LINEAGE			
Early Granulocytic	80H5	85 ± 4.82	68[b] ± 2.21
	82H6	59 ± 6.51	29[b] ± 3.98
	82H1	10 ± 2.48	1[b] ± 0.44
Late Granulocytic		0	0
Monocytic	MO2	0	0
	82H3	70 ± 3.06	65 ± 2.69
MEGAKARYOCYTIC LINEAGE		0	0

[a]The percentage of positive cells was determined by flow cytometry analysis. Values represent the mean ± SE of five separate experiments.

[b]The differences between the means of induced and non-induced (control) cells are significant at the .05 level for Student's t test for paired data.

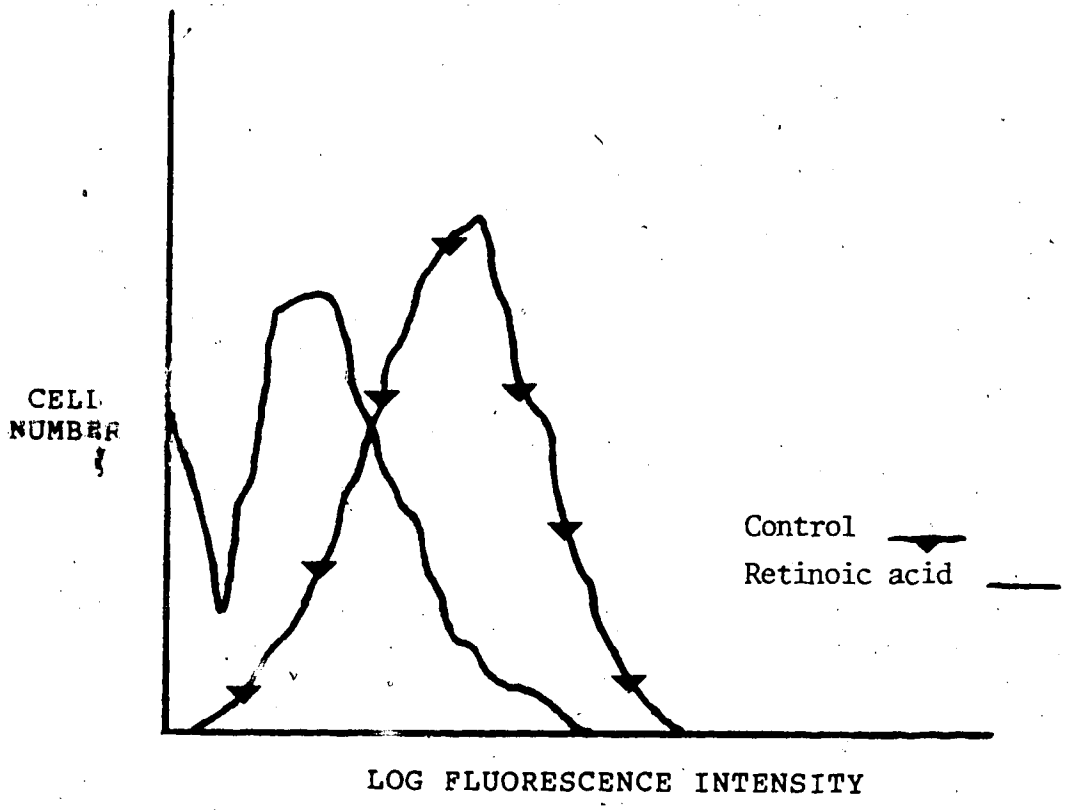


Figure 9. Reactivity of Monoclonal Antibody B357, anti-Glycophorin, with K562 Cells Induced with 1.0 uM Retinoic Acid for 4 Days.

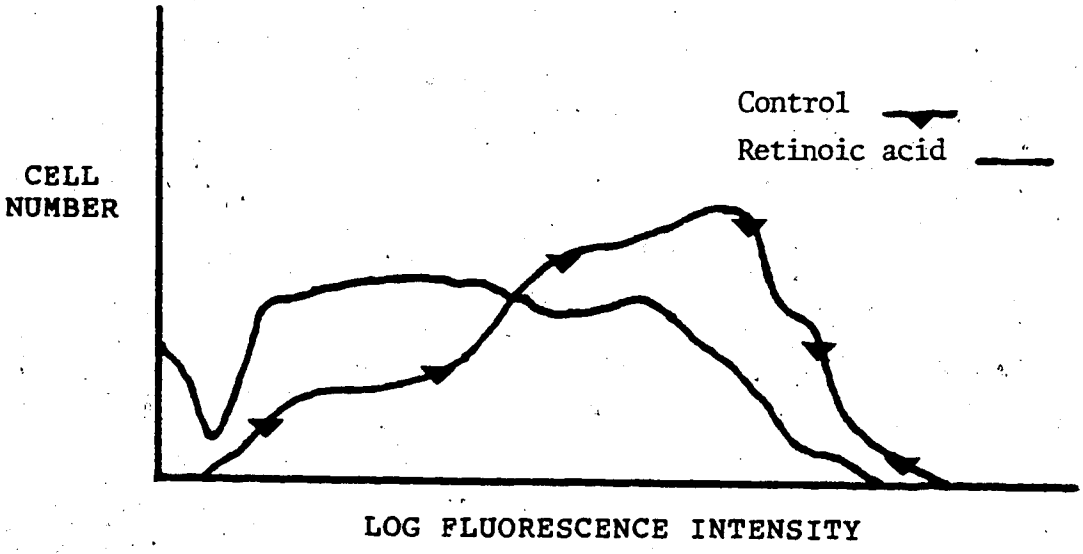


Figure 10. Reactivity of Monoclonal Antibody 80H5, anti-Granulocyte, with K562 Cells Induced with 1 uM Retinoic Acid for 4 Days.

E. Dimethyl Sulphoxide

DMSO triggered some changes in K562 which may be related to megakaryocytic differentiation.

Membrane marker analysis demonstrated the loss of early granulocytic antigens (Figure 11) and the loss of glycophorin A (Figure 12), although this was only observed with the monoclonal antibody B357 and not H85 (Table 7). There were no changes in the expression of monocyte and MHC antigens on the cell membrane (Table 7).

DMSO, like retinoic acid, also inhibited hemoglobin production by K562 cells from a mean of 0.16 +/- a SE of 0.01 pcg/cell to 0.08 +/- 0.006 pcg/cell after four days of exposure.

Exposure to DMSO resulted in a small population of cells that reacted with J15 (Figure 13), an antibody to the platelet glycoprotein IIb-IIIa. In addition, approximately 90% of the cells stained positive for 5'-nucleotidase (PLATE VII). There was also a marked reduction in PAS staining similar to that observed after hemin induction.

Again, there were no gross morphological changes observed in the induced cells, other than a reduction in cell volume.

Although there was no change observed in the concentration and distribution of alpha naphthyl acetate esterase after DMSO induction, the pattern of staining resembles that of megakaryoblasts. These cells show a localized perinuclear positivity which is moderately

sensitive to fluoride inhibition (PLATE VIII).

No significant changes in the distribution of cells in the cell cycle were observed after induction with DMSO. After four days, 52.7% +/- a SE of 3.23 of the control cells were in G1/G0, 31.1% +/- 3.12 in S and 16.2 +/- 0.41 in G2/M. Induced cells were distributed 51.1% +/- 1.78 in G0/G1, 32.2% +/- 1.55 in S and 16.7% +/- 0.8 in G2/M.

Table 7. Membrane Expression of Lineage Specific and Major Histocompatability Antigens on K562 Cells after Induction with Dimethyl Sulphoxide

Membrane Antigens	McAb	Percentage of Positive Cells[a] Control	DMSO
MHC ANTIGENS		0	0
ERYTHROID LINEAGE			
Glycophorin A	H85	63 ± 5.61	61 ± 6.01
	B357	60 ± 4.07	35[b] ± 3.89
MYELOID LINEAGE			
Early Granulocytic	80H5	79 ± 3.48	31[b] ± 3.58
	82H6	45 ± 3.69	11[b] ± 2.80
	82H1	15 ± 3.02	4[b] ± 1.36
Late Granulocytic		0	0
Monocytic	MO2	0	0
	82H3	71 ± 3.35	74 ± 2.89
MEGAKARYOCYTIC LINEAGE	VIII	0	0
	69CA	0	0
	J15	1 ± 0.55	7[b] ± 1.76

[a]The percentage of positive cells was determined by flow cyometry analysis. Values represent the mean ± SE of four separate experiments.

[b]The difference between the means of induced and non-induced (control) cells are significant at the .05 level for Student's t test for paired data.

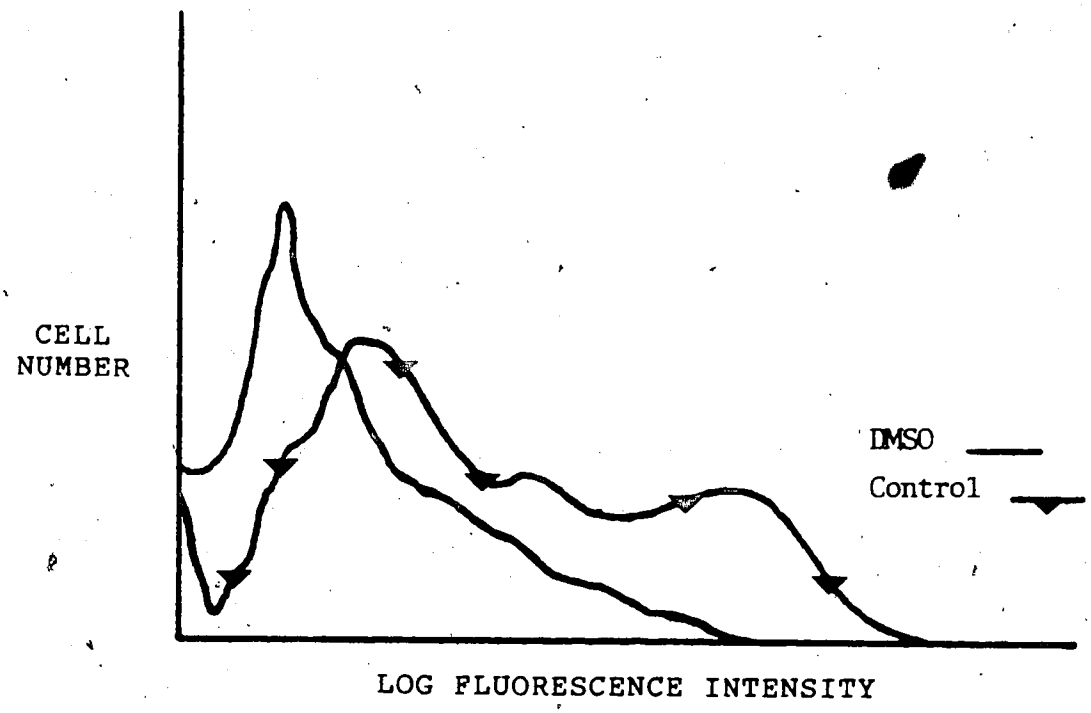


Figure 11. Reactivity of Monoclonal Antibody 80H5, anti-Granulocyte, with K562 Cells Induced with 0.175 M DMSO for 4 Days.

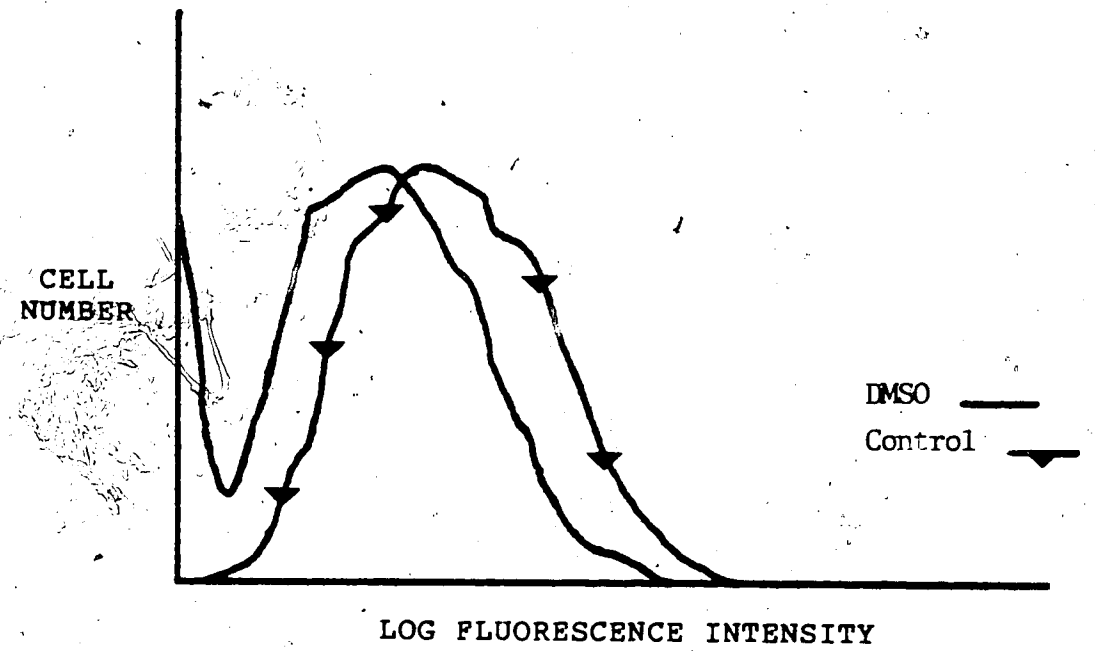


Figure 12. Reactivity of Monoclonal Antibody B357, anti-Glycophorin, with K562 Cells Induced with 0.175 M DMSO for 4 Days.

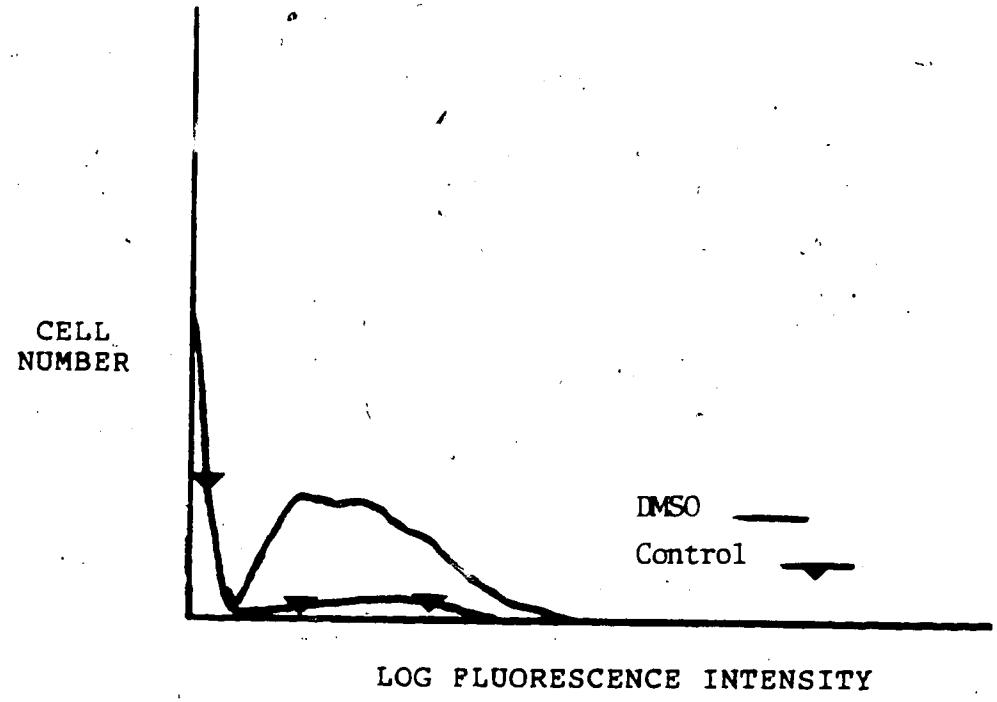


Figure 13. Reactivity of Monoclonal Antibody J15, anti-Platelet Glycoprotein IIb-IIIa, with K562 Cells Induced with 0.175 M DMSO for 4 Days.

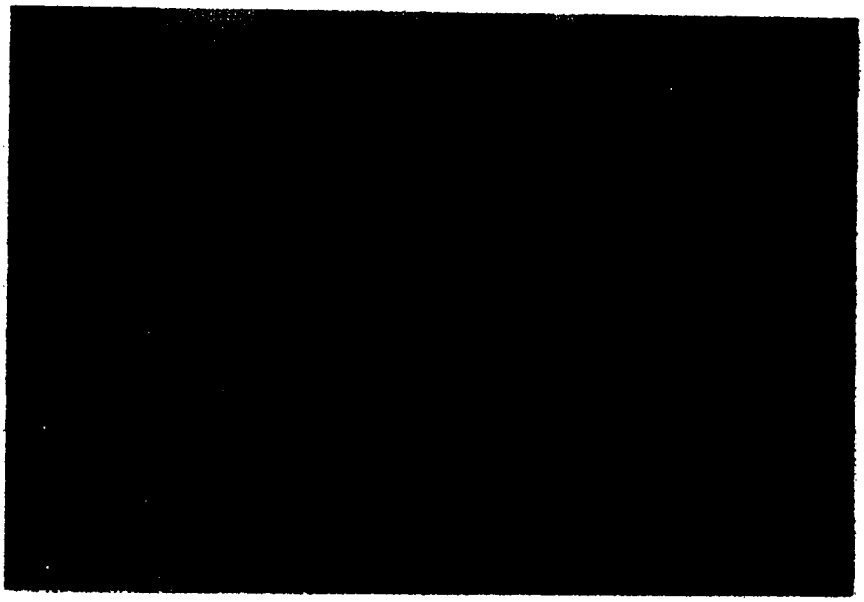
PLATE VII. DEMONSTRATION OF 5'-NUCLEOTIDASE IN K562 AFTER INDUCTION WITH DMSO

A) 100X Magnification of Untreated Cells Stained for 5'-Nucleotidase

B) 100X Magnification of K562 Cells Induced with 0.175 M DMSO for 4 Days Demonstrating the Presence of 5'-Nucleotidase

PLATE VIII. DEMONSTRATION OF ALPHA NAPTHYL ACETATE ESTERASE IN NON-INDUCED K562 CELLS

A) Alpha Napthyl Acetate Esterase Activity in K562 Cells showing Localized Positivity.



B) Sodium Fluoride Inhibition of Alpha Napthyl Acetate Esterase Activity in K562

F. PMA

PMA appeared to induce macrophage-like differentiation in K562 cells.

After induction, the cells became adherent to plastic (PLATE IX), they had a decreased nucleus to cytoplasm ratio, abundant cytoplasm with vacuoles, and a minor percentage of cells showed evidence of nuclear maturation. These cells were able to reduce NBT (PLATE X), and binding with an monocyte-specific MoAb 82H3 (Figure 14) was significantly enhanced. However, the cells were still negative with the monocyte-specific McAb MO2 and there were no changes observed with any of the cytochemical stains.

Like sodium butyrate, PMA induced the synthesis of HLA class I antigens (Figure 15) but not class II.

The erythroid phenotype of the cells was almost completely eliminated. Only a small percentage of cells continued to express glycophorin on their cell membranes (Figure 16) and hemoglobin production was almost completely eliminated. Control cells contained 0.18 pcg +/- a SE of .019 of hemoglobin per cell compared to 0.05 pcg +/- .014 in cells from the cultures treated with PMA for 24 hours.

The expression of granulocytic antigens was also weakened (Figure 17) and there was no evidence of megakaryocytic differentiation observed (Table 8).

PMA was the only inducing agent which completely inhibited cell proliferation (Figure 1) which may suggest that PMA was able to induce terminal differentiation. In

addition the phenotypic changes induced by PMA appeared to be quite stable. After removing PMA, the macrophage phenotype remained essentially unchanged for two weeks (data not shown).

As expected PMA significantly affected the distribution of cells in the phases of the cell cycle. After induction, there was a marked reduction in the number of cells synthesising DNA and an accumulation of cells in the G2 phase. After 24 hours, 41% +/- a SE of 3.87 of control cells were in G0/G1, 36.1% +/- 2.51 in S and 22.9% +/- .52 in G2/M. After induction with PMA for 24 hours, 47.7% +/- 3.04 of the cells were in G0/G1, 11.7% +/- 1.53 in S and 40.6% +/- 4.03 were in G2/M.

Table 8. Membrane Expression of Lineage Specific and Major Histcompatability Antigens on K562 Cells after Induction with 4β- Phorbol 12β Myristate 13α - Acetate

Membrane Antigens	McAb	Percentage of Positive Cells[a] Control	PMA
MHC ANTIGENS			
β2 Microglobulin		2 ± 1.22	73[b] ± 4.51
HLA - ABC Monomorphic		3 ± 1.30	76[b] ± 3.27
HLA - DR Monomorphic		0	0
ERYTHROID LINEAGE			
Glycophorin A	H85	62 ± 3.61	39[b] ± 4.35
	B357	70 ± 5.76	26[b] ± 4.56
MYELOID LINEAGE			
Early Granulocytic	80H5	84 ± 2.66	62[b] ± 3.21
	82H6	51 ± 5.20	19[b] ± 4.72
	82H1	20 ± 4.02	3[b] ± 0.90
Late Granulocytic		0	0
Monocytic	MO2	0	0
	82H3	68 ± 3.57	95[b] ± 1.85
MEGAKARYOCYTIC LINEAGE		0	0

[a]The percentage of positive cells was determined by flow cytometry analysis. Values represent the mean ± SE of four separate experiments.

[b]The differences between the means of induced and non-induced (control) cells are significant at the .05 level for Students t test for paired data.

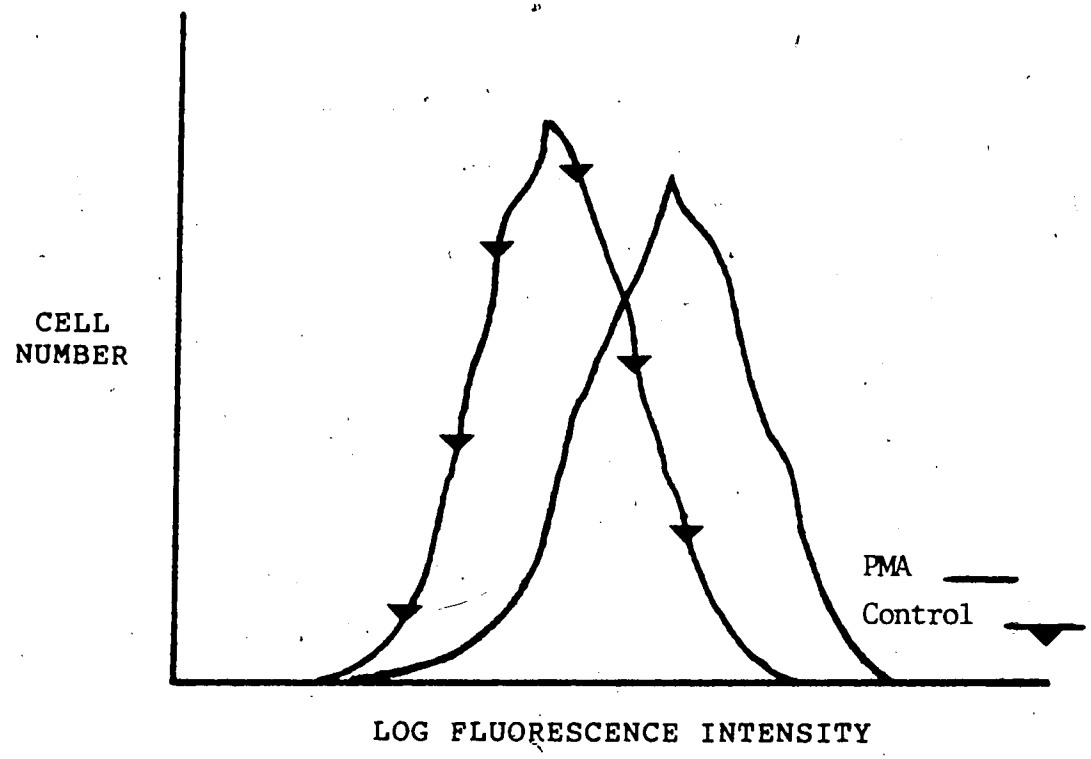


Figure 14. Reactivity of Monoclonal Antibody 82H3, anti-Monocyte, with K562 Cells Induced with 0.5 nM PMA for 24 Hours

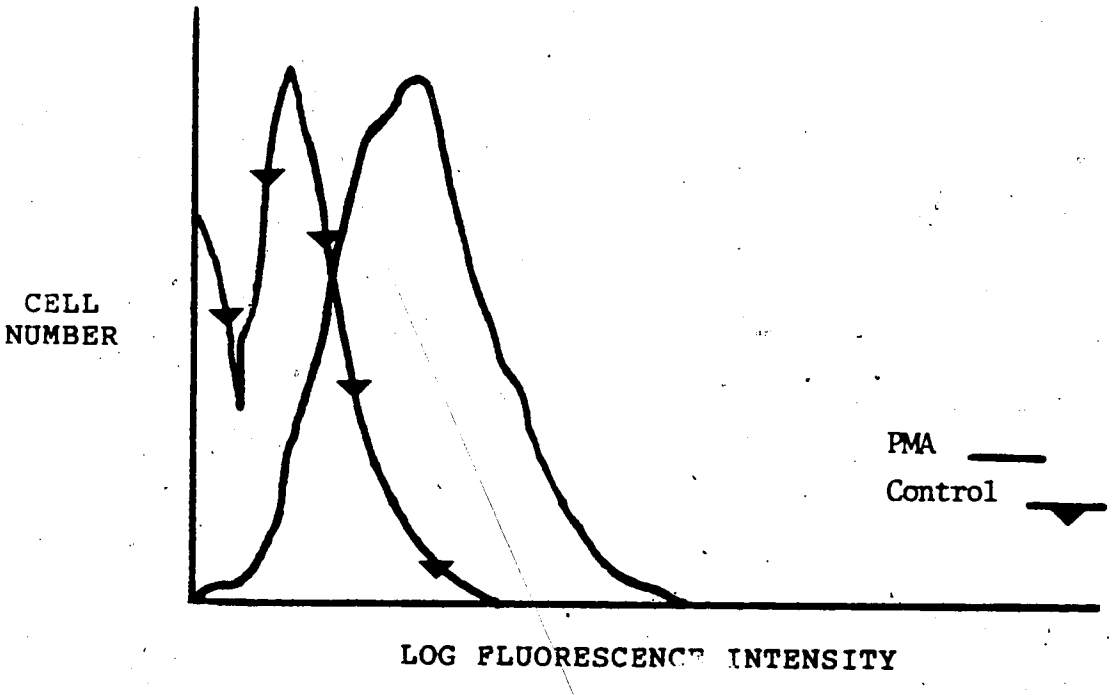


Figure 15. Reactivity of Monoclonal Antibody 81H2, anti-HLA-ABC, with K562 Cells Induced with 0.5 nM PMA for 24 Hours

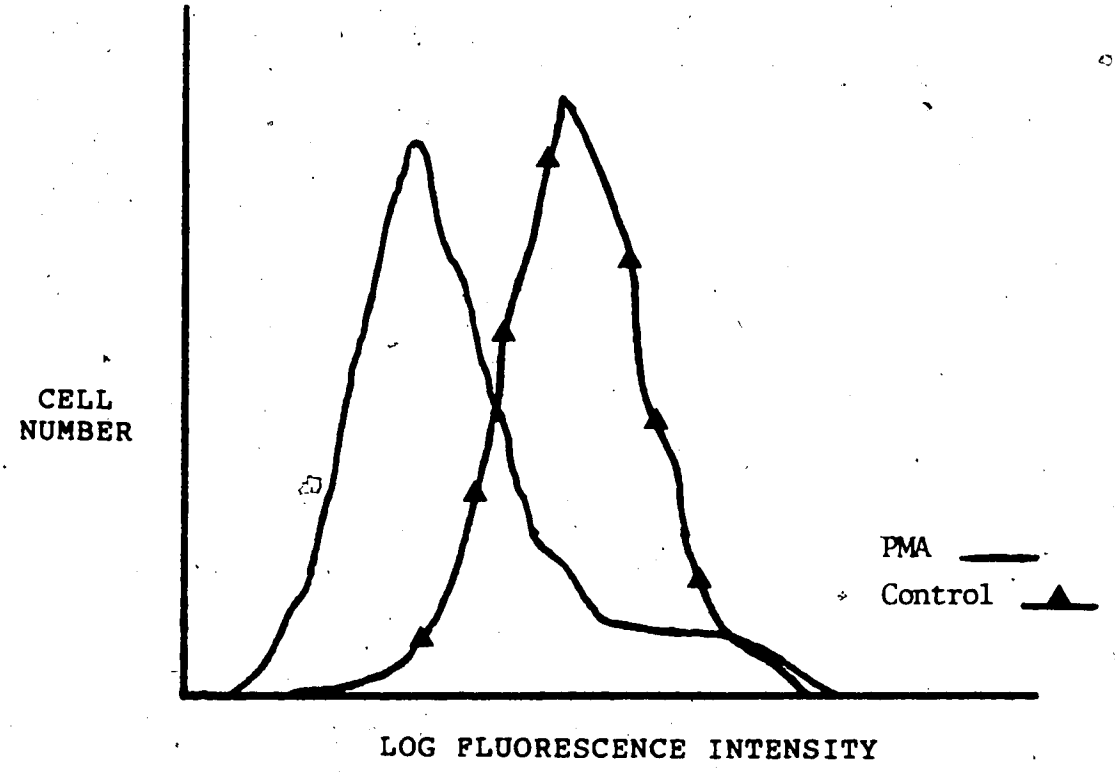


Figure 16. Reactivity of H85, anti-Glycophorin, with K562 Cells Induced with 0.5 nM PMA for 24 Hours.

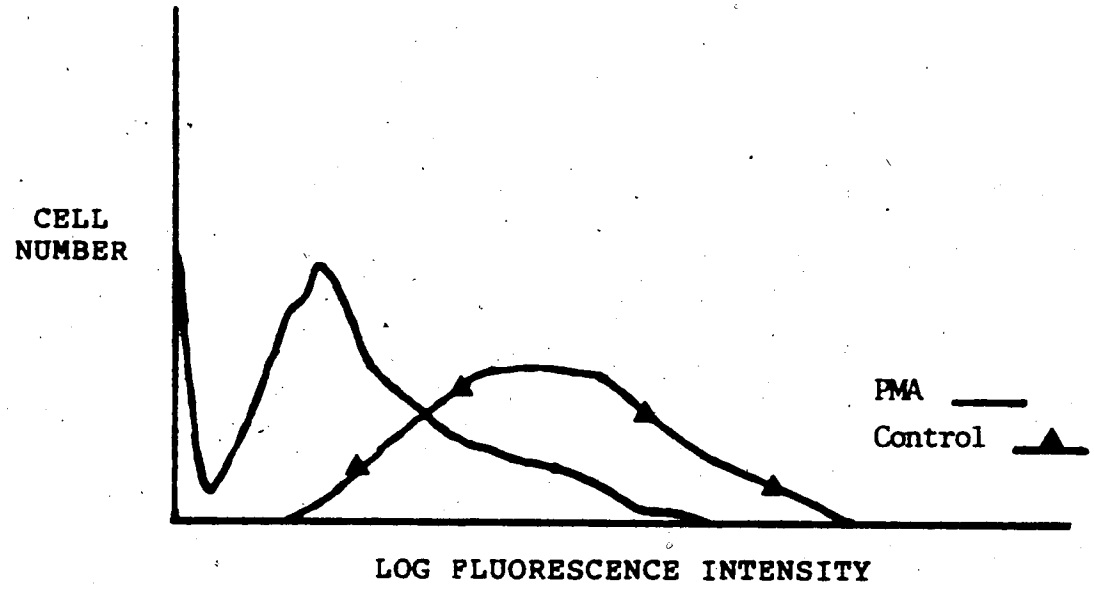
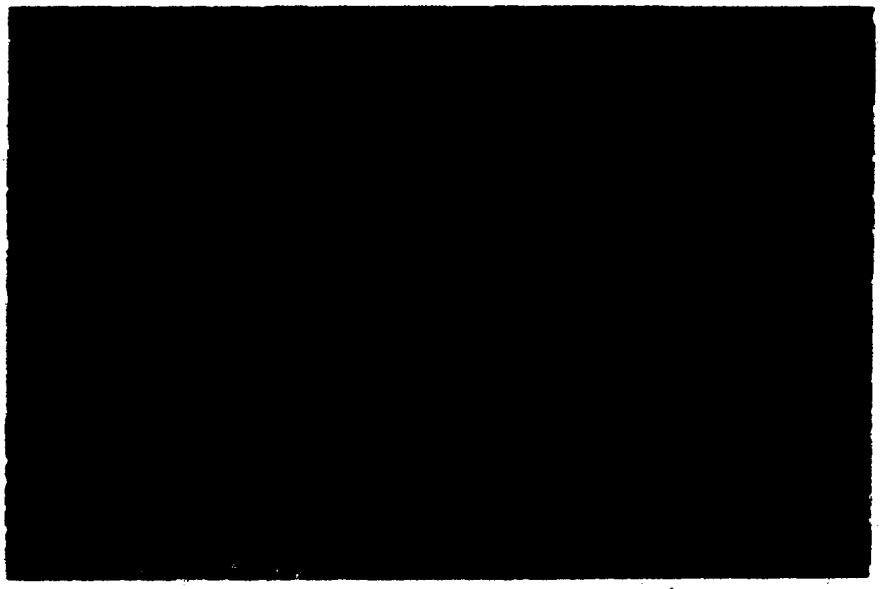
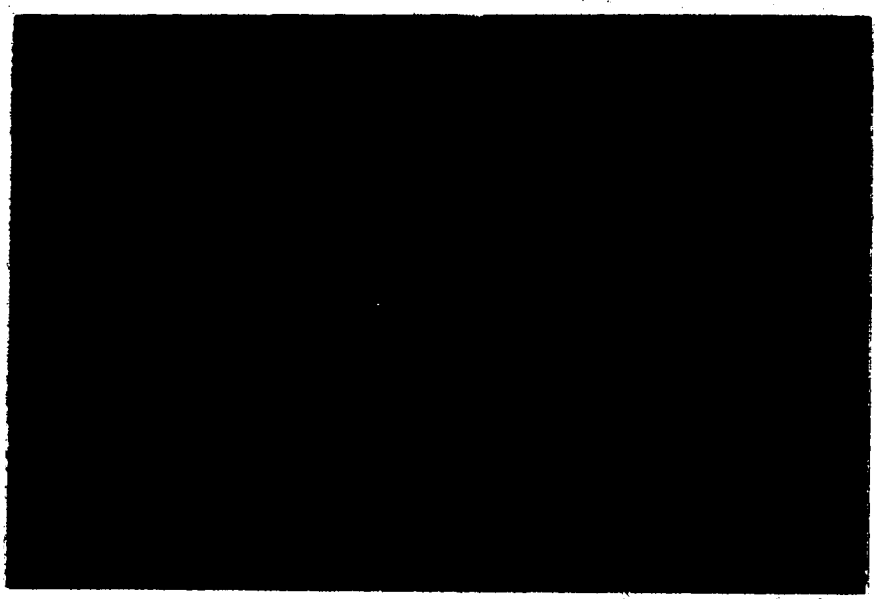


Figure 17. Reactivity of Monoclonal Antibody 82H6, anti-Granulocyte, with K562 Cells Induced with 0.5 nM PMA for 24 Hours

PLATE IX. MORPHOLOGY OF K562 CELLS IN CULTURE

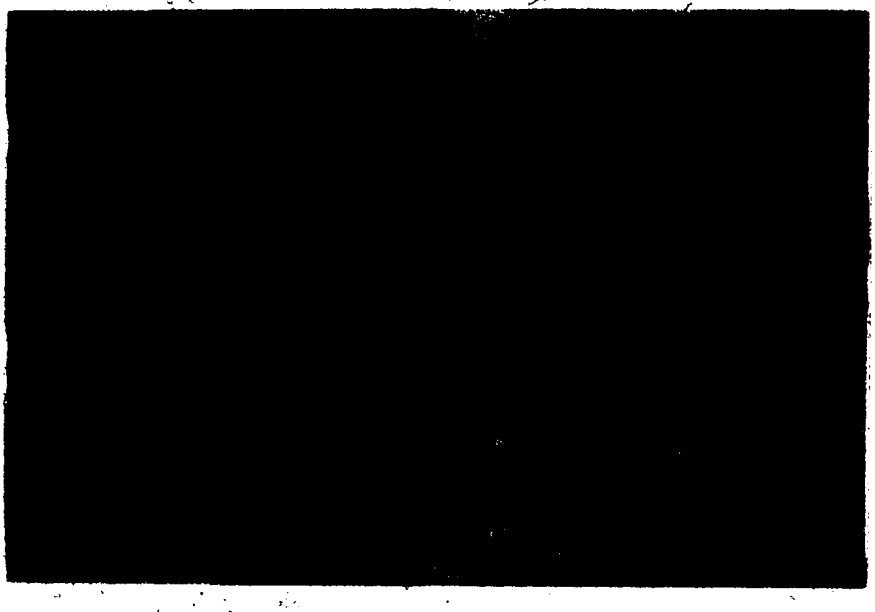


A) Constitutive K562 Cells Growing as a Single Cell Suspension

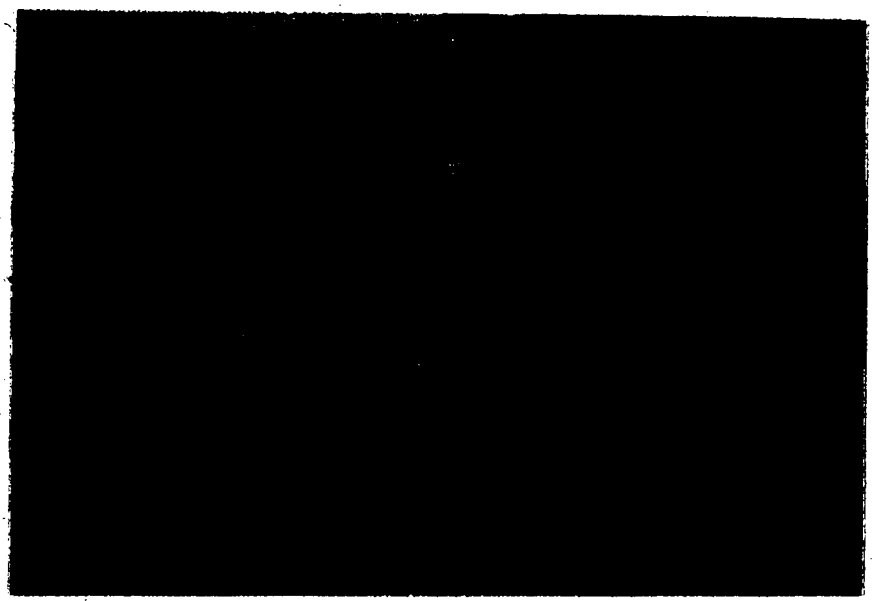


B) K562 Cells showing Adherence to Plastic After Induction with 0.5 nM PMA for 24 Hours

PLATE X. DEMONSTRATION OF NBT REDUCTION BY K562



A) 100X Magnification of Untreated K562 Cells with Negative NBT Reduction



B) 100X Magnification of K562 Induced with 0.5 nM PMA for 24 Hours, Showing Positive NBT Reduction

Table 9. Summary of Responses of K562 to Inducers of Differentiation

PARAMETER		INDUCING AGENT					
	Control	Na. Butyrate	Interferon[c]	Hemin	Retinoic Acid	DMSO	PMA
A. MEMBRANE MARKER							
Class I MHC	-	+	+	-	-	-	+
Class II MHC	-	-	-	-	-	-	-
Glycophorin	+	increase[b]	NC	increase	decrease	decrease[e]	decrease
Early granulocyte	+	decrease	NC	increase	decrease	decrease	decrease
Late granulocyte	-	-	-	-	-	-	-
Monocyte[a]	+	NC	NC	NC	NC	NC	increase
Megakaryocyte	-	-	-	-	-	+[f]	-
B. CYTOCHEMISTRY							
PAS	+	increase	NC	decrease	NC	decrease	NC
Sudan Black B	-	+	-	-	-	-	-
ANAE	+	NC	NC	NC	NC	NC	NC
Alk. Phosphatase	-	+	-	-	-	-	-
5' Nucleotidase	-	-	-	-	-	+	-
C. FUNCTIONAL ASSAYS							
Hemoglobin Prod.	+	increase	increase[d]	increase	decrease	decrease	decrease
NBT Reduction	-	-	-	-	-	-	+
D. CELL MORPHOLOGY	primative blasts	pseudopods, pronounced golgi apparatus	NC	pronormoblast	NC	NC	adherent to plastic

[a] Refers to 82H3 only

[b] only H85 increases

[c] includes alpha and beta

[d] after 4 days exposure

[e] only B357 decreases

[f] refers to J15

NC - no change from control cells

G. Two Parameter Analysis of Cell Cycle and Expression of Lineage Specific Membrane Antigens.

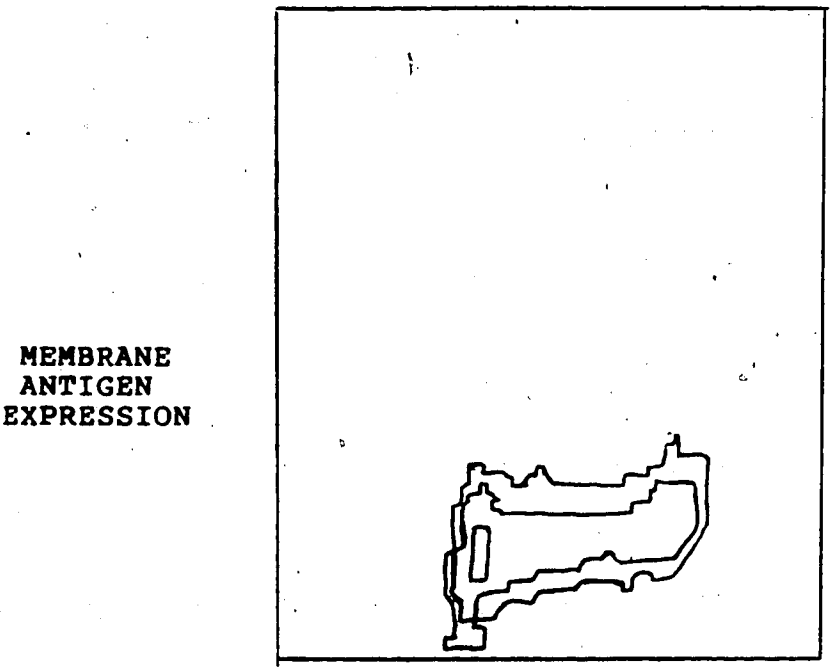


Figure 18. Correlation of the Binding of Monoclonal Antibody 82H3, anti-Monocyte, with DNA Distribution in Constitutive K562 Cells.

The results show no clear cut correlation between DNA distribution and the binding of 82H3. The G1/G0 phase includes a spectrum of cells from negative to strongly positive for this antigen. Cells in S phase were moderately positive for this antigen. Cells in G2 and M phases of the cell cycle included a population of cells strongly binding 82H3 as well as cells moderately positive for this antigen.

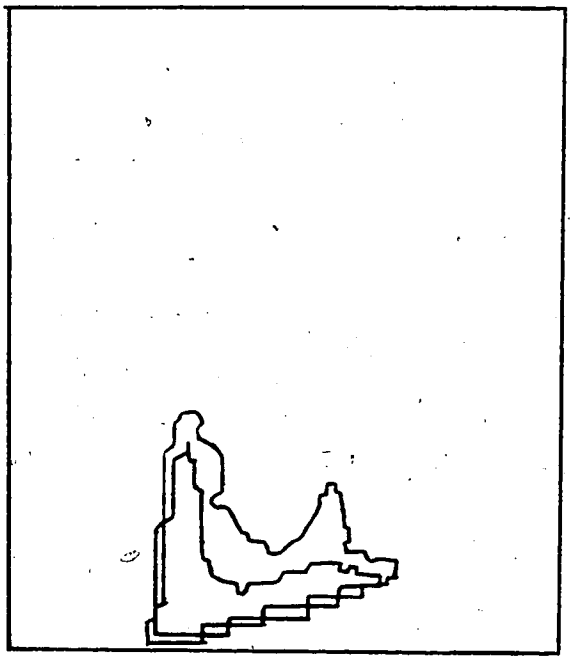


Figure 19. Correlation of the Binding of Monoclonal Antibody 82H6, anti-Granulocyte, with DNA Distribution in Constitutive K562 Cells.

The results again show that the expression of the 82H6 antigen is highly variable in cells in G0/G1. Most of the highly fluorescent cells are in G0/G1. The cells in S phase are much more uniform in the expression of this antigen and the majority of cells appear to be less fluorescent than the G1/G0 cells. As with 82H3 (Figure 18), cells in G2/M express slightly more antigen than the cells in S phase.

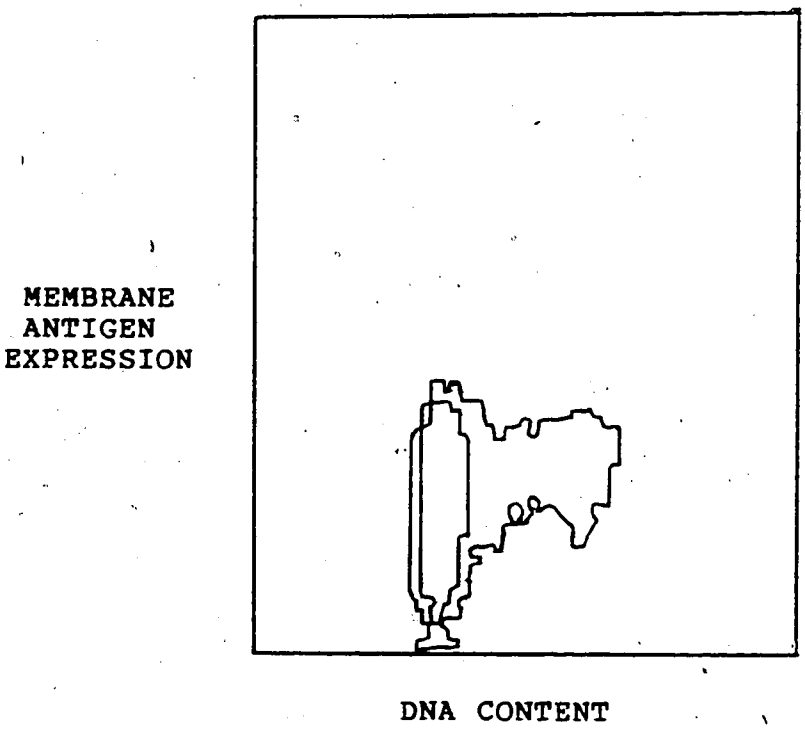


Figure 20. Correlation of the Binding of Monoclonal Antibody 80H5, anti-Granulocyte, with DNA Distribution in Constitutive K562 Cells.

Cells in G0/G1 express varying amounts of this antigen. Cells showing the strongest antigen expression are in G0/G1. Cells in S and G2 phases have a more uniform phenotype. Most of the cells in S and G2 appear to be strongly positive, with the G2/M cells showing slightly more variability.

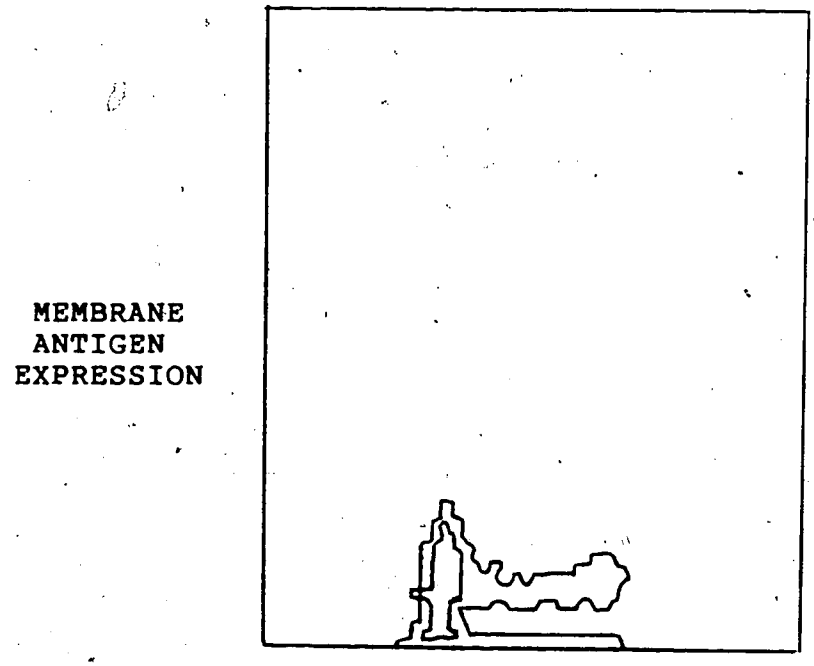


Figure 21. Correlation of the Binding of Monoclonal Antibody B357, anti-Glycophorin, with DNA Distribution in Constituve K562 Cells.

The relationship between cell cycle and membrane expression of glycophorin is similar to that observed with 82H6 (Figure 19), with G1/G0 cells showing a variable staining pattern with anti glycophorin. Cells showing the strongest antigen expression are in G1. The majority of cells in S and G2/M appear to be moderately fluorescent.

CHAPTER V. DISCUSSION

A. K562 - A Pluripotential Stem Cell Line

The results of this study indicated that K562 is a pluripotential stem cell line that has the capacity to express markers and features characteristic of granulocytic, monocytic, erythrocytic and megakaryocytic differentiation when exposed to external stimuli. Each inducing agent was able to trigger a unique, reproducible response, which, in some cases, appeared similar to differentiation observed in normal hematopoietic cells.

Sodium butyrate triggered profound changes in the morphology, expression of membrane antigens, and cytochemistry of K562 cells. As previously reported, hemoglobin production and membrane glycophorin expression was increased (113,114), although the latter change was only detected by one of two monoclonal antibodies specific for glycophorin. At the same time there appeared to be granulocytic differentiation occurring. This statement is supportd by the observations of alkaline phosphatase activity, synthesis of sudanophilic granules and increased PAS positivity.

After sodium butyrate induction, there was a marked decrease in the binding of the McAbs, 80H5, 82H1 and 82H6, which are specific for antigens expressed early in myeloid differentiation. Similar observations were made by Breton - Gorius et al. (121). This can be interpreted in two

ways. These results are consistant with erythroid differentiation where one would expect to see a loss of myeloid features. The alternative explanation is that granulocytic differentiation also occured. The antigens recognized by the McAbs 80H5, 82H1 and 82H6 are expressed on myeloid progenitors and are progressively lost during early granulocyte differentiation (127,128,129). A similar phenomenon has also been observed during granulocytic differentiation of the promyelocytic cell line HL60 (personal communication, Dr. P. Mannoni). Therefore the expression of these antigens appears to be inversely correlated with granulocytic differentiation.
There was no evidence of terminal myeloid differentiation after sodium butyrate induction however, because there was no binding of the monoclonal antibodies 80H3 and 82H7, which recognize late myeloid markers, and the cells were unable to reduce NBT.

Although it has been previously reported that sodium butyrate induces megakaryocytic differentiation in K562 (120), no evidence of this was observed in this investigation. It should be pointed out however that the facilities were not available to detect platelet peroxidase, one of the most sensitive markers of early megakaryocytic differentiation.

Hemin-induced erythroid differentiation in K562 cells was accompanied by increased expression of glycophorin and a large increase in hemoglobin production, confirming results seen in other studies (115,116,117). However, at

the same time an increase in the expression of myeloid specific antigens was observed. A similar observation was made by Breton-Gorius et al. in an independent study (121). The significance of increased expression of myeloid-specific antigens during erythroid differentiation is unknown, but it is consistent with the hypothesis that the expression of these antigens may be inversely correlated with granulocytic differentiation and would therefore be expected to increase during erythroid differentiation.

Hemin also stimulated a marked decrease in PAS staining. A positive PAS reaction is one of the hallmarks of erythroleukemia. However there has been surprisingly little work published correlating PAS staining with differentiation. Attempts to correlate PAS staining of erythroblasts with stages of differentiation would be difficult because no corresponding normal control exists. Normal erythroid progenitors do not stain with PAS (22), but cytoplasmic glycogen can be detected by using electron microscopy. The concentration of cytoplasmic glycogen decreases as erythroid differentiation proceeds (150). These results suggest that the decrease of glycogen observed after hemin induction of K562 indicated a return to more normal erythroid metabolism or that erythroid differentiation had occurred. The observations that PAS staining of leukemic erythroblasts is variable from patient to patient, and often changes during the course of the disease (22), may suggest that erythroid

differentiation is arrested at different stages in these patients and the stage of arrest may change during the course of the disease.

The response of K562 cells to hemin and sodium butyrate is clearly different (Table 6), although both agents have been reported to induce erythroid differentiation in K562 cells (113,114,115). For example, sodium butyrate induced a decreased expression of early myeloid-specific antigens, while induction with hemin induced an increase of these antigens. Sodium butyrate triggered the synthesis of Class I MHC antigens, whereas hemin did not. After induction with sodium butyrate, PAS staining of K562 cells was enhanced, whereas after hemin induction, there was a marked decrease of PAS staining.

Retinoic acid diminished the erythroid phenotype of K562 cells by decreasing hemoglobin production and the membrane expression of glycophorin. There was also a significant loss of the early granulocyte markers. If the premise that the expression of the antigens binding 80H5 is inversely correlated with granulocytic differentiation is correct, there may be some early granulocytic differentiation occuring in K562 cells in response to retinoic acid. There was no evidence of terminal granulocytic differentiation such as synthesis of antigens that appear in mature granulocytes or by the development of functions characteristic of this lineage.

This study also provided evidence from cytochemical staining and analysis of membrane antigens that DMSO can

induce megakaryocytic differentiation in K562 cells while repressing the erythroid phenotype. Previous published studies have reported no response of K562 to DMSO (112) but they were primarily investigating the capacity of DMSO to promote erythroid differentiation as it does in the Friend erythroleukemia line. The induction of megakaryocytic differentiation by DMSO in an "erythroid" cell line has a precedent in the observations by Breton-Gorius of the response of the erythroleukemia cell line, HEL, to DMSO (personal communication).

Constitutive K562 cells exhibit some features of megakaryocytic differentiation such as the expression of platelet glycoproteins on the cell membrane (120,121,122). This study demonstrated a further example. The staining pattern of alpha napthyl acetate esterase resembled that of megakaryoblasts. Megakaryoblasts and K562 cells showed a localized perinuclear positivity which is moderately sensitive to fluoride inhibition (Plate VIII). Andersson describes blasts similar to this in a patient with erythroid blast crisis of CML (151).

The megakaryoblastic pattern of alpha napthyl acetate esterase in K562 cells and in blast cells from erythroid blast crisis of CML, the demonstration of megakaryocytic differentiation in "erythroid" cell lines, and the identification of a bipotent erythroid - megakaryocytic progenitor in mouse bone marrow (35) suggests a possible link in the differentiation pathways of erythroid and

megakaryocytic lineages.

The results of this study should be confirmed with ultrastructural studies to identify platelet peroxidase in the induced cells. The McAb, C17, which binds to an antigen expressed earlier in megakaryocytic differentiation than factor VIII or the IIb-IIIa complex (152), should also be used to confirm the results of this investigation.

As it does in many other cell lines (61,62,64,69), PMA appeared to promote monocytic differentiation in K562 cells. The evidence supporting this statement includes morphological changes such as adherence to plastic, membrane marker analysis showing enhancement of expression of a monocyte specific antigen, and functional assays demonstrating the ability to reduce NBT. It should be pointed out that the induced cells were unable to bind the McAb, MO2, one of the classical markers of the monocyte lineage, and that there was no apparent change in the distribution or concentration of alpha napthyl acetate esterase in the induced cells.

Monocytic differentiation has not previously been reported for K562 cells and several investigators have reported that cells blocked at the stage of a myeloblast such as KG-1 and K562 are completely resistant to PMA-induced differentiation and that the proliferation of these cells is not affected by phorbol diesters (153).

An important observation about PMA induced macophage differentiation in K562 and other cell lines is that it does not require DNA synthesis (154,155). This violates

one of the central dogmas about the requirement of mitosis for differentiation. In recent years this relationship between proliferation and differentiation in hematopoietic cells has been the subject of considerable controversy.

Another important observation is that PMA, like sodium butyrate, induced the synthesis of Class I MHC antigens but not that of HLA-DR antigens in K562 cells. Synthesis of Class I MHC antigens was not observed after induction with hemin, retinoic acid, or DMSO.

After replacing PMA-containing medium with fresh medium, the macrophage phenotype remained essentially unchanged for two weeks, suggesting that PMA was able to induce the majority of cells to undergo terminal differentiation. In contrast, cultures treated with the other inducing agents reverted to their original phenotype after approximately 5 days in fresh media. This indicated that not all the cells were responsive to the inducing agent so that the proliferation of the unresponsive cells in the fresh media may have masked the more differentiated cells. To test this hypothesis one could use flow cytometry analysis of membrane markers to identify those cells that appear to be the most and the least differentiated, and then examine the stability of their phenotypes and their proliferative capacity in fresh media. Alternatively, the reversion of the induced cultures back to their pre-induced phenotype may suggest that differentiation is reversible.

With the exception of sodium butyrate, each of the inducing agents was able to trigger some degree of differentiation while repressing the expression of features of the other lineages. This is consistent with the concept that differentiation involves a gradual narrowing of the spectrum of gene products that a cell can produce. It is also consistent with Till's hypothesis (104) of a multiply marked pluripotential hematopoietic stem cell that, through differentiation, gradually loses features of all lineages except one. However, after treatment with inducing agents, the K562 cells expressed some antigens, for example, platelet specific markers, that were not present in the untreated cells. This response is consistent with McCulloch's view of pluripotent stem cells which synthesise and express antigens characteristic of specific lineages during differentiation (92).

Sodium butyrate appears to be unique in that it induced features of erythroid, myeloid and possibly megakaryocytic (120) lineages simultaneously in K562 cells. Cell sorting experiments and multi-labelling techniques could be used to determine if markers of all these lineages appear on the same cell, or if there is evidence of lineage fidelity with increased differentiation.

Does K562 represent a model of normal hematopoietic differentiation ? Within the erythroid system, the K562 cell line exhibits many of the features of early erythroid differentiation, including hemoglobin production, globin mRNA accumulation, glycophorin and spectrin production and

erythroid-like patterns of enzymes of heme metabolism. The phenotype of K562 cells is compatible with embryonic or fetal erythroblasts. For example, the lactose dehydrogenase (LDH) isoenzymes are produced in a fetal pattern, the i antigen is present and only embryonic and fetal hemoglobins are produced (116). Like normal embryonic cells, K562 does not express the globin gene although the gene is present and intact (117). However, erythroid maturation is not normal in these cells. They lack ABO and Rh antigens and carbonic anhydrase (112), and to this point, terminal erythroid differentiation has not been demonstrated in these cells.

Similarly, K562 cells synthesise megakaryocytic markers, including platelet peroxidase, 5'-nucleotidase and platelet glycoproteins; IIa, III, and IIIa. Other early specific markers, such as platelet glycoproteins Ib and IV, and Factor VIII related antigen, have not yet been detected in K562 cells and terminal differentiation has not yet been demonstrated.

Another major discrepancy with normal hematopoietic differentiation is that K562 cells lack the DR antigens. Recent studies have shown that pluripotential progenitors express HLA-DR (A. Keating, Toronto, Communication at the AACHT meeting, Chicago, November 1983, and Dr. Maraninchi personal communication).

In conventional lineage representations of hematopoietic differentiation, K562 cells may represent the

leukemic counterpart of the stem cell which gives rise to cells of erythroid, myeloid, and megakaryocytic lineages. If lymphocytic differentiation can be demonstrated for this cell line, K562 cells could represent the leukemic form of a more primitive stem cell.

In summary, the K562 cell line appears to be a useful model for limited stages of hematopoietic differentiation, including embryonic erythropoiesis and early stages of megakaryocytic differentiation. The study of both of these events is hampered by insufficient quantities of cells from normal donors. K562 cells cannot be considered representative of normal pluripotential progenitors because of their inability to complete differentiation in any lineage.

K562 cells appear to be a valid model of leukemic pluripotential stem cells - particularly those observed in CML blast crisis. Morphologically, CML blasts and K562 cells are similar; both frequently have karyotypic abnormalities and show evidence of lineage infidelity. Blasts from patients in CML blast crisis, with similar binding patterns of monoclonal antibodies and cytochemical staining profiles to K562 cells have been observed (98,151).

Measurement of membrane antigens with specific antibodies, and particularly monoclonal antibodies, to monitor hematopoietic differentiation is a relatively new technique. Within the past five years literature in this area has expanded tremendously. The function and

physiological significance of cell surface antigens defined by the vast majority of the antibodies available remains unknown. An important research project is to correlate the modulation of membrane markers that can be observed during differentiation with a particular function. An example is the presence of transferrin receptors on the membrane of erythroid precursors. Their function is to incorporate iron from the plasma into cells for use by mitochondria for the synthesis of heme. Other examples of membrane receptors, detected by monoclonal antibodies, that have specific functions include; T cell receptors, T4 or T8 antigens, and receptors for interleukin 2 (156). In terms of differentiation, the goal of such research would be to determine if the changes observed in membrane antigen expression can be attributed to the activation or inactivation of genes involved in regulation of hematopoiesis.

It is also possible that some differentiation-associated and lineage-specific membrane antigens may not have functional significance within the hematopoietic system. For example, the antigens of the ABO system do not appear to be important to the development and function of erythrocytes.

The use of chemical agents and physiological materials to stimulate hematopoietic differentiation in leukemic cell lines is now a common technique. However, one of the problems which has not been addressed is whether the

changes that can be measured, particularly in the expression of membrane antigens, are artifacts resulting from perturbing the growth pattern of the cell line. The answer is not obvious. Within this study, this problem was investigated by using flow cytometry analysis of DNA distribution to analyze the effects of the inducing agents on the growth cycle of K562 cells. In addition, two parameter analyses of membrane antigen expression and DNA distribution on untreated K562 cells was done. The results show that some of the inducers did affect the distribution of cells in the various phases of the cell cycle. It is not possible to say from this study whether cellular differentiation influenced growth kinetics or whether changes in growth kinetics were due to a non-specific action of the inducing agent.

The two parameter analyses revealed a complex relationship between cell cycle and membrane antigen expression. It seems unlikely that the changes observed in the cell cycle of the induced cells would be sufficient to account for the modulation of membrane antigen expression observed in this study. The coincident observations of morphological, cytochemical and functional maturation that are similar to events observed in leukemic and normal hematopoietic differentiation also suggest that the changes observed in membrane markers in this study are not artifacts.

B. Applications of Inducers of Differentiation to Leukemia Therapy

Clinical trials investigating applications of differentiation inducing agents to treatment of well-differentiated leukemias and pre-leukemic conditions are now underway in several centers. Preliminary studies indicate that retinoic acid may be clinically effective for inducing terminal differentiation in patients with promyelocytic leukemia (69). This study suggests that cancer therapy based on induction of differentiation may also be applicable to patients with relatively undifferentiated leukemias. Although terminal differentiation was never achieved in this study, with the possible exception of PMA induction, proliferation rates of treated cultures were reduced and, in some cases, cells acquired functions characteristic of specific lineages.

In a recent study (157), K562 cells, which were resistant to the cytotoxic action of vincristine, were still able to undergo differentiation when induced with hemin or sodium butyrate. Patients who are refractory to conventional chemotherapy may benefit from this approach to therapy.

It has also been recently suggested that patients with a preleukemic condition may also be responsive to therapy with differentiation inducers (69). Current data suggest that preleukemic cells can mature in vitro, and that, while their differentiation is abnormal, it is closer to normal

than observed in AML cells. Currently there is no effective therapy for these patients.

Another approach worth exploring is the use of inducing agents to sensitize leukemic cells to cytotoxic drugs. It is interesting to note that many of the cytotoxic drugs currently in use are able to induce differentiation of several leukemic cell lines (69,140).

One of the problems that has been suggested in treating cancer by promoting differentiation, is that karyotypic abnormalities present in many tumours may prevent normal differentiation. It has been pointed out that chromosomal abnormalities may be compatible with normal differentiation (9).

This study also provided additional evidence supporting the role of monoclonal antibodies and the 5'-nucleotidase stain, in conjunction with conventional cytochemistry, for classifying poorly differentiated leukemias. The 5' nucleotidase stain, which has not previously been used as a tool for investigating differentiation in cell lines, was used in this study as a cytochemical marker of megakaryocytic differentiation. The results indicated that this stain may be useful in an analogous way to the acetylcholinesterase stain in murine systems (158). It may also provide a less expensive substitute for ultrastructural studies used in diagnosing megakaryoblastic leukemia and for studying megakaryocytic differentiation.

C. The Relationship between Expression of MHC Antigens, Malignancy, and Differentiation

One of the most exciting results of this investigation was that K562 cells express class I MHC antigens under certain conditions. There is growing evidence that MHC antigens play a role in cell differentiation, including hematopoiesis and in the immune response to malignancy (159,160,161,162,163).

After induction with sodium butyrate or PMA, K562 cells expressed class I MHC antigens. This was confirmed for sodium butyrate treated cells by immune precipitation and by identification of Class I MHC mRNA in experiments done by Marc Fellous and Frederic Rosa at the Institut Pasteur in Paris. These results are unique for several reasons. K562 cells have been described as lacking Class I and II MHC antigens (112,116,164). This is the first report of expression of HLA-ABC and β2 microglobulin in K562 cells. Secondly, although interferon enhances the expression of MHC antigens in several cell types (165,166), this is the first report that a substance other than a lymphokine can trigger the synthesis of these antigens.

To further investigate the induction of class I antigens, K562 cells were treated with alpha, beta, and gamma interferons. K562 cells were able to sythesize Class I antigens under these conditions as well, but as was seen in similar experiments with sodium butyrate, none of the interferons stimulated the synthesis of HLA-DR antigens.

It should be pointed out that since the preliminary experiments, there have been passages of K562 in which weak binding of the McAbs 81H2 and M18 (anti-HLA class I) was observed in uninduced cultures. There are several possible explanations for this phenomenon. In the literature, cytotoxicity assays have been the primary test for membrane expression of class I antigens on K562 cells. This study indicates that this is an inappropriate test with K562 cells because of their anticomplementary properties. This was demonstrated by the observation that the McAbs 9H1, 83H1, and 81H2, which are lymphocytotoxic and which bind to K562 after sodium butyrate or interferon induction, were unable to kill K562 cells in a cytotoxicity assay. Therefore, it is possible that some K562 cells do transiently express HLA-ABC antigens and β2 microglobulin when cultured for long periods of time. This may be attributed to clonal evolution, or to the spontaneous induction of discrete stages of differentiation. Alternatively, the sporadic expression of these antigens in K562 cells may be an artifact. The McAbs specific for class I antigens may be cross reacting with other antigens unrelated to HLA that are transiently expressed on the cell membrane. For example, the W6/32 McAb specific for a monomorphic human HLA antigen has been shown to cross react with a mouse tumor associated antigen (167).

The mechanisms of induction of Class I antigens by sodium butyrate and interferon appear to be different. The evidence supporting this is that there appears to be an

additive relationship as measured by production of mRNA and cytoplasmic antigen in K562 after double induction with sodium butyrate followed by interferon. These results were particularly striking for gamma interferon (Plates I,II). The observation that sodium butyrate decreased the expression of class I MHC antigens on normal T lymphocytes (Figure 4), and on the cell lines HEL and HL60 (data not shown), while interferon enhanced antigen expression (Figure 4), also corroborated the hypothesis that sodium butyrate and interferon act by different mechanisms.

To try and explain the relationship between these compounds, it was postulated that sodium butyrate may be acting by increasing the number of receptors for interferon on the cell membrane of K562, or by stimulating the production of interferon by K562. In experiments done by M. Fellous and F. Rosa, (personal communication) attempts to block the action of sodium butyrate failed, when an antibody to interferon was added to the culture media. This suggests that sodium butyrate does not influence the production of, or response to, interferon by K562 cells.

Using immune fluorescence, the K562 cells induced with interferon or sodium butyrate were typed and found to express A2 and B12 antigens. Because the HLA phenotype of the woman from whom K562 originated is unknown, it is not possible to say whether this typing reflects her phenotype. It would be desirable to type the induced cells with a larger panel of HLA antisera to see whether a complete

typing could be obtained, or whether her cells would react to most antisera, which is characteristic of many leukemic blasts.

Although the involvement of class I MHC antigens in the regulation of cell interactions in the immune system is well established, the widespread distribution of these antigens in the body suggest that they may have a wider role in regulating cell interactions.

Within the hematopoietic system, class I and II MHC antigens are expressed in all cell lineages at some point, with modulation occurring during differentiation. Class II antigens are lost during early stages of development of all blood cells except for B lymphocytes and monocytes. Mature erythrocytes and perhaps platelets lack both class I and II MHC antigens. As well as quantitative differences (159) between cell lineages, there also appear to be qualitative differences. A recent paper reports that the expression of HLA-DR antigens on lymphoid cells differs from those on myeloid cells (160). The authors suggest this lineage-restricted variation provides the basis for tissue specific recognition signals required for cell interactions and differentiation.

The variable effects that interferons have on differentiation in many cell lines may be attributed to their profound effects on the expression of MHC antigens in these cells.

In this study, the expression of class I MHC antigens in K562 cells after treatment with well known inducing

agents may not be a coincidence. The two agents, sodium butyrate and PMA, which triggered the most profound changes in K562 cells and were the most effective inhibitors of cell proliferation, were also able to induce the synthesis of HLA-ABC antigens and β2 microglobulin in K562. It is difficult to equate the synthesis of class I MHC antigens by induced K562 to events in normal hematopoiesis because it is not known whether primitive pluripotential stem cells express these antigens.

To further explore the relationship between differentiation and expression of class I antigens in this model system, K562 cells could be treated with interferon to stimulate MHC antigen production prior to induction with retinoic acid, DMSO or hemin. HL60 has been shown to be more responsive to inducing agents under these conditions (74), but this cell line constitutively expresses class I MHC antigens.

In a recent paper that explores the relationship between expression of class I MHC antigens and malignancy, the authors report that there is often a substantial decrease or virtual absence of these antigens in a wide variety of tumours (161,162). There is also a clear inverse relationship between the expression of MHC class I antigens and the degree of malignancy in some tumors (163).

Because neoplasms appear to be disorders of cell differentiation, these observations further implicate the involvement of the MHC antigens in the regulation of cell

differentiation.

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