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ASSESSMENT OF ERYTHROCYTIC AND GRANULOCYTIC COLONY FORMATION IN AN *IN VIVO* PLASMA CLOT DIFFUSION CHAMBER CULTURE SYSTEM

By

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A Dissertation Submitted to the Graduate Faculty in Biology in Partial Fulfillment For the Degree of Doctor of Philosophy, The City University of New York.

1978

SECTION XII - FORM L

Howard Steinberg

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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TABLE OF CONTENTS: PAGE

PAGE

ACKNOWLEDGEMENTS

It is with sincere appreciation and respect that I acknowledge Dr. Eugene S. Handler's contribution toward my intellectual growth and development as a research scientist. While encouraging independence, his constant availability to discuss data, share insights and provide concrete and supportive criticism were critical to this development.

I also wish to express my gratitude to Dr. Evelyn E. Handler for her guidance and support.

I am truly indebted to Betty Semel for her excellent technical assistance, important advice and suggestions.

To my parents, Alex and Esther, and parents-in-law, Michael and Hilda, for their concern, support and love.

Finally, I wish to dedicate this thesis to my wife Shaindy and son Jeffrey Aaron for their unwavering love, encouragement, understanding and tolerance and for truly adding substance and meaning to my life.

ABSTRACT:

Suspensions of normal rat bone marrow cells seeded into a plasma clot diffusion chamber (PCDC) developed into erythrocytic and granulocytic colonies *in vivo*. Chambers implanted into the peritoneal cavity of normal hosts showed erythrocytic colony (CFU-E) numbers reaching an initial peak on day 2, declining on days 3 to 5, and increasing in a secondary growth phase on day 7. Day 2 colonies were evenly dispersed in the clot matrix; day 7 colonies were grouped into discrete areas defined as bursts (BFU-E). The secondary growth phase on day 7 corresponds with an increase in the number of bursts. Granulocytic colony numbers (CFU-C) reached a peak on day 4 and gradually declined through day 7. Cells in various stages of differentiation can be detected in both colony types. Both erythroid and myeloid colonies appear clonal since the numbers observed are proportional to the number of bone marrow cells seeded into the chamber. Host animals treated with phenylhydrazine induced a marked increase in erythroid colony numbers and size on days 2 and 7, and a decrease in granulocyte colony formation. Host rats treated with endotoxin suppressed erythroid colonies while

increasing granulocytic colony size. Preincubation of normal bone marrow with 0.25U erythropoietin/ml cells prior to PCDC seeding and implantation into normal host animals, resulted in peak erythroid colony formation on day 1 of culture. The numbers of CFU-E increased to twice normal while day 7 BFU-E were not observed. Granulocytic colonies were significantly depressed. Normal bone marrow cells seeded into PCDC cultures and implanted into day 0, 4 and 6 posttransplanted leukemic animals showed a significant ($P < 0.0005$) depression in granulocytic colony formation of between 25% and 65% with a mean of 35%. The size of the developing colonies were reduced to under 100 cells, but the maturation of the cells within the colonies appeared unaffected. Erythroid colonies were depressed in the early stages of the leukemia, stimulated to 150% of control between days 7 and 8 of the leukemia, and were inhibited to 50% to 65% of control in the later stages of the disease. Burst formation was also inhibited in the late leukemic stages. Since the normal bone marrow cells were compartmentalized within the PCDC, the suppression of erythroid and myeloid progenitor cell proliferation and differentiation was directly due to the release of inhibitory substances from

leukemic cells. Suppression of colony formation was, therefore, not the result of a dilutional or "crowding out" effect by the expanding leukemic cell population. The results suggest that, with the progression of the leukemia, imbalances in the serum levels of inhibitory substances are established resulting in either a direct effect on progenitor cell development or the loss of progenitor cell responsiveness to their particular stimulatory regulators (e.g. colony stimulating factor and erythropoietin). Fluctuations in normal bone marrow erythroid and myeloid colony growth in some leukemic animals reflect the influence of a variety of changing physiological parameters during the course of the leukemia on the growth and development of normal hematopoietic elements.

INTRODUCTION:

Methods are presently available for the quantitative assessment of hematopoietic cells (Till and McCulloch, 1961; Bradley and Metcalf, 1966; Pluznik and Sachs, 1965; Robinson and Pike, 1970; Axelrad et al., 1973; McLeod et al., 1974). These procedures depend upon the formation of observable, discrete colonies of progeny cells whose numbers and morphological characteristics can be readily determined. The development of these cloning techniques for the culture of bone marrow cells has played an important role in the understanding of the developmental architecture of the hematopoietic system and the factors and mechanisms regulating the production of mature erythrocytic and granulocytic cells. The formation of functionally mature hematopoietic cells involves a "three tier system of interlinked cell populations" consisting of the pluripotential and committed stem cell compartments and the histologically identifiable maturing "end" cells (Lajtha and Schofield, 1974; Lajtha, 1975; 1976). Since these maturing "end" cells have no self-renewal properties (Lajtha, 1976), the formation of new mature blood cell elements, to replace dying cell populations or

compensate for increased physiological demands (e.g. anemia, neutropenia), is derived from the differentiation of the less differentiated pluripotential and committed stem cell compartments. Depending upon the physiological demands of the organism (e.g. neutropenia, anemia), the pluripotential stem cell will differentiate either into the erythroid, myeloid or thrombocyte lines forming stem cells committed to a single direction of development (Till and McCulloch, 1961). Committed stem cells give rise to histologically recognizable maturing erythroid, myeloid and megakaryoid cells which then complete maturation to form functionally mature elements. Both the pluripotential and committed stem cells possess the capacity for self-renewal, thus, they keep their own pool size constant, and concomitantly differentiate into maturing elements. The pluripotential stem cell has been assayed in vivo using the spleen colony method. Colonies of erythrocytic, granulocytic and megakaryocytic cells form in the spleens of lethally irradiated (Till and McCulloch, 1961) and genetically anemic [W/W^v] (McCulloch et al., 1964) mice injected with a suspension of normal bone marrow cells. These colonies are derived from a single pluripotential CFU-S (Becker, 1963; Wu et al., 1967; Chen and Schooley, 1968;

Trentin et al., 1969). The factors controlling pluripotential stem cell differentiation may involve both short range cell-cell interactions encountered in a hematopoietic inductive microenvironment [HIM] (Trentin, 1976; Tavassoli, 1975) and/or long range chemical signals acting through specific feedback loops (Breivik et al., 1971; Boyum et al., 1972b; Tyler et al., 1972; Breivik and Chervenick, 1973). Committed precursors of the granulocytic (CFU-C) and erythrocytic (BFU-E, CFU-E) lines have been assayed in vitro in agar colony (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966) and plasma clot (Axelrad et al., 1973; McLeod et al., 1974) culture systems. While the differentiation of both myeloid and erythroid stem cells may partially depend upon cell-cell interactions (Haskill et al., 1972), it is apparent from in vitro studies that both erythropoiesis and granulopoiesis are under the control of stimulatory and inhibitory chamical regulator substances found circulating within the animals [e.g. colony stimulating factor, erythropoietin, chalones inhibitors etc.] (Pluznik and Sachs, 1965; Axelrad et al., 1973; Rytomaa, 1976). These regulators act as chemical signals operating through specific positive and negative feedback loops that influence the differentiation of the progenitor stem cell compartments.

The fine interplay between these physiological regulators may indeed be responsible for the maintenance of constant numbers of mature elements in the peripheral blood and the adjustment to these numbers during times of increased demand (e.g. infection, anemia). Imbalances in the physiological levels of these stimulatory and inhibitory regulators may represent a part of the pathogenesis of myelodysplastic disorders and leukemia (Handler et al., 1974). A detailed understanding of the role of these regulators may lead to an understanding of these disorders. Toward this end, the use of various model systems of leukemia (Handler and Handler, 1970b) have contributed to the knowledge of normal regulatory mechanisms controlling hematopoiesis. Since leukemia represents a lesion in the normal pattern of differentiation, its study serves as a useful tool to develop a concept of normal control mechanisms. Therefore, through the comparative study of the regulatory events in normal and leukemic animals the mechanisms involved in hemic cell differentiation can be further elucidated.

While contributing important information on the hematopoietic system and its regulation, the in vitro and

in vivo cloning techniques have limitations. *In vitro* cultures reduce the known variables by isolating a specific system in a petri plate. However, conditions for colony growth are not always physiological. Therefore, physiological changes occuring within normal and hematopoietically perturbated animals (e.g. anemia, neutropenia, leukemia) can only be inferred by results obtained from non-physiological culturing of bone marrow, assaying for specific serum activity or specific cellular interactions in these animals. Colony formation in the spleen retains the physiological environment but isolation of transplanted bone marrow for analysis is difficult. The recent use of the diffusion chamber (DC) to compartmentalize hematopoietic elements has enabled progenitor cells to grow in a more physiological environment (Benestad, 1970; Boyum and Borgstrom, 1970). In this system, a suspension of bone marrow cells is compartmentalized within a chamber consisting of two millipore filters (0.45μm or 0.22μm porosity) glued to a lucite ring. The chamber is then sealed and surgically implanted within the peritoneal cavity of a recipient host animal. Growth of bone marrow cells within the diffusion chamber thereby occurs within a more physiological environment and is dependent upon the diffusion

of physiological levels of gases, nutrients and specific serum regulators. Perturbations induced within the host animals can influence diffusion chamber cell growth in a manner that more closely approximates conditions in situ. In addition, cellular isolation within the DC eliminates the problems of stem cell migration which may complicate interpretations of dats from in vivo animal systems (Rencricca et al., 1970). Using this technique, the proliferation and kinetics of mouse, rat and human bone marrow cells have been evaluated in normal, experimentally treated and leukemic hosts (Boyum et al., 1972a; 1972b; Petersen et al., 1974; Boyum and Breivik, 1973; Benestad, 1972; Squires, 1975; Breivik and Benestad, 1972; Laissue et al., 1975; Tyler et al., 1972; Rothstein et al., 1971; 1973; Vilpo et al., 1973).

While a highly useful technique, the diffusion chamber, as such, is limited in its ability to directly quantitate the number of progenitors and evaluate their changes in proliferation and differentiation within the chamber. Determination of stem cell development in chambers depends upon indirect methods of statistical analysis based on histological evaluation of chamber contents [limiting dilution

technique] (Boyum and Borgstrom, 1970; Breivik, 1971) or recloning of harvested chamber cells into secondary spleen colony or agar colony assay systems to assess CFU-S and CFU-C (Tyler et al., 1972; Boyum et al., 1972b; Breivik and Chervenick, 1973; Quesenberry et al., 1974; MacVittie and McCarthy, 1974; Breivik et al., 1971; Symann et al., 1976; Hoelzer, 1976; Pfeffer and Boyum, 1977). By combining the advantages of both in vitro cloning techniques (agar or plasma clot with physiological growth of bone marrow cells in in vivo diffusion chamber cultures, a direct method for assessing progenitor cell proliferation within the chamber can be accomplished. Conceptually, colony formation, not normally seen in diffusion chamber suspension cultures, may be observed with an architectural support matrix similiar to that of in vitro cultures. If the bone marrow cells are seeded within the chamber and then immobilized within a supportive semisolid matrix (e.g. agar, methylcellulose, plasma or fibrin clots), differentiating progeny cells would remain localized and develop into discrete, quantifiable colonies. applying this concept, Gordon developed and used an agar diffusion chamber technique (ADC) to support the growth of granulocyte/macrophage colonies and the study of ADC colony

formation in normal, treated and leukemic host animals (Gordon, 1974; Gordon and Blackett, 1975; Gordon and Lindop, 1975). Jacobsen characterized the development of human granulocyte, lymphocyte and megakaryocyte colonies in a fibrin clot matrix and described the changes occuring in colony formation in irradiated host animals (Jacobsen, 1974; Jacobsen and Fauerholdt, 1976; Jacobsen, 1977). In both techniques colony formation was clonal and therefore represented the in vivo proliferation of progenitor cells within the diffusion chamber. However, in neither of these techniques is the growth of erythroid colonies observed. In the present study a plasma clot diffusion chamber technique (PCDC) is described which allows for the direct assessment of both erythroid and myeloid colonies in the same culture system, and the in vivo regulators influencing their development. The PCDC system, therefore, more closely simulates hematopoiesis of the intact animal in which there exists competitive demands on the pluripotential stem cell leading to the differentiation along the erythroid or myeloid lines. In the present study, the usefulness of the PCDC technique for assaying erythroid and myeloid progenitor cells will be demonstrated by evaluating the clonal origin of each,

examining changes in the pattern of colony formation and their developmental potential in response to specific hematopoietic perturbations induced within the host animal (e.g. neutropenia, anemia, leukemia). For example, conditions of anemia induced by phenylhydrazine in host rats will alter the growth pattern of erythroid colonies in terms of numbers and morphological characteristics. This will reflect the functional state of erythroid stem cells (BFU-E and CFU-E) and the influence of erythropoietin on them. Similiar studies involving endotoxin treatment of the host will allow for the evaluation of the factors influencing alterations in colony formation and the functional state of the CFU-C. Therefore, alteration in PCDC colony formation will reflect changes in the levels of specific physiological regulators (both stimulatory and inhibitory) and represent fluctuations in progenitor cell populations <u>in situ</u>. Since the pluripotential stem cell acts as a common precursor for both erythroid and myeloid lines, depending on competitive demands, the ability of the PCDC technique to assay for the CFU-S will also be evaluated.

The PCDC technique will also be used to answer questions regarding the effect of cell-cell interactions versus long

range chemical products of cells on stem cell proliferation and differentiation. The question is particularly applicable to an understanding of the events leading to the reduction in the normal populations of erythroid and myeloid cells observed in experimental acute myelogenous leukemia (Handler et al., 1974; Handler and Handler, 1976). Whether this reduction is due to a decline in pluripotential or committed stem cells due to a direct leukemic cell-cell interaction and/or the loss of their functional capacities resulting from the influence of leukemic cell inhibitory products will be evaluated.

In the present study the Shay chloroleukemia has served as a model system of leukemia and abnormal hematopoiesis. The Shay chloroleukemia (SCL) was initially induced in rats by gastric instillation of 20-methylcholanthrene (Shay et al., 1951; 1952). Intravenous transplantation of Shay chloroleukemia cells into healthy Long-Evans rats results in a predictable alteration of medullary histology (Handler and Handler, 1970b). Leukemic myeloblasts can be detected in the bone marrow from 3 to 5 days after the initial injection of SCL cells. The bone marrow pathology is characterized by a progressive decrease in cellularity (Varsa et al., 1965),

increase in percent leukemic myeloblasts (Handler et al., 1968) and a decrease in normal erythroid and myeloid elements (Handler et al., 1968). Hepatosplenomegaly occurs in the late stages of the disease (Handler and Handler, 1970b). The progressive increase in the percent bone marrow myeloblasts is the most consistent feature of the leukemic pathology and has therefore been used in staging the course of the leukemia (Varsa et al., 1965; Handler and Handler, 1970b) and correlating changes in various physiological parameters at different stages of the disease. Infiltration and/or proliferation of leukemic myeloblasts in the spleen follows a less predictable course.

In summary, using the PCDC technique, the physiological properties of the hematopoietic cells, the associations which exist between progenitor cells, humoral control agents and the development of progeny cells within the competitive complexities of the hematopoietic system will be studied.

MATERIALS AND METHODS:

I. EXPERIMENTAL ANIMALS:

A. BREEDING AND MAINTENANCE:

All animals used were male, black hooded, Long-Evans rats weighing 250g to 350g and maintained on a diet of Purina Laboratory Chow and water ad libitum. Normal rats served as a source of bone marrow cells for culture procedures.

B. TREATMENT:

Host rats implanted with either plasma clot diffusion chamber (PCDC) or diffusion chamber suspension (DC) cultures are either normal, treated with endotoxin (induced neutropenia), phenylhydrazine (induced hemolytic anemia) or have an induced acute myelogenous leukemia.

1. INDUCTION OF NEUTROPENIA IN LONG-EVANS RATS:

receive a single intraperitoneal injection of 20μg or 100μg

receive a single intraperitoneal injection of 20μg or 100μg Salmonella typhosa lipopolysaccharide dissolved in sterile non-pyrogenic saline.

2. INDUCTION OF HEMOLYTIC ANEMIA IN LONG-EVANS RATS:

Host rats receiving PCDC cultures are rendered anemic by two intraperitoneal injections of 50mg/kg body weight

phenylhydrazine hydrochloride dissolved in sterile non-pyrogenic saline; one 24 hours and the second three to four hours prior to PCDC implantation. Hematocrits on free flowing tail blood are taken daily for seven days to follow the course of the anemia.

3. INDUCTION OF ACUTE MYELOGENOUS LEUKEMIA IN LONG-EVANS RATS:

Rats are made leukemic by the intravenous (i.v.) injection of a $10\text{-}15x10^6$ cell suspension of Shay chloroleukemic cells (SCL) obtained from a subcutaneous chloroma tumor as described in section IIC. These leukemic rats are implanted with normal PCDC cultures either on the same day of i.v. transplantation or on days 4 or 6 post-transplantation (leukemia days 0, 4 and 6). Leukemic rats receiving DC suspension cultures are implanted on the fourth and seventh days post-transplantation. In each experiment the progression of the disease is monitored by the increasing appearance of myeloblasts as assessed from smears prepared from the femoral bone marrow. In the host, evaluation of the percent myeloblasts in the femoral bone marrow has been used to determine the progression of the and stage of the disease. The percent leukemic myeloblasts are expressed as follows:

$$\% \text{ leukemic blasts} = \frac{\text{number of leukemic myeloblasts}}{\text{total number of nucleated cells}} \times 100.$$

The smears are stained with 3,3'-dimethoxybenzidine and counterstained with Wrights'-Giemsa. Benzidine positive (Benz +) cells are also evaluated as follows:

$$\% \text{ Benz + cells} = \frac{\text{number of Benz + cells}}{\text{total number of nucleated cells}} \times 100.$$

Prior to sacrifice of leukemic hosts, hematocrits are taken of free flowing tail blood. In addition, spleens are removed and weighed.

II. CELL SUSPENSIONS, MEDIA AND INSTRUMENTS:

A. MEDIA AND INSTRUMENTS:

All surgical equipment and glassware are dry heat sterilized at 220°c for three hours. Preparation of all cell suspensions and surgical procedures are performed under a vertical laminar air flow unit. Media for normal bone marrow cell suspensions consist of 20% fetal calf serum heat inactivated for 30 minutes at 56°c or 60°c in McCoy's 5A media. The complete media is sterilized by filtration through a 0.2μ Nalge filter unit to prevent bacterial contamination.

Media for *in vitro* bone marrow culture is prepared as follows:

1. Methylcellulose is prepared by adding 4g of powdered methylcellulose to 125ml of still boiling autoclaved water. Upon cooling, 125ml of 2x McCoy's 5A medium is added. The resulting medium is stirred for 2 days, frozen and then thawed to remove granularity.

2. To 50ml of methylcellulose, 50ml of McCoy's 5A supplemented with essential and non-essential amino acids, glutamine, serine, asparginine, vitamines, sodium pyruvate, sodium bicarbonate, fetal calf serum and antibiotics are added. The proportions of the above ingredients are detailed in Appendix C.

3. Plating: To 100ml of the above completed media a total of 3.3×10^6 cells are added, giving a final concentration of 1×10^5 cells in 3ml media/petri plate. Using a 10ml sterile pipette, 3ml of media plus cells are plated in a 35x15mm petri plate. To each plate rat sera prepared from freshly bled untreated rats and sterilized by millipore filtration is added. Completed plates are incubated in a humidified incubator at 37°c with 7% CO_2. On days 7 and 14 the plates are removed and evaluated for colony formation using an inverted stage microscope.

B. NORMAL BONE MARROW CELL SUSPENSIONS:

Both femurs of normal rat donors are asceptically removed and then flushed with 5ml cold media using a 10ml hyperdermic syringe with a 21 gauge needle. A suspension of cells is prepared by passage through a 44.0μ nylon mesh filter. An aliquot is removed for total nucleated cell counts using a hemocytometer and 2% acetic acid as diluent. Cells are diluted to the appropriate concentration with media and stored on ice until use. Normal bone marrow cell suspensions are used in the following ways:

1. Determination of *in vivo* growth patterns of normal cells by seeding into diffusion chamber suspension cultures. These are implanted into normal, days 4 and 7 leukemic host rats.

2. Determination of *in vivo* erythroid and myeloid colony forming potential by seeding $5x10^5$ normal cells into PCDC's that are implanted into normal, endotoxin and phenylhydrazine treated and leukemic host rats.

3. Preincubation with erythropoietin: Prior to seeding PCDC cultures, normal cells ($5x10^5$ cell/ml) are incubated for 3 hours at 37°c with 0.25U erythropoietin/ml cell solution. The cells are then centrifuged at low speed and the cell pellet

resuspended in cold medium to a concentration of 4.2×10^5 cells/ml.

C. LEUKEMIC CELL SUSPENSIONS:

Rats with well formed, highly vascularized subcutaneous tumors are chosen as leukemic cell donors. The subcutaneous chloroma tumor and non-necrotic, well vascularized pieces are removed asceptically and placed in a sterile 30ml hand held homogenizer containing 20ml non-pyrogenic saline. The leukemic blast cells are then released from the connective tissue matrix by gently moving a loosely fitting ground down teflon pestle up and down several times. The resulting suspension is then filtered twice through glass wool to remove the connective tissue debris. The concentration of this debris free suspension of SCL cells is determined by hemocytometer using 2% acetic acid as diluent. The prepared SCL suspension is used as follows:

1. Maintenance of the SCL line by subcutaneous injection of 20×10^6 SCL cells into healthy 40-60g male rats. This procedure results in a well localized solid tumor at the site of injection. well formed tumors develop within 7 to 10 days. Animals bearing massive tumors which have broken through the skin are not chosen for experimental use.

2. Induction of acute myelogenous leukemia: Procedure described in sectionIB3.

3. Determination of *in vivo* growth pattern by seeding $1.0x10^5$ cells into DC suspension cultures which are then implanted into host animals.

III. DIFFUSION CHAMBERS:

A. CONSTRUCTION AND STERILIZATION:

Diffusion chambers are constructed by attaching, with millipore filter cement #1, 0.22μ GS type filters to both sides of a lucite ring using a diffusion chamber construction apparatus. The completed chambers are tested for leaks by injecting air into them while submerged under water. Sterilization is accomplished either by a two hour exposure to ultraviolet light or leaving the chambers under a laminar air flow unit for 2 to 3 days rpior to use. The volume capacity of these chambers is 150μl.

B. DIFFUSION CHAMBER SUSPENSION CULTURES:

1. PREPARATION AND HARVEST:

Each chamber is wetted with media and then seeded with $1.0x10^5$ normal bone marrow cells in 100μl of media. The chambers are stoppered with a plastic plug, heat sealed with

a hot spatula and placed in ice cold media until implantation. Alternatively, chambers are sealed with tensol cement smeared over the plastic plug. Each recipient host animal is surgically implanted with 4 diffusion chambers into the peritoneal cavity. At varying intervals up to seven days the animals are sacrificed, the chambers removed and cleaned of adhering tissue and placed into a phosphate buffered saline solution (pH 7.2) containing 5% pronase and 5% ficoll at room temperature for 10 minutes. The chambers are punctured and the cells removed by Pasteur pipette, counted by hemocytometer and smears prepared for differential analysis.

2. RECLONING INTO IN VITRO METHYLCELLULOSE CULTURES:

To determine the growth of committed granulocytic precursor cells (CFU-C) in DC suspension cultures, $1x10^6$ cells are seeded and implanted into normal and days 4 and 7 leukemic hosts. Cells harvested on days 3, 4 and 5 of DC culture from normal and leukemic hosts are recloned into in vitro methylcellulose cultures as described in section IIa3.

C. PLASMA CLOT DIFFUSION CHAMBER CULTURES (PCDC):

1. PREPARATION:

Each chamber is wetted and seeded with 120µl of either

$5x10^5$ normal bone marrow cells or $5x10^5$ cells preincubated for 3 hours with 0.25U erythropoietin/ml cells. This is followed by injection of 20μl citrated bovine plasma into the chamber, which is then immediately stoppered with a plastic plug, quickly heat sealed or cemented, and shaken vigorously to ensure a uniform distribution of cells within the chamber. Each completed chamber is set aside for 5 to 10 minutes to allow for clot formation, after which the PCDC is transferred to ice cold media where it remains until implantation.

2. HOST ANIMALS:

PCDC containing normal bone marrow cells are implanted into normal, endotoxin and phenylhydrazine treated and leukemic host rats as outlined in sections IB1, IB2 and IB3. PCDC's seeded with bone marrow cells preincubated with erythropoietin are implanted only into normal hosts.

3. HARVEST AND FIXATION:

Diffusion chambers are removed from host animals at intervals over a period of 7 days. The chambers are cleaned of adhering tissue and placed in ice cold medium. Using a thin, sharp, disposable microtome knife, one millipore filter is carefully removed without disturbing the clot and examined under the microscope. It is routinely found to

be free of cells. The remaining filters and their adhering clots are then removed from the plastic ring by careful trimming with a blood stylet. The clot, still attached to the filter, is placed face down on a precleaned slide and allowed to settle for 3 to 5 minutes. Leaving the clot attached to one of the millipore filters insures ease of handling, maintains the integrity of the clot and does not interfere with the final examination of colonies on a permanent slide. Excess fluid is absorbed from the clot by the repeated application of a Whatman #1 filter paper to the millipore filter. The clot is pressed lightly to insure flattening. The preparation is then fixed with 5% gluteraldehyde in 0.01M phosphate buffer (pH 7.0-7.2). Using a Pasteur pipette, the fixative is added to the filter paper overlying the clot preparation and allowed to stand for 6 minutes. The clot is then removed from the slide and placed in distilled water for 8 minutes. Excess water is removed by gentle blotting with filter paper and the clot is then stained with benzidine and hematoxylin. The clots are placed face up on the slide (the millipore filter adheres to the slide), and the stains are added directly with a Pasteur pipette. Clots are throughly dried with a cold air hair drier. To reduce

shrinkage and cracking, clots are sandwiched between fine wire gauze during the dring period. The dried preparation is then placed into immersion oil until the millipore filter appears transparent. A permanent slide is then prepared, the cover slip weighted to insure a flat preparation and allowed to set for 2 days prior to microscopic evaluation.

4. SCORING OF COLONIES:

Clot preparations are evaluated at x400 magnification. Colonies are examined according to type, number of cells per colony and the degree of cell maturation within each colony. Myeloid colonies are scored when consisting of 20 cells or more. Erythroid colonies are scored as benzidine positive units of 4 to 7 cells or greater. Two classes classes of erythroid colonies are counted depending upon the spatial orientation of the erythroid colonies within the clot matrix. Erythroid colonies appearing on day 2 of culture are randomly dispersed throughout the clot matrix while those occuring on day 7 are grouped into larger aggregates called bursts. Each burst is counted as a single large colony consisting of 6 to 8 smaller erythroid units. Both the individual colonies comprising the burst as well as the burst itself (larger aggregate) are evaluated separately.

RESULTS:

I. PLASMA CLOT DIFFUSION CHAMBER COLONY FORMATION IN NORMAL, ENDOTOXIN AND PHENYLHYDRAZINE TREATED HOST RATS:

The proliferation of normal bone marrow cells seeded into plasma clot diffusion chamber cultures (PCDC's) and implanted into the peritoneal cavity of various host rats is characterized by the formation of discrete erythrocytic and granulocytic colonies. The pattern of colony formation over a time course of 7 days in normal hosts is given in figure #1. Pretreating recipient hosts with endotoxin (figures 2 and 3) and phenylhydrazine (figure #4) markedly alters both erythroid and myeloid colony formation.

A. GRANULOCYTIC COLONIES:

Granulocytic colony growth in normal hosts is characterized by an increase in colony numbers from days 1 to 4. Linear regression analysis indicates a coefficient of correlation of $r = 0.97$ with a slope of $m = 39.2$ colonies per day. The peak number of colonies is observed on day 4, declining significantly ($P < 0.05$) on day 5 and leveling off through day 7. Small colonies containing approximately 20 to 100 cells, consisting of myeloblast-promyelocyte and myelocyte-metamyelocyte cells could be detected on days 2 and

3 (photoplate #1). By day 4 a wide range of colonies, in terms of size and degree of maturation could be observed (photoplate #2). Most of the colonies are of the myelocyte-metamyelocyte type containing several hundred cells (photoplate #3). Having reached the metamyelocyte-mature neutrophil stage of differentiation, many colonies by day 5 are in the process of breaking up. Individual cells appear to migrate away from the colony resulting in a diffuse aggreate of cells (photoplate #4). This is in sharp contrast to the tight groups of cells seen in day 4 colonies (photoplates # 2 and 3). The period between days 5 and 7 is characterized by the migration of cells from colonies, the development of new colonies (characterized by the appearance of early cell types) and the maintenance and growth of some old colonies. In figure #5 a good correlation exists between the number of granulocytic cells grown in diffusion chamber suspension cultures and the granulocyte colony formation in PCDC cultures. The increase in cell number is therefore closely related to the increase in colony formation and the differentiation of precursor stem cells into maturing progeny.

Cultures grown in 20μg and 100μg endotoxin treated hosts

(figures #2 and #3) also display a linear increase in the number of granulocytic colonies up to a peak at day 4. However, this linear increase occurs after a lag period of one day. Despite this delay, the numbers of colonies observed on day 4 in both 20μg and 100μg treated hosts are similiar to that seen in normal hosts. Compared with controls, the rate of colony formation is increased as shown by the slopes of the growth curves of m = 57.9 for 20μg and m = 60.4 for 100μg treated host rats. In addition, many of the colonies observed on day 4 are approximately twice the size of those which develop in normal hosts. There also appears to be an increase in the rate of maturation within the colonies, with many more myelocyte-metamyelocyte colonies appearing on harvest day 3 as compared to the number observed in normal hosts. In both 20μg and 100μg endotoxin treated hosts there is no leveling off of colony numbers between days 5 and 7, but rather a sharp decline to levels half that scored on day 7 in cultures grown in normal hosts. This is due to the greater number of colonies breaking up and fewer numbers of new early colonies being developed.

In phenylhydrazine treated hosts (figure #4) the numbers of granulocytic colonies assessed on day 4 is significantly

($P < 0.001$) lower than seen in normal hosts. The rate of growth ($m = 43.7$) and the size of the colonies observed appear normal up to day 3 of culture. Thereafter, colony levels decline and remain constant through day 7.

Between days 5 and 7 in normal, endotoxin and phenylhydrazine host rats, the presence of colonies composed of eosinophilic leukocytes is observed. Eosinophilic colonies consist of cells in different stages of maturation and are distinguished from predominant granulocyte colonies containing neutrophils on the basis of staining reaction. Although the appearance of such colonies has been noted, quantitative assessment was not undertaken. However, eosinophilic colonies semm yo be more apparent in endotoxin and phenylhydrazine treated hosts. It is interesting to note that in the time period studied (7 days) no mononeuclear colonies are detected in any of the recipient rats. Macrophages are observed but only as single cells.

B. ERYTHROCYTIC COLONIES:

The formation of erythrocytic colonies in chambers implanted into differently treated host rats follow patterns influenced by pretreatment. In all host rats an initial

increase in the numbers of colonies reaching a peak on day 2 is observed. This is followed by a significant decline and a secondary wave of erythroid colony development on days 6 to 7. In normal hosts (figure #1), 188 erythroid colonies are scored on day 2. This number is significantly ($P < 0.05$) increased in anemic hosts to 275 colonies and reduced to 36 and 16 colonies in 20μg and 100μg endotoxin treated hosts respectively. Colonies can be grouped according to two separate criteria, number of cells or maturational state of cellular elements. Colonies are scored as benzidine positive aggregates consisting of 4 to 7 cells and greater than 8 cells. The number of cells per colony up to day 2 rarely exceeds 20 cells. Early erythroid colonies, consisting of basophilic normoblasts lightly stained with benzidine, are scored separately. Table #1 shows the size and maturation distribution of colonies counted daily after chamber implantation.

It is evident from figure #4 and table #1 that phenylhydrazine induced anemia in host animals elicits an increase in colony formation with augmented numbers of both early basophilic normoblasts and orthochromatic and polychromic normoblasts in larger colony units. The development

of anemia is indicated by the decrease in the percent hematocrit throughout the culture period (figure #6). Endotoxin treatment (figures #2 and #3), on the other, depresses erythroid development in all catagories. The decline in colony numbers from day 3 through day 6 is due primarily to the maturation of erythroid elements into enucleated red blood cells. Such mature red blood cells are observed in aggregates, but are not scored as colonies in this study. Erythrocytic colonies persist longer in anemic hosts as shown by both the number and size of the colonies observed on days 3, 4 and 5. On occasion, colonies are seen with greater than 100 cells in anemic rats whereas normal hosts never induce colonies of that size.

A second wave of colony growth is observed on day 7 in normal hosts. However, these colonies differ from those of day 2 in their spatial orientation within the clot matrix. Similiar to the bursts described by Axelrad et al., 1973, individual erythroid colonies are grouped into discrete aggregates consisting of 6 to 8 identifiable erythroid colonies (photoplates #5 and #6) whereas day 2 colonies are randomly dispersed throughout the clot matrix (photoplate #7). In addition, these larger entities (bursts) can be counted

effectively without scoring the individual colonies within them. Thus, the secondary increase in the total number of colonies is concomitant with the linear increase in the number of bursts quantitated starting on day 5 and reaching 28 bursts on day 7. In anemic hosts this secondary wave of differentiation starts on day 4, a day earlier as compared to normal hosts. The peak number of colonies is reached on day 6 and is maintained on day 7. Approximately twice the number of colonies as well as bursts are observed in this secondary wave as compared to normal hosts. The numbers of colonies per burst remain 6 to 8. However, the number of cells per colony is markedly increased. In endotoxin treated hosts, the secondary wave of colony formation is minimal as is the number of corresponding bursts. Within all host animals studied, each colony comprising the burst displays predominantly ortho-chromatic and polychromatic normoblasts (Table #1, day 7).

C. CLONAL ORIGIN OF PCDC COLONIES:

To determine the relationship between the numbers of bone marrow cells cultured and the numbers of colonies observed on peak days, 0.5, 1.0 and 5.0×10^5 normal bone marrow cells are implanted into normal host rats. Figure #7 shows a linear

relationship for granulocytic colonies scored on day 4 (peak colony formation) with a coefficient of correlation of r = 0.99. Figure #7 also shows a linear relationship for erythrocytic colonies scored on peak day 2 with a coefficient of correlation of r = 0.99. These studies suggest that both erythroid and myeloid colonies are clonal in origin.

D. SUMMARY:

The number of committed stem cells per 10^5 cells cultured and calculated from peak colony formation is given in Table II. Comparable numbers of CFU-E on days 2 and 7 are noted in normal hosts. Anemic hosts induce an increase in CFU-E with elevated values on day 7 and a twofold increase in BFU-E. Endotoxin treated hosts effectively suppress the growth of CFU-E and BFU-E. Numbers of CFU-C are slightly suppressed in anemic hosts and mildly elevated in endotoxin treated hosts. The number of cells per colony in these endotoxin treated hosts is markedly increased.

II. PCDC COLONY FORMATION IN NORMAL BONE MARROW CELLS PREINCUBATED WITH ERYTHROPOIETIN (EPO) PRIOR TO IMPLANTATION INTO NORMAL HOST RATS:

In a preliminary experiment the direct action of EPO on erythroid colony formation in PCDC cultures is determined. Normal bone marrow cells preincubated *in vitro* with 0.25U EPO/ml cells for 3 hours prior to seeding in PCDC cultures are implanted into normal host rats. The results are shown in figure #8. A shift to the left occurs in day 2 CFU-E with maximal growth occuring on day 1. The number of colonies is increased to twice that observed in untreated marrow. The secondary growth phase seen in untreated marrow (figure #1) is eliminated due to lack of burst formation. Myeloid colony formation is dramatically reduced as indicated by the slope $m = 16.9$. While this experiment is not properly controlled, it suggests that the PCDC culture system may be valuable in studying the direct effects of regulatory substances on committed progenitor cells.

III. PCDC COLONY FORMATION IN HOST RATS WITH ACUTE MYELOGENOUS LEUKEMIA:

In studying normal PCDC myeloid and erythroid colony formation in leukemic host rats, experiments are designed to evaluate the growth of CFU-C, CFU-E and BFU-E at various

stages of the leukemia. PCDC cultures containing $5x10^5$ normal bone marrow cells are implanted into leukemic host animals on days 0, 4 and 6 after induction of the leukemia by i.v. transplantation of SCL cells. Based on histological characterization of the bone marrow throughout the course of the disease, days 0, 4 and 6 of the leukemia are selected to represent PCDC implantation at early, middle and late stages of the disease. Within 3 to 5 days after i.v. transplantation of SCL tumor cells, leukemic myeloblasts begin to appear in the bone marrow and increase in percentage with the progression of the disease. The increase in percent medullary myeloblasts is characteristic of the leukemia and has been used as a reliable parameter for staging the progression of the disease and monitoring physiological changes during the course of the disease.

PCDC cultures implanted into leukemic hosts are harvested over a 7 day period in each experiment and colony formation evaluated. It can be seen (figures #9 and #10) that days of PCDC's in culture correspond to varying stages of the leukemia as follows:

1. Day 0 leukemic host: Harvest days 1 through 7; early and middle stage.

2. Day 4 leukemic host: Harvest leukemia days 5 through 11; middle to late stage.

3. Day 6 leukemic host: Harvest leukemia days 7 through 13; late stage.

For example, PCDC cultures harvested on day 4 of culture from days 0, 4 and 6 leukemic hosts represent colony growth on days 4, 8 and 10 of the leukemia. Thus, granulocyte colony formation occuring at a peak on day 4 of culture in normal hosts is evaluated at early, middle and late stages in the progression of the leukemia. The concept is similiar for the assessment of erythroid colony formation (day 2 CFU-E; day 7 BFU-E).

Myeloid and erythroid colony formation in leukemic hosts (figures #9 and #10) are represented as percent of control PCDC cultures implanted into normal host rats. The bars represent mean percents of all the chambers evaluated from several leukemic animals sacrificed on the same day of leukemia. Each point represents one animal sacrificed with 2 to 4 chambers harvested and assessed for colony formation. In day 0 and 4 leukemic recipients (figure #9), significant inhibition ($P < 0.003$; $P < 0.005$) of granulocytic colony formation ranging between 10% to 60%

with a mean of 36% is observed over the entire course of the disease. While early inhibition is evident in day 0 recipients, it is not statistically significant. In terms of cell number, the size of those developing myeloid colonies is dramatically reduced to under 100 cells as compared with colonies consisting of several hundred cells observed in normal hosts. Maturation of the cells within the colony is unaffected with the appearance of normal metamyelocyte-mature neutrophil colonies. Granulocyte colony formation showed a mild stimulation ($P < 0.005$) on day 10 of the leukemia in day 6 recipient hosts with colonies appearing normal in size and maturation.

Erythroid colonies (figure #10) are mildly depressed ($P < 0.03$) in the early days of the leukemia, significantly stimulated ($P < 0.05$; $P < 0.0005$) to 100% to 350% of control between days 7 and 8 and significantly inhibited ($P < 0.01$; $P < 0.003$) to 50% to 65% in the late stages of the disease. Burst formation is similiarly inhibited in the later stages of the leukemia. The numbers of individual colonies comprising a burst as well as the number of cells within each colony is noticeably reduced . In contrast to the inhibition on leukemia day 10 observed in day 4 recipients,

there is a significant stimulation ($P < 0.05$) of erythroid colony formation on day 10 of the leukemia in day 6 recipients.

Both erythroid and myeloid colony formation show very low correlations to either percent leukemic myeloblasts (figures #11 and #12) or percent benzidine positive cells (figures #13 and #14) in the bone marrow. These graphs indicate that either inhibition or stimulation of both myeloid and erythroid colonies can occur during early (low percent leukemic blasts) or late (high percent leukemic blasts) stages of the disease. This indirect relationship between PCDC colony formation and the progression of the leukemia may reflect individual variations in the complex formation of the disease.

Inhibition of myeloid colony growth in PCDC cultures is supported by recloning experiments. Normal bone marrow cells seeded into diffusion chamber suspension cultures are implanted into leukemic rats 4 and 7 days after leukemia induction. Control chambers are implanted into normal hosts. On days 3, 4 and 5 of the culture (days 7, 8 and 9; days 10, 11 and 12 of the leukemia), chambers are harvested and the cells recloned into secondary <u>in vitro</u> methylcellulose cultures for CFU-C evaluation. Colonies are scored after

7 days incubation at 37°c. In both 4 and 7 day leukemic recipients CFU-C development in diffusion chambers is inhibited between 28% and 88% with a mean of 44%.

In figure #15 the growth of normal and SCL tumor cells are compared in diffusion chamber suspension cultures implanted into normal animals. SCL cells show a linear increase in the cell number reaching a peak on day 6 and declining thereafter. SCL cells show a higher proliferative rate reaching a number ten times greater than normal cells. The decline in SCL growth is believed to be related to the increasing density within the chamber. Cell death is determined by dye exclusion with trypan blue.

DISCUSSION:

I. GENERAL CHARACTERISTICS OF THE PLASMA CLOT DIFFUSION CHAMBER CULTURES:

The *in vivo* plasma clot diffusion chamber technique described above combines the advantages of the *in vitro* plasma clot culture (Axelrad *et al.*, 1973) and the *in vivo* diffusion chamber system (Benestad, 1970; Boyum and borgstrom, 1970). Bone marrow cells seeded into this culture system sre immobilized in a supportive semisolid matrix so that differentiating progeny cells remain localized and develop into discrete, quantifiable colonies of erythroid and myeloid cells. The clonal nature of the observed colonies allows for the quantitative assessment of the number of progenitor cells seeded into the chamber. The number and morphological characteristics of the cells comprising each colony provide a means of evaluating the proliferative and developmental potential of the stem cells. The system circumvents problems of stem cell migration noted in spleen colony assays as well as the need for exogenous humoral factors (e.g. EPO, CSF). The host animal, within an experimental design, can provide a physiological environment to supply the nutritive as well as the humoral regulators requisite for cell growth

and differentiation.

II. GRANULOPOIESIS IN PLASMA CLOT DIFFUSION CHAMBER CULTURES:

The linear relationship established between the concentration of bone marrow cells seeded into PCDC cultures and the number of erythroid and myeloid colonies formed suggests the clonal origin of these colonies. This is also supported by the observation of day 0 harvested cultures which show an even distribution of seeded cells with no cellular aggregation occuring within the clot matrix. PCDC colonies therefore result from the differentiation of a single pluripotential and/or committed stem cell. Although the clonal origin of granulocytic cells grown in diffusion chamber suspension culture has not as yet been directly established, the increase in the cellularity in both normal and treated hosts, and the appearance of newly formed granulocytes is believed to be due to the proliferation and differentiation of a diffusion chamber progenitor cell [DCPC] (Boyum et al., 1972b; Petersen et al., 1974). The concentration of DCPC within the chamber is determined by limiting dilution technique. In concept, when each chamber is seeded with a homogeneous cell suspension, the distribution of stem cells in the chambers can be calculated from the

Poisson probability law (Boyum and Borgstrom, 1970; Breivik, 1970). If the cell suspension is diluted, there is a finite probability that some diffusion chambers will not receive any stem cells resulting in "empty chambers". Chambers are scored as "empty" when no new granulocyte formation is observed at the end of a 7 day culture period. The probability, P(0), of getting no stem cells in a chamber is given by the equation: $\lambda = -\ln P(0)$, where,

$$P(0) = \frac{\text{number of empty chambers}}{\text{total number of chambers}} .$$

Boyum and Borgstrom (1970) report 46 ±6 DCPC's/10^5 cells or one progenitor in 2000 bone marrow cells in C_3H x DBA mice. One progenitor in 2700 to 4900 marrow cells is found in white outbred mice. Gordon reports one CFU-C in 1000 mouse marrow cells in the agar diffusion chamber system. In the present study, 27 ± 3.3 myeloid colonies per 10^5 bone marrow cells are observed in PCDC cultures, representing one CFU-C per 3700 bone marrow cells seeded. This incidence is in the reported range and represents expected variations in growth potential between rat and mouse marrow in diffusion chambers (Petersen et al., 1974). In addition, the close correlation between the pattern of granulocytic cell growth in diffusion

chamber suspension cultures and myeloid colony formation in PCDC cultures (figure #5) indicates that the increase in chamber cellularity is due to the differentiation of granulocytic precursor stem cells into maturing progeny. Paran et al., 1969 has noted one CFU-C in 1500 mouse bone marrow cells assayed in vitro. Metcalf and Moore (1971) places a figure closer to one CFU-C in 500 marrow cells. In adult Long-Evans rats one CFU-C in approximately 2200 bone marrow cells is plated. While in vitro assays demonstrate the growth of both granulocyte and macrophage colonies (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966), only the growth of granulocytic colonies is observed up to 7 days in PCDC cultures. Macrophages, although seen as isolated cells in the backround matrix, never appear as colonies. This observation is in sharp contrast to studies indicating that granulocytes and macrophages are closely related cell populations derived from a common CFU-C (Moore et al., 1972; Metcalf, 1971; Paran and Sachs, 1969). Whether macrophage colonies appear over longer period of PCDC culture has not been determined in this study.

In diffusion chamber suspension cultures, the number of differentiating myeloid elements and the proliferation of

myeloid precursors are increased in neutropenic hosts. Chamber DCPC as well as CFU-S and CFU-C, assayed by recloning harvested chamber cells into secondary spleen colony or agar colony culture systems, increase in host animals rendered neutropenic by irradiation (Petersen et al., 1974; Boyum et al., 1972b), cyclophosphamide (Tyler et al., 1972) and endotoxin (Rothstein et al., 1973) treatment. Granulocytic colony formation is increased in response to irradiation induced neutropenia in agar (Gordon et al., 1974) and fibrin clot (Jacobsen and Fauerholdt, 1976; Jacobsen, 1977) diffusion chamber culture systems. The increased pattern of cell growth and proliferation in perturbated hosts is attributed to the humoral factors colony stimulating factor [CSF] (Gordon and Blackett, 1975) and/or diffusible granulopoietic stimulator [DGS] (Rothstein et al., 1973).

In vitro, the presence of CSF is an absolute requirement for the formation of granulocyte/macrophage colonies. CSF is isolated from a variety of animal and human tissues (Bradley et al., 1971; Sheridan and Stanley, 1971; Sheridan and Metcalf, 1972; Austin et al., 1972; Pluznik and Sachs, 1966) is present in serum (Robinson et al., 1967; Foster et al., 1968) and is excreted in the urine (Stanley and

Metcalf, 1969; Robinson and Pike, 1970; Chan, 1970). A number of studies show that partially purified human urinary CSF is a sialic acid containing glycoprotein with a moleculer weight of approximately 45,000 daltons (Austin et al., 1971; Stanley and Metcalf, 1969; Stanley et al., 1975). Murine CSF shows similiar properties (Stanley et al., 1975). A low molecular weight substance with CSF activity is isolated from the lung (Price et al., 1973; Sheridan and Metcalf, 1973). The in vivo significance of these activities is unknown.

In murine systems the growth of in vitro granulocyte/macrophage colonies shows an absolute dependence on CSF (Metcalf and Foster, 1967; Para and Sachs, 1968). In humans and other species bone marrow cells form colonies in the absence of exogenous CSF. A variety of studies show that such autostimulation is due to a specific population of CSF producing cells (Moore and Williams, 1972; Moore et al., 1972; Chan and Metcalf, 1972; Messer et al., 1973; Haskill et al., 1972). When CSF producing cells are removed by differential centrifugation, The CFU-C exhibit a similiar requirement for exogenous CSF as mouse bone marrow cells (Moore and Williams, 1972; Moore et al., 1972). Robinson suggests

that the mature granulocyte is the CSF producing cell and therefore regulates CFU-C differentiation via positive feedback control (Robinson and Mangalik, 1972; Mangalik and Robinson, 1973). However, such a mechanism cannot correct for neutropenia and therefore would provide a constant stimulus during granulocytosis. A number of studies demonstrate that the CSF producing cells belong to the monocyte-macrophage line (Golde and Cline, 1972, Chercenick and LoBuglio, 1972; Golde et al., 1972). These cells have the ability to produce CSF in vitro upon exposure to endotoxin (Cline et al., 1974; Eaves and Bruce, 1974). Since the CFU-C is the common progenitor for both granulocyte and mononuclear cells, the control of CFU-C differentiation by mononuclear cell production of CSF would still operate via a positive feedback regulation.

Despite its isolation from various tissues, serum and urine, the role of CSF as an in vivo physiological regulator of granulopoiesis is still uncertain. However, early studies on irradiated mice with shielded hindlimbs show an inverse relationship between blood neutrophil count and both serum CSF levels and marrow CFU-C in the shielded hindlimb (Morley et al., 1971; Rickard et al., 1971). Granulocyte

proliferation is enhanced in mice injected with CSF (Metcalf and Stanley, 1971). CSF levels are elevated in animals made neutropenic by irradition (Morley et al., 1971; Rickard et al., 1971), endotoxin (Quesenberry et al., 1973; Shadduck, 1974), cyclophosphamide (Shadduck and Nunna, 1971) and administration of antineutrophilic serum (Shadduck and Nagabhushanam, 1971). The alterations in CSF output in humans with cyclic neutropenia also supports an in vivo role for CSF (Greenberg et al., 1976).

While serum levels of CSF are indeed augmented in animals made neutropenic, Rothstein et al., (1973) demonstrate that CSF is different from a diffusible granulocytic stimulator (DGS) affecting granulopoiesis in diffusion chambers in endotoxin treated hosts. Serum CSF levels peak at 24 hours, whereas DGS levels are elevated 72 hours after endotoxin treatment. In the present study, granulocyte colony size but not number is increased in hosts experiencing a mild transient neutropenia. Whether this is Due to CSF and/or DGS has not been determined. The approximate doubling in colony size suggests an extra division at some time after the colony is formed, perhaps at the mitotically active myelocyte stage of differentiation. The mild, though non-significant increase

in the number of myeloid colonies formed may indicate the need for the induction of a more sustained neutropenia in the host animal. However, the increased rate of myeloid colony formation is consistent with a shortened generation time in neutropenic hosts as demonstrated by Niskanen et al., (1974).

III. ERYTHROPOIESIS IN PLASMA CLOT DIFFUSION CHAMBER CULTURES:

Erythroid progenitor cells do not grow well in diffusion chamber suspension cultures. Erythropoiesis tends to be minimal, with few normoblasts observed beyond day 3 (Boyum et al., 1972b) The addition of the plasma clot supportive matrix allows for the growth of erythroid colonies in a manner closely resemblig the pattern observed in in vitro studies. The in vitro plasma clot culture introduced by Stephenson et al., (1971) and improved by McLeod et al., (1974) has allowed for the growth and the quantitative assay of benzidine positive erythroid colonies derived from a committed stem cell (CFU-E). Erythroid colony formation of human as well as mouse bone marrow has also been characterized in plasma clot (Tepperman et al., 1974) and methylcellulose culture systems (Iscove et al., 1974; Iscove and Sieber, 1975; Ogawa et al., 1976). Two classes of erythropoietic precursors

have been demonstrated with the plasma clot system. The first class designated as the erythrocyte colony forming unit (CFU-E), form small erythrocytic colonies consisting of 8 to 32 cells at 2 days of culture (peak growth), and because of their limited proliferation in culture, are considered to be at late stages of erythrocytic development (Stephenson et al., 1971; McLeod et al., 1974; Gregory et al., 1973). The formation of day 2 CFU-E is highly dependent on the addition of erythropoietin to the medium. In PCDC cultures peak erythroid colony formation occurs on day 2 with colonies morphologically similiar to those found in vitro. 38 CFU-E per 10^5 normal bone marrow cells are observed compared with in vitro studies in the same rat strain showing 200 CFU-E per 10^5 marrow cells (Handler and Handler, 1976) and 300 CFU-E per 10^5 marrow cells in mice (McLeod et al., 1974; Iscove and Sieber, 1975). The fewer numbers of CFU-E seen in the PCDC may reflect the still less favorable growth conditions for erythropoiesis in the chamber and/or the important differences in the actual amount of EPO the cultured cells are exposed to. While PCDC erythroid colony formation is dependent on physiological levels of EPO, in vitro cultures are exposed to high, non-physiological doses

of EPO. The clonal origin of erythroid colonies in both *in vitro* (McLeod *et al.*, 1974; Ogawa *et al.*, 1976; Iscove and Sieber, 1975; Prchal, 1976) and PCDC cultures is suggested by the linear relationship between the number of colonies formed and the number of cells plated. Using time-lapse cinematography, Cormack (1976) elegantly demonstrates the formation of an erythroid colony from a single cell.

Axelrad *et al.*, (1973) identified a second class of erythropoietic precursor cells on day 8 after repeated additions of high concentrations of EPO to the culture medium. The precursors, termed burst forming units (BFU-E), are distinguished from day 2 colonies not only by their EPO dependency but also by the spatial orientation of the developing colonies in the culture plate. While day 2 CFU-E are randomly dispersed, day 8 BFU-E are grouped into large macroscopic colonies made up of discrete units of CFU-E. The secondary increase in CFU-E observed on day 7 in PCDC cultures corresponds to the linear increase in the number of BFU-E quantitated and is due to the differentiation of the BFU-E into CFU-E. 5 BFU-E per 10^5 normal adult rat bone marrow cells are observed compared with 5 BFU-E per 10^5 marrow cells reported by Axelrad *et al.*, (1973) in mouse.

These bursts are morphologically similiar to those grown in vitro. Iscove and co-workers report 25 BFU-E per 10^5 adult mouse bone marrow cells in an in vitro methylcellulose system (Iscove and Sieber, 1975). Bursts are characterized as single macroscopic colonies consisting of 10^4 cells (Iscove et al., 1974). The supporting matrix (plasma clot versus methylcellulose) may play an important role in the morphology of bursts and the number formed. The presence of BFU-E in normal hosts indicates that the added "push" required in vitro by repeated additions of high EPO concentrations is not necessary in the more physiological environment of the host rat.

Although the clonal origin of the BFU-E remains to be proven, sedimentation velocity studies indicate that BFU-E and CFU-E are two discrete and separable entities (Axelrad et al., 1973; Heath et al., 1976). Burst formation, therefore, does not result from the aggregation of CFU-E. This is supported by the lack of colonies between days 3 and 5 in PCDC cultures. Based on the following evidence, the BFU-E is believed to be the precursor of the CFU-E:

A. BFU-E have a greater proliferative potential producing a greater number of progeny over a longer period

of time in culture than the CFU-E.

B. Each burst observed *in vitro* and in the present study is composed of smaller groups of colonies which morphologically resemble erythroid colonies derived from CFU-E.

C. The numbers of CFU-E are greater than BFU-E in normal mouse marrow and spleen cultured *in vitro* and in adult rat bone matrow cultured in the PCDC.

D. Hypertransfusion reduces the number of CFU-E but has no effect on the BFU-E.

E. New BFU-E develop when marrow cells from hypertransfused mice are cultured under conditions favoring burst formation (high EPO concentration). These new BFU-E are demonstrated by the production of erythroid colonies at low EPO in secondary cultures (Axelrad *et al.*, 1973; Heath *et al.*, 1976; Iscove and Sieber, 1975).

The differences between BFU-E and CFU-E in sensitivity to EPO, in physical (sedimentation velocity) and morphological (colony formation) characteristics provide evidence for the concept of two different EPO responsive cell populations within the committed stem cell compartment. Recently, Gregory has proposed the existence of a third stem cell between the BFU-E and CFU-E in the erythropoietic hierarchy

(Gregory, 1976). The age structure of the committed stem cell compartment is consistent with the model proposed by Gregory (Gregory et al., 1974), kinetic analysis of spleen colony growth (Lajtha et al., 1971) and studies involving the regeneration pattern of EPO responsiveness following busulfan treatment of hypertransfused mice (Reissmann and Samorapoompichit, 1970; Reissmann and Udupa, 1972).

The increase in PCDC erythroid colony formation in phenylhydrazine induced anemic hosts is consistent with previously reported increases in diffusion chamber erythropoiesis. Berman and Newby (1967) have shown increased ^{59}Fe incorporation in mouse bone marrow cultures in diffusion chambers in the presence of erythropoietin. Meints and Van Zant (1969) demonstrate elevated erythropoiesis in DC's implanted into hypoxic hosts. Boyum et al., (1972) show a shift in chamber cellularity toward the erythroid line in host animals made anemic by hypoxia or hemorrhage and treated with EPO. As measured in vitro, the proliferative and differentiative activity of CFU-E and BFU-E in situ is increased in response to anemia induced by hemorrhage (McLeod et al., 1974) and phenylhydrazine (Hara and Ogawa, 1976). The elevation in BFU-E and CFU-E in PCDC cultures

suggests the stimulatory influence of increased titers of erythropoietin induced by the severe hemolytic anemia in phenylhydrazine treated rats.

The existence of a humoral factor (EPO) that regulated red cell production is first postulated by Carnot and Deflandre (1906) at the turn of the century. This is confirmed by experiments on parabiotic rats in which hypoxia induced in one of the pair led to stimulation of erythropoiesis in both members. EPO has been highly purified and characterized as a glycoprotein with a molecular weight of 45,000 daltons and 11% sialic acid content (Goldwasser and Kung, 1968; Goldwasser, 1975). EPO is produced in the kidney (Jacobson et al., 1957) in response to tissue hypoxia (Erslev, 1971). The red cell mass also influences EPO production but to a lesser extent. In the present study, the direct effect of EPO on PCDC erythroid precursors is suggested. In this study, pretreatment of bone marrow cells in vitro prior to PCDC seeding leads to the increased proliferation of CFU-E and the differentiation of BFU-E into CFU-E.

Aside from its affects on committed stem cell differentiation, EPO affects the proliferation rate of erythroblasts by shortening the mean transit time of the

proliferating erythroblast compartment (Hodgson, 1970). The increased rate of CFU-E and BFU-E colony formation as well as their earlier apperance in anemic hosts may, therefore, represent an EPO mediated shortening of the generation time in the committed stem cell compartment. In addition, the dramatic increase in the number of erythroid cells per colony is consistent with an increase in the red cell mass after prolonged injection of EPO (Schooley, 1965). EPO is also responsible for the rapid release of reticulocytes from the bone marrow into the peripheral blood (Gordon et al., 1962; Hodgson, 1970).

At the molecular level EPO has been shown to affect macromolecular systhesis (Goldwasser, 1975). The affect of EPO on protein synthesis is most reliably measured by hemoglobin synthesis. ^{59}Fe incorporation into heme is taken as an index of hemoglobin synthesis with over 90% of the assessed heme produced being attributable to hemoglobin synthesis (Gallien-Latigue and Goldwasser, 1964). ^{14}C-glucosamine incorporation into stroma of the rat bone marrow cells (Dukes and Goldwasser, 1965) and the synthesis of delta-aminolevulinic acid synthetase, the rate limiting enzyme of heme formation (Bottomley and Smithee, 1969), is

increased in response to EPO. The synthesis of RNA is shown to be an early response, possibly the initial response, to EPO in rat bone marrow (Kranz and Goldwasser, 1965) and in mouse fetal liver (Djaldetti et al., 1972). It is postulated that EPO might act to induce mRNA synthesis and thereby initiate new protein synthesis. Within 5 minutes after exposure to EPO rat bone marrow cells synthesize a 150S type RNA, the role of which in red cell differentiation is unknown (Gross and Goldwasser, 1969; 1971). The synthesis of 9S, 55S to 65S and 4S to 6S species of RNA are also enhanced. Inhibition of DNA synthesis with cytosine arabinoside suggests that the formation of RNA in response to EPO is independent of prior DNA synthesis and that EPO acts on the CFU-E during the G_2 phase of the cell cycle (Bedard and Goldwasser, 1976).

IV. DEVELOPMENT OF THE PLURIPOTENTIAL STEM CELL IN PLASMA CLOT DIFFUSION CHAMBER CULTURES:

The similiarities established between erythroid and myeloid colony formation in *in vivo* PCDC and *in vitro* cultures indicates that PCDC colonies are derived from committed stem cells. However, committed stem cells for the erythroid and myeloid lines are presumably derived from the common pluripotential stem cell (CFU-S) as assayed by spleen colony formation in irradiated mice (Till and McCulloch, 1961). Shifts in the populations of committed progeny cells reflect competitive proliferative demands on the subsequent differentiation of the CFU-S. Decreases in diffusion chamber progenitor cells (DCPC), as assayed by limiting dilution technique, are noted in hypoxic animals (Boyum *et al.*, 1972b). Injection of erythropoietin during the regenerative phase of transplanted bone marrow cells into lethally irradiated mice results in a decrease in granulopoiesis (Hellman and Grate, 1967). Reduction in CFU-C in the bone marrow under conditions which augment erythropoiesis is used as further evidence for competing pathways from a common stem cell origin (Rickard *et al.*, 1971; Metcalf, 1967). The reduced erythropoiesis observed following endotoxin treatment supports a similiar conclusion (Fruhman, 1967; Schade and Fried, 1976). This

same pattern of erythroid-myeloid directional competition is observed in the present study in perturbated host rats, suggesting the proliferation and differentiation of the CFU-S within the PCDC chamber. Studies on the nature of the DCPC support this conclusion. Whether the DCPC represents the multipotential CFU-S, the committed CFU-C or both is uncertain. Using the spleen colony and the diffusion chamber assay systems, the pattern of depletion and regeneration of the CFU-S and DCPC after treatment with cyclophosphamide (Boyum et al., 1974), busulfan (Jovasen and Boyum, 1973), vinblastin (Breivik, 1972) and X-ray inactivation (Petersen et al., 1974) suggests that the DCPC is probably a mixture of both CFU-S and CFU-C. Recloning of harvested chamber cells into secondary spleen colony assay systems indicates that both CFU-S and CFU-C proliferate within the diffusion chamber (Boyum et al., 1972b; Tyler et al., 1972; Breivik et al., 1971; MacVittie and McCarthy, 1974; Quesenberry et al., 1974; Symann et al., 1976; Hoelzer, 1976; Pfeffer and Boyum, 1977; Breivik and Chervenick, 1973). The nature of the stem cells leading to the formation of myeloid colonies in agar and fibrin clot diffusion chamber culture systems has not been clarified. Since the normal bone marrow is isolated within

the PCDC culture system, directional hematopoiesis cannot be ascribed to shifts in cell populations due to stem cell migration.

The proliferation of CFU-S in PCDC and DC suspension cultures provides evidence for the long range control of CFU-S differentiation. Since cells are compartmentalized within the chamber, eliminating cell-cell interactions, the increased proliferation of CFU-S in neutropenic host animals (Tyler et al., 1972; Niskanen et al., 1974; Boyum et al., 1972) is attributed to a circulating factor. Gregory suggests that an ∝-macroglobulin is released after treatment of animals with cyclophosphamide and causes stimulation of stem cell growth (Gregory et al., 1971). Byron, in a serias of experiments reports on a number of agents given in vivo or added in vitro to marrow cell suspensions that influence the influence the irradiated mouse spleen colony formation (Byron, 1975). In the present study, the directional differentiation of CFU-S in treated rats suggests the direct control of CFU-S differentiation into the granulocytic or erythrocytic line by CSF and EPO.

Humoral control of CFU-S may involve a model similiar to the one proposed by Goldwasser (1975). In this model

Goldwasser regards the EPO responsive cells (CFU-E) as well as the other "committed" stem cells as being the same cells but in different phases of the cell cycle. These cells (CFU-E, CFU-C and thrombocyte), are defined by the presence of specific receptors for EPO, CSF and thrombopoietin. The receptors have a short life span on the surface of the cell. The appearance of these receptors would occur in a specific part of the cell cycle making the cells responsive to specific stimulators. It is possible that Goldwasser's proposed "committed' stem cell is actually the pluripotential precursor. At present it is not clear if the transition from CFU-S to committed precursor is a one step or multiple step change. Recent studies with the alkylating agent isopropyl methane sulfonate raised the possibility that the CFU-S may exist in two potential states: one which can reproduce itself, the other, also a CFU-S, which possesses the capacity to form spleen colonies of either type, but which cannot reproduce itself (Schofield and Lajtha, 1973). Therefore, the decendents of this second CFU-S are potentially committed. This stem cell may represent the cell in Goldwasser's model that is responsive to several humoral factors.

The control of CFU-S differentiation by humoral factors does not preclude the importence of short range cell-cell interactions in this control. Accumulating evidence indicates that both short range cell-cell interactions as well as long range humoral influences are importent. Induction of severe anemia in rodents by phenylhydrazine results in the migration of CFU-S from the bone marrow to the erythropoietically more favorable environment in the spleen (Rencricca et al., 1970). The CFU-S remaining in the depleted CFU-S compartment enter cycle. Thymidine suicide experiments have shown the CFU-S to be in a non-proliferative G_0 state from which it can be triggered into differentiation along the erythroid or myeloid cell lines (Lajtha et al., 1969). This is similiar to the effect seen in the regenerating tissue after irradiation or transplantation of normal bone marrow cells into irradiated recipients (Becker et al., 1965). When the critical compartment size is reached the CFU-S return to G_0. The CFU-S that migrate into the spleen remain in G_0. These results are taken as evidence for CFU-s control by cell-cell interaction. Trentin and others have further developed this concept of cell-cell control by proposing the concept of a hematopoietic inductive microenvironment (HIM)

in which the local milieu of the particular hematopoietic organ (bone marrow or spleen) will influence the direction of CFU-S differentiation (Trentin, 1970; Trentin et al., 1974; Trentin, 1976; Tavassoli, 1975). Evidence for the existence of a myeloid and erythroid HIM and its control and micro-anatomy are demonstrated in a number of studies. For example, erythroid colonies predominate in the spleen having an erythroid/granulocytic ratio (E:G) of approximately 3, while myeloid colonies predominate in the bone marrow with an E:G of 0.5 (Curry et al., 1967; Wolf and Trentin, 1968). Whole spleens transplanted subcutaneously support hematopoiesis and give rise to spleen colonies with the same E:G colony ratio as does spleen in situ, i.e., 3.5 (Wolf and Trentin, 1968). Pieces of marrow stroma transplanted into the spleen support hematopoiesis with an E:G ratio similiar to that of bone marrow in situ. Single colonies (clones) growing across the junction of marrow and spleen stroma show abrupt transition of hematopoieitc type, with erythropoiesis in spleen stroma and granulopoiesis in marrow stroma (Wolf and Trentin, 1968). The existence of two genetically anemic strains of mice, Sl/Sl^{d} and W/W^{v} supports the concept of a HIM and indicates that the microenvironment and production of normal

CFU-S are under genetic control. Sl/Sl^d mice have normal stem cells and levels of EPO (Bernstein et al., 1968). Anemia results from a defective microenvironment (McCulloch, 1970) as indicated by the fact that these mice are not cured by transfusion of marrow or spleen cells, but by subcutaneous transplantation of whole spleens from non-anemic littermates (Bernstein, 1970; Trentin, 1971). W/W^v mice, on the other hand, have defective stem cells since they are cured by marrow cells transfused from Sl/Sl^d mice (Bernstein et al., 1968; Bernstein, 1970). The microanatomy also contributes to the microenvironment and is morphologically composed of a microvascular compartment, a connective tissue compartment, and neutral elements associated with both the blood vessels and stroma (McCuskey et al., 1972). These compartments are involved in the regulation and transfer of metabolites to the microenvironments influencing and modifying cellular reactions or responses.

V. GRANULOPOIESIS IN PCDC CULTURES IMPLANTED INTO ACUTE MYELOGENOUS LEUKEMIC RAT HOSTS:

The decrease in normal myeloid elements in the bone marrow and peripheral blood of humans and animals with AML is associated with disturbances in *in vitro* colony formation (Senn *et al.*, 1967; Greenberg *et al.*, 1971; Moore *et al.*, 1973a; 1973b; 1973c; Brown and Carbone, 1971; Robinson and Entringer, 1973; Iscove *et al.*, 1971; Handler *et al.*, 1974). *In vitro* cultures of bone marrow from patients with AML are predominantly characterized either by the lack of colony formation or the formation of large numbers of small abortive clusters composed of poorly differentiated cells (Greenberg *et al.*, 1971; Iscove *et al.*, 1971; Moore *et al.*, 1973a; 1973c). In cases where some degree of normal colony formation occurs, the ratio of clusters to colonies is abnormally high when compared to the ratio found in normal humans. Karyotypic analysis (Moore and Metcalf, 1973; Duttera *et al.*, 1972), bouyant density studies and cell cycle analysis (Moore *et al.*, 1973a; 1973c) demonstrate that AML clusters are generated by leukemic colony forming cells that are representitive of the leukemic cell population. These leukemic CFU-C have an abnormally light bouyant density and a reduced sensitivity to the suiciding effect of tritiated thymidine. The change

in cell cycle response suggests that the leukemic CFU-C have a longer cell cycle time or are predominantly in a non-cycling G_0 state. Remission of the AML is associated with the following:

1. Normal *in vitro* colony formation with a restoration of the normal cluster to colony ratio (Harris and Freidreich, 1970; Moore *et al.*, 1973a; 1973b).

2. Return of normal *in vitro* proliferation and differentiation of the CFU-C.

3. Restoration of normal cell cycle parameters as well as a return of the CFU-C to normal bouyant density (Moore *et al.*, 1973a; 1973c).

4. Restoration of normal cytogenetic status as assessed in marrow preparations and from cells developing in *in vitro* colonies (Moore and Metcalf, 1973; Duterra *et al*, 1972).

The comparison of *in vitro* colony formation, bouyant density distribution, cell cycle and cytogenetic studies of *in vitro* colony and cluster forming cells during AML in relapse and remission suggests the coexistence of populations of normal and leukemic cells in AML marrow. Remission is due to the reemergence of a normal progenitor cell population that has

apparently remained dormant as the leukemic clone expanded during relapse.

The maintenance of normal hematopoietic stemm cell populations during AML, responsible for normal hematopoiesis in remission, argues against the reduction of normal in vitro colony formation as a result of a dilutional or "Crowding out" effect by the expanding leukemic cell population. This is supported by the observation of defective colony development in preleukemia in which a decline in colony formation is detected long before leukemic cells become predominant in the bone marrow (Greenberg et al., 1971; Senn and Pinkerton, 1972). A number of studies support the concept of active suppression of normal CFU-C proliferation and differentiation by the release of inhibitory substances from leukemic cells, suggesting a disturbance in the negative feedback regulation observed in normal granulopoiesis. The suppressive effect of leukemic cells on colony forming ability is demonstrated in single and double layer in vitro cultures in which leukemic cells from patients with AML are either coplated with normal bone marrow cells or serve as feeder layers (Bull et al., 1973; Morris et al., 1975; Chiyoda et al., 1975; Knudtzon and Mortensen, 1976). In these experiments no colony formation occurs. Maximal

inhibition occurs at leukemic cell concentrations of 5% to 10% (Bull et al., 1973). Conditioned media of leukemic cells (Chiyoda et al., 1976; Iscove et al., 1971; Knudtzon and Mortensen, 1976) and serum from AML patients (Mintz and Sachs, 1973) also supress the colony forming ability of normal bone marrow cells.

Leukemic suppression of normal hematopoiesis via release of humoral inhibitory factors is supported in animal leukemia studies. Normal colony formation is significantly reduced in agar diffusion chamber cultures implanted into RFM mice (Gordon, 1975). Coculture of normal marrow cells with c1498 mouse AML cells in diffusion chambers results in the complete inhibition of normal granulocyte growth (Miller et al., 1976). A similiar reduction in normal granulocyte proliferation is observed in DC cocultures of normal bone marrow cells obtained from patients with chronic myelogenous leukemia (Boyum et al., 1976). Proliferation of normal bone marrow cells are reduced by 33% in DC's implanted into Shay chloroleukemic animals [SCL] (Vilpo et al., 1973). In vitro colony formation of bone marrow from late stage SCL rats is significantly inhibited with the development of a large number of tight abortive clusters (Handler et al., 1974). Sera from

these late stage leukemic animals and media conditioned with leukemic cells inhibited *in vitro* colony formation by normal bone marrow cells (Handler *et al.*, 1974).

In the present study, abnormal colony formation in *in vivo* PCDC cultures implanted into SCL rats is consistent with disturbances in colony formation observed *in vitro* (Senn *et al.*, 1967; Greenberg *et al.*, 1971; Moore *et al.*, 1973a; 1973b; 1973c; Brown and Carbone, 1971; Robinson and Entringer, 1973; Iscove *et al.*, 1971; Handler *et al.*, 1974). Both the reduction in the total number of CFU-C as well as the formation of abortive colonies are observed. Since the normal bone marrow cells are compartmentalized within the chamber, the suppression of normal CFU-C proliferation and differentiation is directly due to the release of inhibitory substances from leukemic cells and thus, is not the result of a dilutional or "crowding out" effect by the expanding leukemic cell population. The reduction in CFU-C in the early stages of the leukemia ($<$10% marrow blasts) tends to support this conclusion and is in agreement with observation of reduced colony development in preleukemic states (Greenberg *et al.*, 1971; Senn and Pinkerton, 1972). While clusters are believed to be derived from leukemic elements in human AML, the origin

of tight clusters observed *in vitro* from SCL rats is uncertain (Handler *et al.*, 1974). The present study suggests a normal cell origin. Clusters developing in isolated PCDC cultures are derived from the normal bone marrow cells seeded into them. The formation of abortive colonies *in vitro* and in PCDC may, therefore, be linked to an effect of leukemic inhibitory substances on normal cells in the later stages of their differentiation (e.g. myelocyte).

In recent years the work of Rytomaa and others have brought into focus the role of granulocytic chalone in the negative feedback control of normal hematopoiesis and its relationship to suppression of normal granulopoiesis during the pathogenesis of leukemia (Rytomaa, 1973; 1976; Vogler and Winton, 1975; Lozzio, 1975). In general, chalones are tissue specific, species non-specific regulator substances which are produced within the same tissue upon which they act, and which inhibit cell proliferation in a reversible, non-cytotoxic manner (Bullough and Rytomaa, 1965; Bullough, 1965; Maugh, 1972). Granulocytic chalone extracted from mature granulocytes and isolated from serum and spleen, is partially characterized as a low molecular weight (< 5000 daltons) polypeptide with a chain length of 20 to 30 amino acids

(Rytomaa and Kiviniemi, 1968a; 1968b; Rytomaa, 1973; Paukovits, 1971; 1973; Paukovits and Paukovits, 1975). The specificity and mode of action of chalone is examined by various in vitro techniques as well as the in vivo diffusion chamber (Rytomaa, 1973; 1976; Vogler and Winton, 1975).

Early studies on chalone activity are demonstrated in autoradiographic and scintillation analysis of ^{3}H-thymidine uptake by bone marrow cells in short term in vitro cultures (Rytomaa 1968a; 1968b; 1968c). The granulocytic chalone significantly decreases the number of labelled granulocyte precursor cells without changing the labelling index of other bone marrow cells [e.g. erythroid precursors] (Rytomaa, 1968; 1968b, 1968c). Extracts of erythrocytes only decrease the labelling index of erythrocyte precursors (Kivilaakso and Rytomaa, 1971). Paukovits demonstrates that a population of ficoll density fractionated bone marrow cells, composed of 80% immature granulocytic cells, are strongly inhibited by chalone (Paukovits, 1973). The other subfractions show no inhibition. Lord studies changes in the structuredness of the cytoplasmic matrix (SCM) in response to chalone treatment (Lord et al., 1974a; Lord, 1975). The SCM as measured by flourescence polarization, depends on the physical state of

organization of the cytoplasmic molecules, and its changes reflect the proliferative state of the cell (Cercek et al., 1972; 1973). As cells move into cycle from G_0 or G_1 there is a decrease in flourescence polarization associated with a decrease in SCM. Exposure to chalone results in an increase in SCM compatable with reduced proliferation (Lord et al., 1974a). This effect is substantiated by decreased autoradiographic labelling index in vivo. In addition, granulocytic extracts only affect the SCM of granulocytic cells while lymphocyte and erythrocyte extracts affect only their respective cell types. Reversibility of the chalone effect is demonstrated by the return of SCM and the in vivo labelling indices to pretreatment levels following a cellular wash (Lord et al., 1976).

The effect and specificity of the granulocytic chalone is also examined in in vivo diffusion chamber studies using exogenous and endogenous chalone. In two studies injection of granulocytic chalone into mice implanted with DC's containing normal bone marrow cells results in a 26% and 15% to 30% inhibition of ^{3}H-thymidine uptake into proliferating granulocytes (Benestad et al., 1973; Laerum and Maurer, 1973). No effect on lymphocytes or macrophages is evident. In

addition, epidermal chalone has no effect on myelopoietic cell proliferation in DC cultures (Laerum and Maurer, 1973). MacVittie and McCarthy (1975) demonstrate a 37% decrease in DC cellularity in host animals receiving multiple injections of chalone. This reduction in cell number is correlated with a decrease in the number of proliferating CFU-C per chamber as determined by recloning harvested cells into secondary in vitro agar cultures, suggesting a direct effect of chalone on the CFU-C. No effect on chamber CFU-S as assayed by in vivo spleen colony formation is evident. Vilpo et al., (1973) implant chambers containing normal or leukemic cells into chloroleukemic rat hosts in which the endogenous levels of chalone are high (Rytomaa and Kiviniemi, 1968c; 1968d; 1970). The number of normal and leukemic cells recovered from these chambers is 33% lower than control values. Chambers containing mastocytoma and HeLa cells implanted into the same chloroleukemic hosts remained unaffected in growth. As measured by fraction labelling mitosis, the proliferative activity of chloroleukemic cells is inhibited when grown in DC cultures implanted into chloroleukemic versus normal host rats (Ferris et al., 1973). Similiar labelling curves are observed in normal and chloroleukemic hosts. Taken together, the

studies described provide evidence for the cell specificity, reversibility and non cytotoxicity of chalone activity.

Although the granulocytic chalone has been shown to reduce DNA synthesis, little is known about the phase of the cell cycle in which chalone exerts its influence or mode of action. Using automatic flourescence cytophotometry, to measure the DNA content of single cells and ^{3}H-thymidine labelled mitosis, Laerum and Maurer demonstrate that chalone arrests DC cultured cells in G_1 phase but not in G_2 or M phase (Laerum and Maurer, 1973). This G_1 effect is supported by autoradiographic analysis (Rytomaa and Kiviniemi, 1968a). These studies also indicate an effect on S phase in which cells that are not arrested in G_1, pass through S phase very slowly. Support for a chalone effect on S phase is demonstrated by a significantly prolonged S phase in chloroleukemic cells cultured in DC's implanted into leukemic animals (Ferris et al., 1973). However, the primary effect of chalone appears to be on the G_1 phase.

The existence of the inhibitory chalone as a normal regulator of granulopoiesis indicates that the stability of the granulopoietic system depends upon the balanced interplay of stimulatory and inhibitory factors (Rytomaa, 1975; Vogler

and Winton, 1975; Lozzio, 1973). Therefore, as part of its pathogenesis, the suppression of normal granulopoiesis in human and animal AML may be related to imbalances in the relationship between stimulatory and inhibitory regulators. Due to the excessive loss of chalone from leukemic cells, the endogenous levels of chalone in the serum of Shay chloroleukemic rats are abnormally high (Rytomaa and Kiviniemi, 1968c; 1968d; 1970). While granulocytic chalone is produced by both normal and SCL cells, chloroleukemic cells contain only 1/10 to 1/40 the amount found in normal granulocytes. A progressive increase in the percent leukemic myeloblasts in the bone marrow during the course of the SCL leads to a concomitant elevation in the serum level of chalone. Inhibition of normal bone marrow proliferation and the development of abortive clusters *in vitro* in late stage leukemic animals is related to the increase in percent medullary leukemic myeloblasts (Handler *et al.*, 1974). In the present study, the suppression of normal CFU-C proliferation and differentiation in PCDC cultures implanted into SCL rats is due to the imbalances established in the serum levels of inhibitory chalone with the progression of the disease. In contrast to *in vitro* studies (Handler *et al.*, 1974), abnormal colony formation is

observed in early (< 10% leukemic blasts) as well as late stage leukemic animals indicating that maximal chalone levels for suppression of normal CFU-C proliferation and differentiation are reached early in the disease. This is consistent with the observations of *in vitro* co-cultures of normal and leukemic cells in which maximum inhibition of mormal colony formation is reached at a leukemic cell concentration of 5% to 10% (Bull *et al.*, 1976). The 35% reduction in CFU-C proliferation and differentiation observed in PCDC cultures is in close agreement with, and explains, the 33% decline in cellularity in chambers harvested from SCL rats (Vilpo *et al.*, 1973). In chamber bearing animals multiply injected with chalone a 37% reduction in chamber cellularity is observed (MacVittie and McCarthy, 1975). This reduction is correlated with a decrease in the number of proliferating CFU-C per chamber as determined by recloning into secondary *in vitro* agar cultures. While this suggests a direct effect of chalone on the CFU-C, the reduction of normal PCDC colony formation

in SCL rats provides a direct evidence for the control of CFU-C proliferation and differentiation by chalone. In addition, the formation of abortive clusters suggest a regulatory effect on maturing cells (e.g. myelocyte). The normal colony formation observed in a minority of SCL animals may reflect individual variations in the establishment of complex physiological changes which influence PCDC colony formation during the development of the disease. This is supported by the large variation in percent stimulation of myeloid colonies seen in day 6 implanted leukemic hosts.

Aside from granulocytic chalone a number of other inhibitors of granulopoiesis have been characterized. Their in vivo role, however, is uncertain. The formation of granulocyte/macrophage colonies in vitro is blocked by high molecular weight, non-dialyzable, ether extractable, heat labile (55° for 30 minutes) lipoprotein inhibitors found in the sera of humans and mice (Stanley et al., 1968; Chan and Metcalf, 1970; Chan et al., 1971). A possible in vivo role

is demonstrated by fluctuations in inhibitor levels in various hematological disorders (Chan and Metcalf, 1970; Chan, 1971; Metcalf et al., 1971) in single dose and chronically irradiated mice (Chan and Metcalf, 1975; Beran, 1974). The levels of inhibitor as determined by in vitro colony assay decreases 6 hours after irradiation. A second decrease is observed at a time when CSF production by adherent bone marrow cells and CFU-C are increasing (Chan and Metcalf, 1973). Although a direct effect on the CFU-C cannot be ruled out, these inhibitors may act by modulating the production of CSF from colony stimulating cells (Beran, 1975). Metcalf describes a dialyzable, non-ether extractable, heat stable inhibitor which inhibits in vitro colony development (Metcalf, 1971). The physiological significance of this inhibitor is uncertain. Inhibitors with various characteristics have been isolated from mature granulocytes and shown to inhibit colony formation in vitro (Paran et al., 1969; Chervenick and LoBuglio, 1972; Shadduck, 1976;

Broxmeyer et al., 1977). Cell fractionation studies substantiate the ability of the mature granulocyte to inhibit in vitro colony formation (Haskill et al., 1972; Chervenick and LoBuglio, 1972; Baker et al., 1975; Broxmeyer et al., 1976). Since the mature granulocyte is derived from the differentiation of the precursor CFU-C, inhibition of the CFU-C by the mature granulocyte represents a negative feedback regulation. Whether these inhibitors are the same as the granulocytic chalone is uncertain.

VI. ERYTHROPOIESIS IN PCDC CULTURES IMPLANTED INTO ACUTE MYELOGENOUS LEUKEMIC RAT HOSTS:

The development of anemia in human (Killman, 1968), mouse (Lajtha, 1973) and rat (Hoelzer and Harriss, 1973; Handler and Handler, 1976) leukemia is believed to be an erythropoietic lesion in the stem cell compartment. In the Shay chloroleukemic rat, the reduction in nucleated erythroid elements is associated with the progressive increase in leukemic myeloblast content in the bone marrow (Handler et al., 1968). This decrease in the pattern of medullary erythropoiesis is related to the reduction in the numbers and/or the functional capacity of the CFU-E as reflected in the reduction in erythroid colonies formed in in vitro plasma clot cultures (Handler and Handler, 1976). A similiar decline in erythroid colony formation is observed in the present study in PCDC cultures implanted into SCL animals. Both CFU-E and BFU-E are depressed in SCL rats receiving PCDC cultures on days 0 and 4 after leukemia induction. In vitro, decreased erythroid colony forming capacity occurs during the early stages of the disease when leukemic cell infiltration in the marrow is minimal, suggesting that factors other than a dilutional or "crowding out" effect due to the

encroachment by the expanding leukemic cell population are responsible for inhibition of *in vitro* erythroid precursor cell proliferation and differentiation. The reduction of erythroid colony formation in compartmentalized PCDC cultures, in the early as well as the later stages of the leukemia, supports this view and suggests that the suppression of the CFU-E and BFU-E is due to the elaboration of inhibitory substances from leukemic cells. This possibility is demonstrated by the suppression of *in vitro* CFU-E by media conditioned with SCL tumor cells (Bacon and Handler, unpublished). Whether *in vitro* suppression is due to inhibitors specific for erythroid precursors or to non-specific toxic factors is presently under study. The release of specific erythroid inhibitors from leukemic cells explains the early shutdown in erythropoiesis (Handler and Handler, 1970a; 1972; 1976; Handler *et al.*, 1974) and provides a mechanism for the reduction of *in vitro* and PCDC erythroid colony formation. Whether the reduction in erythroid colony development is due to an absolute decrease in the number of CFU-E or depression in erythropoietin responsiveness is unclear.

Although reduced heme synthesis in vitro (Handler and Handler,1972; Chiyoda *et al.*, 1974), reduced ^{59}Fe incorpora-

tion in vivo (Handler and Handler, 1970a; Harriss and Hoelzer, 1974) and reduced RNA synthesis in response to EPO (Handler et al., 1974) suggest a specific suppressive mechanism for erythropoiesis, results in the present study indicate that the mode of inhibition operates via decreased CFU-E responsiveness to EPO. While the loss of EPO responsiveness may be due to an abberant EPO-generating system, the high circulating levels of EPO observed during the pathogenesis of rat SCL (Handler and Handler, unpublished) and human AML (Kranz and Jacobson, 1970) is suggestive of a functioning EPO-generating system. As a result, it is postulated that medullary erythroid cells become refractory to high circulating levels of endogenous EPO induced by the anemia (Thorling, 1965; Zaizov and Matoth, 1971; Chiyoda et al., 1974; Kranz and Jacobson, 1970). The results in the present study are consistent with view. The early inhibition observed in PCDC colony formation is reversed at days 7 and 8 of the leukemia leading to a 150% stimulation in colony formation. This significant increase occurs in response to the hifh circulating levels of EPO induced by the anemia and confirms the existence of a functioning EPO-generating system. Therefore, due to the presence of an inhibitory substance, the

erythroid progenitors in PCDC cultures are suppressed, remain dormant and do not respond to EPO until the levels of EPO are high enough to override the effects of the inhibitor. The sensitivity if the CFU-E to physiological levels of EPO is thus decreased. If the absolute numbers of CFU-E are responsible for reduced erythroid colony formation in PCDC cultures, increased colony development would not be observed in response to high circulating levels of EPO on days 7 and 8 of the leukemia. This view argues against an absolute decline in the number of medullary CFU-E as the mode of erythrocytic inhibition.

The presence of both an intact EPO-generating system and potentially active medullary erythroid progenitors suggests that <u>in situ</u> the ability of CFU-E and BFU-E to undergo differentiation may be related to the unavailability of EPO due to an altered erythropoietic microenvironment. The existence of specific granulocytic and erythrocytic microenvironments in the bone marrow and the spleen and their importence in determining the direction of pluripotential stem cell differentiation has been previously discussed in this thesis. The microanatomy, morphologically composed of a microvascular compartment, a connective tissue compartment

neutral elements associated with both the blood vessels and the stroma, plays an important role in the erythroid microenvironment (McCuskey et al., 1972). These compartments are involved in the regulation and the transfer of metabolites to the microenvironments influencing and modifying cellular reactions and responses. McCuskey et al., (1972) suggest that stem cells committed to the erythroid line will complete their development only in a microenvironment that is highly vascularized, has a high rate of blood flow and contains neutral mucopolysaccharides. During the course of the Shay chloroleukemia, progressive alteration in the bone marrow sinuses are observed (Chen et al., 1972). The destruction of the erythropoietic microenvironment may, therefore, result in the inability of the BFU-E and CFU-E to complete differentiation due to the lack of proper environment and the inability of EPO to reach potentially responsive erythroid stem cells. The present study indicates that inhibitory substances released from leukemic cells may be responsible for the alteration in erythroid microenvironment. This would explain the early decline in erythropoiesis prior to leukemic cell infiltration in the bone marrow.

The decrease of BFU-E and CFU-E in PCDC cultures in the

later stages of the leukemia suggests the decline in the circulating EPO levels due to:

1. Increased levels of circulating inhibitors causing a shift in the balance between stimulatory and inhibitory substances in favor of the latter.

2. Decline in EPO production due to leukemic cell infiltration into the kidney or effects of increasing levels of inhibitors on the EPO-generating system.

3. Direct inactivation of EPO by inhibitors.

4. Increased rate of plasma clearance similiar to that observed in mice infected with Rauscher leukemia virus (Okunewick and Erhard, 1974).

The apparent contrast in percent stimulation of erythroid colonies on day 10 of the leukemia in rats implanted with PCDC cultures on days 4 and 6 after disease induction reflects the individual variations in the establishment of complex physiological changes which influence erythroid colony development during the course of the disease. This is supported by the lack of correlation between percent stimulation of erythroid colonies and percent medullary leukemic blasts and the variation in percent stimulation of erythroid colonies on day 10. In day 6 implanted rats, approximately 40%

of the animals assayed on day 10 show inhibition of PCDC colony formation comparable to that of day 4 implants.

FIGURE # 1:

Number of colonies counted per clot in chambers removed daily from normal host rats. Each point represents the mean of 4 to 10 samples; verticle lines S.E.m.

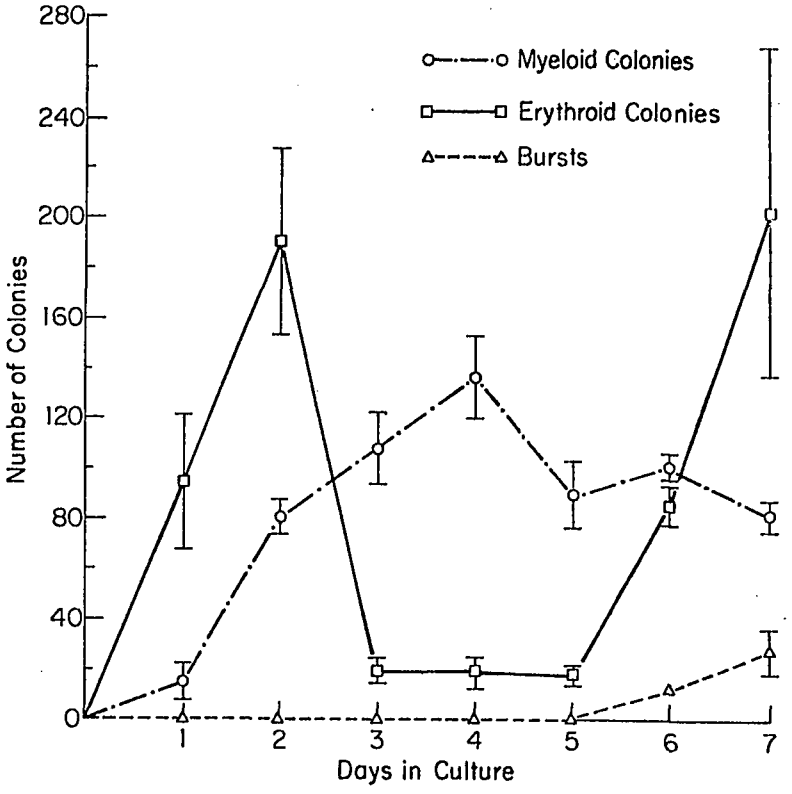

Myeloid Colonies
Erythroid Colonies
Bursts
Number of Colonies
280
240
200
160
120
80
40
0
1
2
3
4
5
6
7
Days in Culture

FIGURE #2:

Numbers of colonies counted per clot in chambers removed daily from rats receiving 20μg endotoxin 4 to 5 hours prior to chamber implant. Each point represents the mean of 3 to 5 samples; verticle lines, S.E.m.

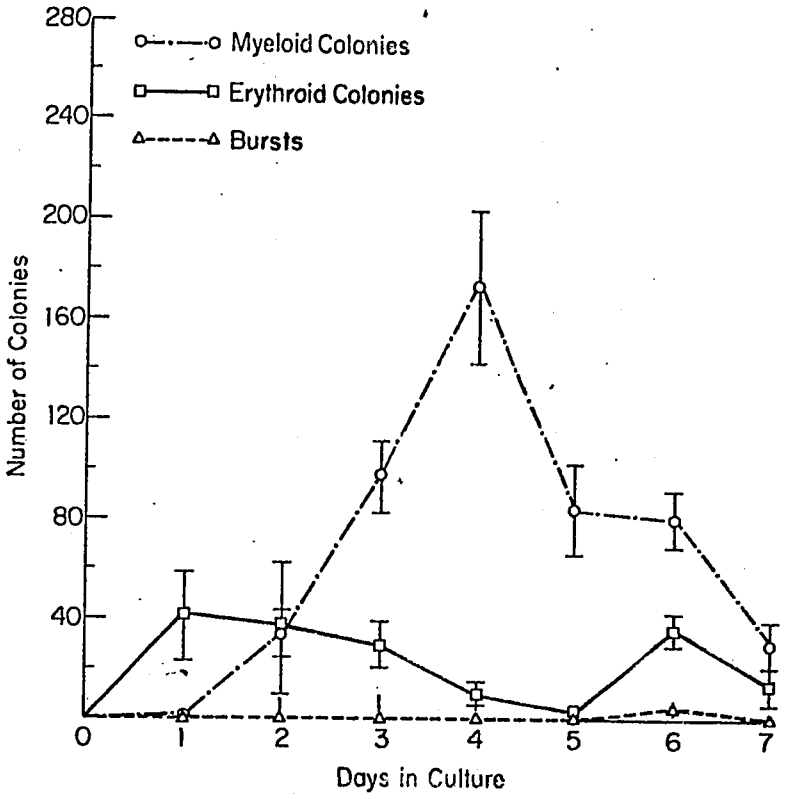

Myeloid Colonies
Erythroid Colonies
Bursts
Number of Colonies
280
240
200
160
120
80
40
0
1
2
3
4
5
6
7
Days in Culture

FIGURE #3:

Numbers of colonies counted per clot in chambers removed daily from rats receiving 100μg endotoxin 4 to 5 hours prior to chamber implant. Each point represents the mean of 3 to 5 samples; verticle lines, S.E.m.

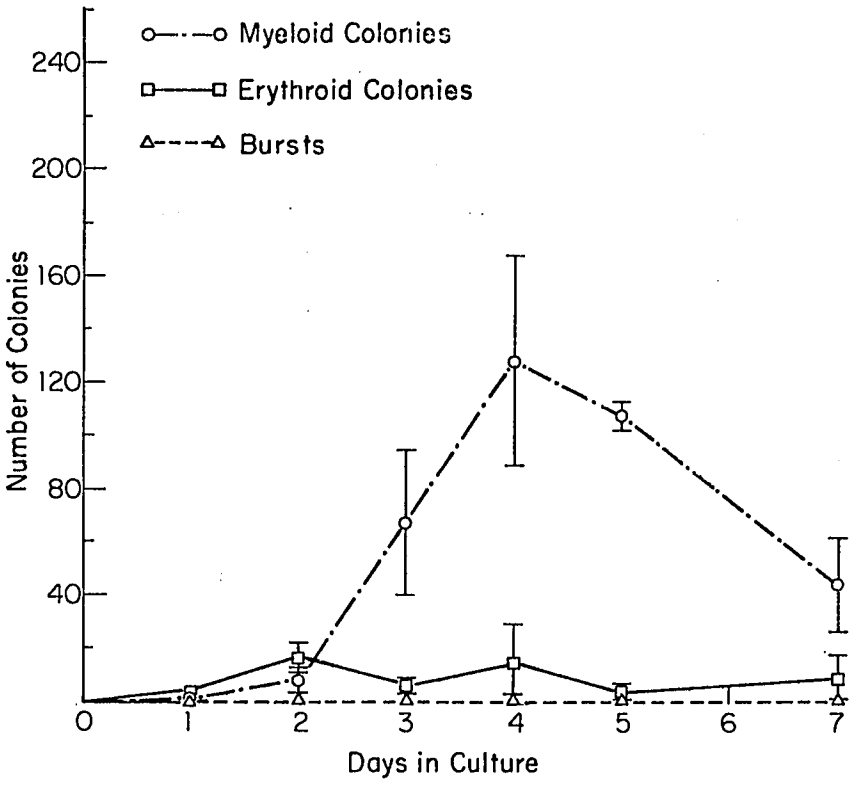

Myeloid Colonies
Erythroid Colonies
Bursts
Number of Colonies
240
200
160
120
80
40
0
1
2
3
4
5
6
7
Days in Culture

FIGURE # 4:

Number of colonies counted per clot in chambers removed daily from rats receiving two injections of 50mg/kg phenylhydrazine; one 24 hours and the second 3 to 4 hours prior to chamber implant. Each point represents the mean of 3 to 12 samples; verticle lines, S.E.m.

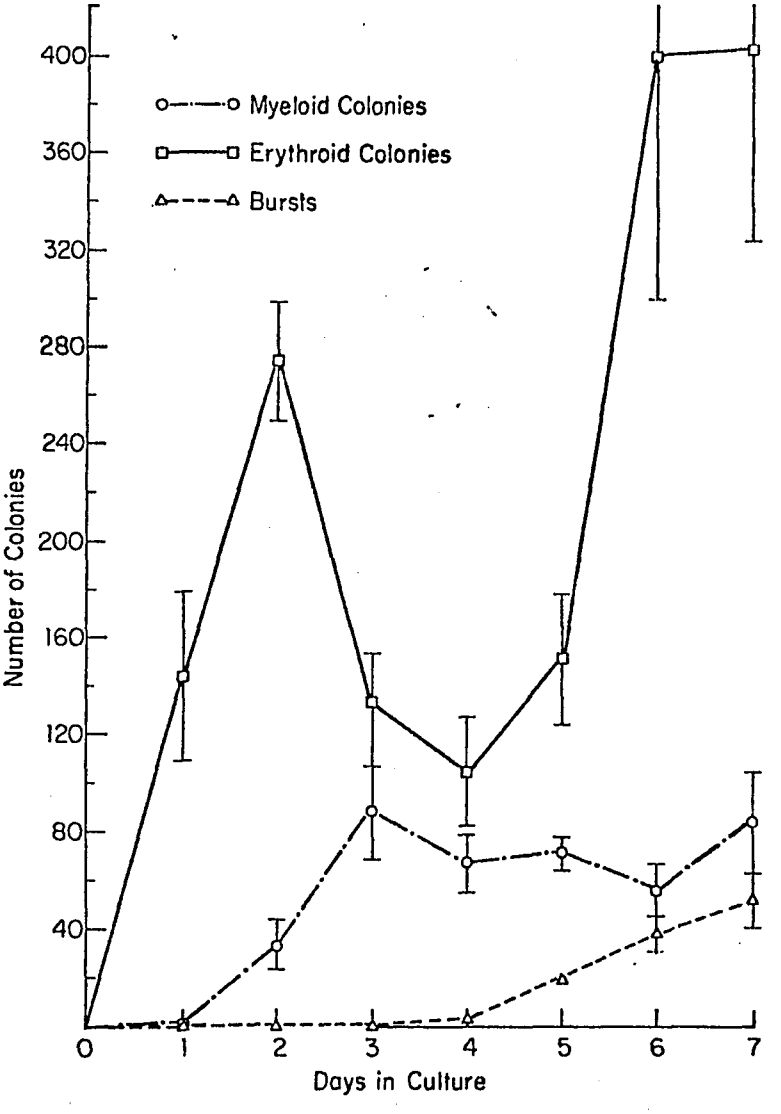

Myeloid Colonies
Erythroid Colonies
Bursts
Number of Colonies
400
360
320
280
240
200
160
120
80
40
0
1
2
3
4
5
6
7
Days in Culture

FIGURE #5:

Relationship between the number of cells harvested from diffusion chamber suspension cultures and the number of myeloid colonies/10^5 normal bone marrow cells cultured in plasma clot diffusion chamber cultures.

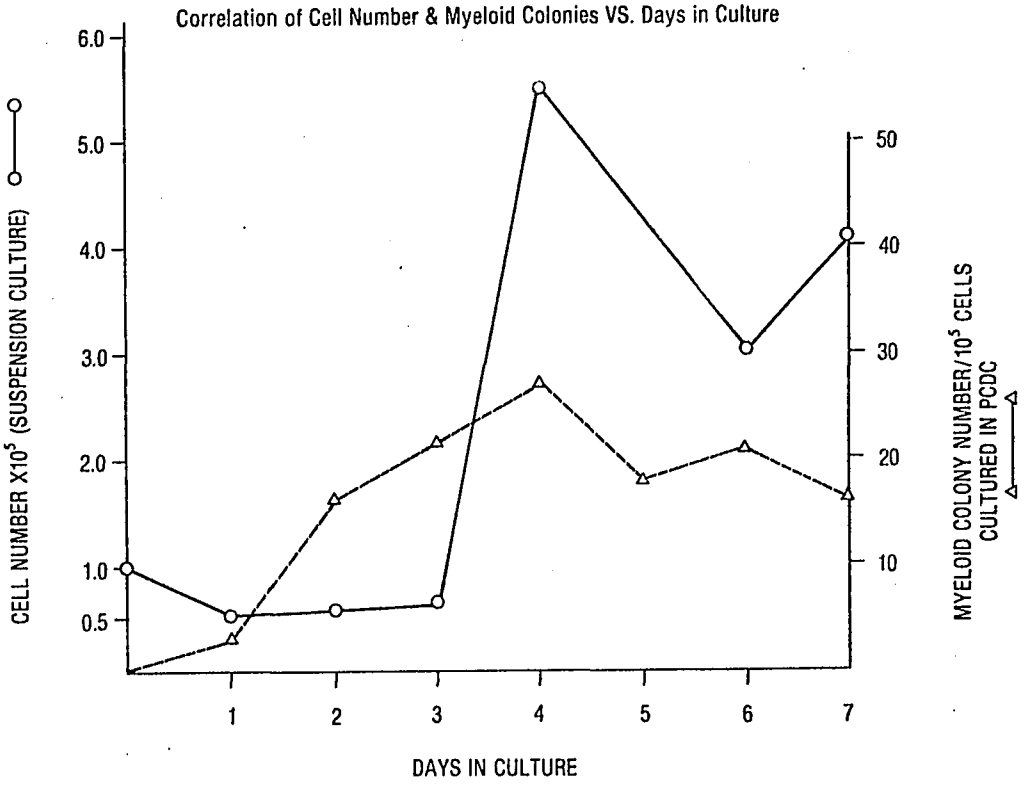

Correlation of Cell Number & Myeloid Colonies VS. Days in Culture
CELL NUMBER X10^5 (SUSPENSION CULTURE)
6.0
5.0
4.0
3.0
2.0
1.0
0.5
50
40
30
20
10
MYELOID COLONY NUMBER/10^5 CELLS CULTURED IN PCDC
1
2
3
4
5
6
7
DAYS IN CULTURE

TABLE #1:

Maturation and size distribution of individual erythrocytic colonies counted per plasma clot in different host animals.

Maturation and Size Distribution of Individual Erythrocytic Colonies Counted per Plasma Clot in Different Host Animals

	Normal			Phenylhydrazine			Endotoxin		
	Early*	Matured†		Early	Mature		Early	Mature	
Day	<8	4–7	>8	<8	4–7	>8	<8	4–7	>8
1	9.0 ± 2.7	46.5 ± 9.0	38.8 ± 13.8	43.3 ± 17.8	50.7 ± 3.8	50.7 ± 10.6	11.3 ± 4.9	19.8 ± 7.3	10.0 ± 5.6
2	1.7 ± 1.2	111.7 ± 20.3	75.0 ± 9.0	23.0 ± 6.9	111.8 ± 13.4	139.8 ± 9.7	3.8 ± 4.3	17.7 ± 7.2	13.7 ± 10.8
3	3.6 ± 1.4	6.1 ± 1.3	8.4 ± 3.0	7.8 ± 3.0	49.3 ± 9.6	73.7 ± 13.0	1.8 ± 0.8	13.4 ± 6.0	13.8 ± 3.9
4	4.4 ± 1.7	6.3 ± 1.5	8.1 ±2.0	10.3 ± 2.7	27.5 ± 4.9	67.1 ± 15.4	2.0 ± 0.4	3.8 ± 1.7	4.0 ± 1.8
5	5.3 ± 1.5	3.6 ± 0.8	5.7 ± 2.2	12.2 ± 3.9	27.8 ± 3.8	111.2 ± 23.7	0	1.2 ± 0.4	1.0 ± 0.5
6	12.1 ± 2.6	0.7 ± 0.1	73.0 ± 12.1	55.7 ± 16.2	50.3 ± 11.9	293.7 ± 83.7	14.0 ± 2.6	6.3 ± 1.5	15.3 ± 4.1
7	28.5 ± 6.5	6.0 ± 0.2	172.5 ± 43.5	29.0 ± 2.1	28.3 ± 5.9	346.3 ± 68.9	3.5 ± 3.5	2.0 ± 2.0	7.5 ± 6.5

*Early denotes colonies composed of basophilic normoblasts that are randomly dispersed within the clot matrix and stain lightly with benzidine.

†Mature colonies are composed of cells in later stages of erythroid development which appear to fall into two size classes.

FIGURE #6:

Daily time course of the changes occuring in the percent hematocrit after two injections with phenylhydrazine; one 24 hours and the second 3 to 5 hours prior to diffusion chamber implant. Untreated animals served as controls.

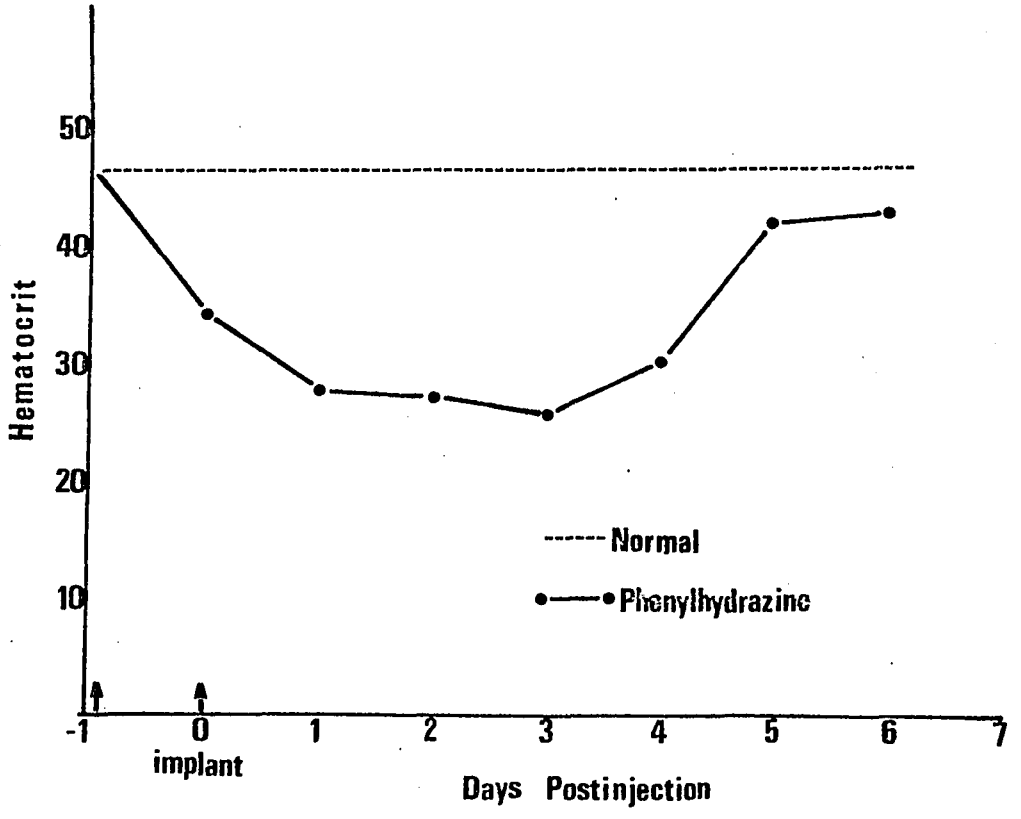

Hematocrit
50
40
30
20
10
Normal
Phenylhydrazine
-1
0
implant
1
2
3
4
5
6
7
Days Postinjection

FIGURE # 7:

Relationship between the numbers of granulocytic colonies counted on day 4 and erythrocytic colonies counted on day 2 clot cultures and the numbers of bone marrow cells seeded into diffusion chambers in normal hosts. Verticle lines, S.E.m.

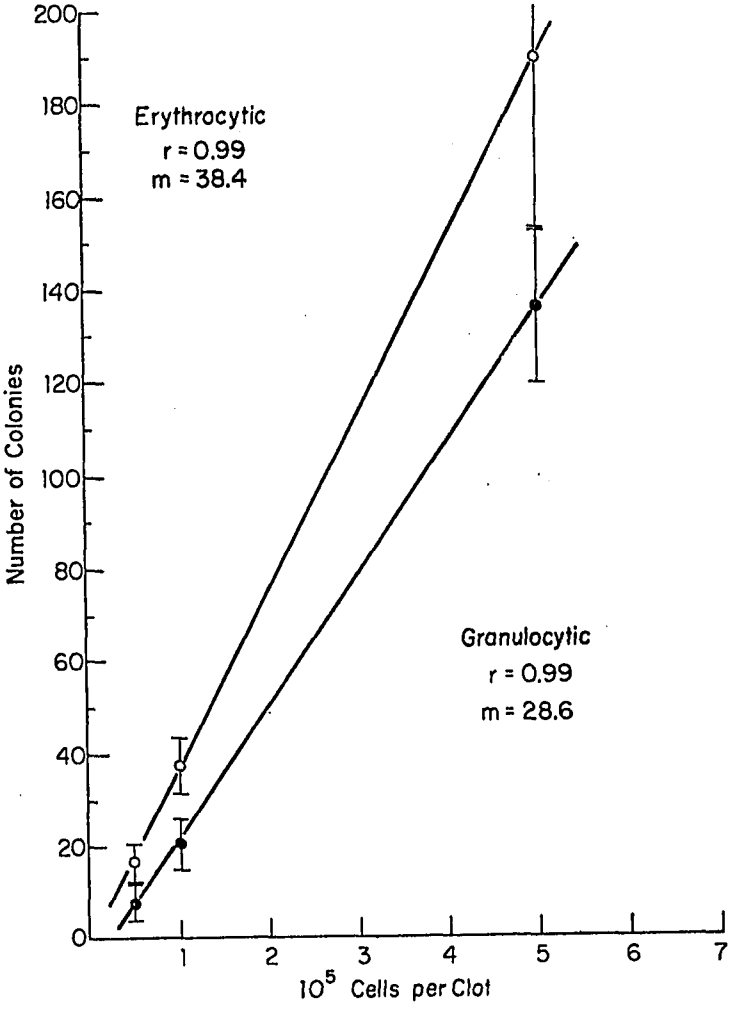

Erythrocytic
r = 0.99
m = 38.4
Granulocytic
r = 0.99
m = 28.6
Number of Colonies
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
10^5 Cells per Clot

TABLE #2:

Number of committed stem cells per 10^5 cells seeded into plasma clot diffusion chamber cultures

HOSTS	CFU-E DAY 2	CFU-E DAY 7	BFU-E DAY 7	CFU-C DAY 4
NORMAL	38.1 ±6.9* (6:2)**	41.1 ±11.6 (6:2)	5.5 ±1.9 (6:2)	27.3 ±3.3 (10:4)
PHENYL-HYDRAZINE	54.9 ±4.9 (6:2)	80.7 ±16.6 (6:2)	10.5 ±2.3 (6:2)	17.8 ±2.4 (12:4)
ENDOTOXIN (20μg)	8.2 ±5.6 (5:2)	7.1 ±1.2 (5:2)	0.8 ±0.5 (5:2)	34.6 ±6.2 (5:2)
ENDOTOXIN (100μg)	3.3 ±2.1 (6:2)	3.8 ± 0.7 (6:2)	0.4 ±0.3 (6:2)	25.6 ±4.1 (6:2)

* MEAN ± S.E.m.

** NUMBER OF CHAMBERS SCORED:NUMBER OF EXPERIMENTS

FIGURE #8:

Preincubation of normal bone marrow cells with 0.25U EPO/ml cells for 3 hours prior to chamber seeding of $5x10^5$ treated cells into plasma clot diffusion chamber cultures. PCDC cultures are implanted into normal host rats and harvested daily up to 7 days. Each point represents the mean of 4 to 10 samples (one experiment); verticle lines, S.E.m.

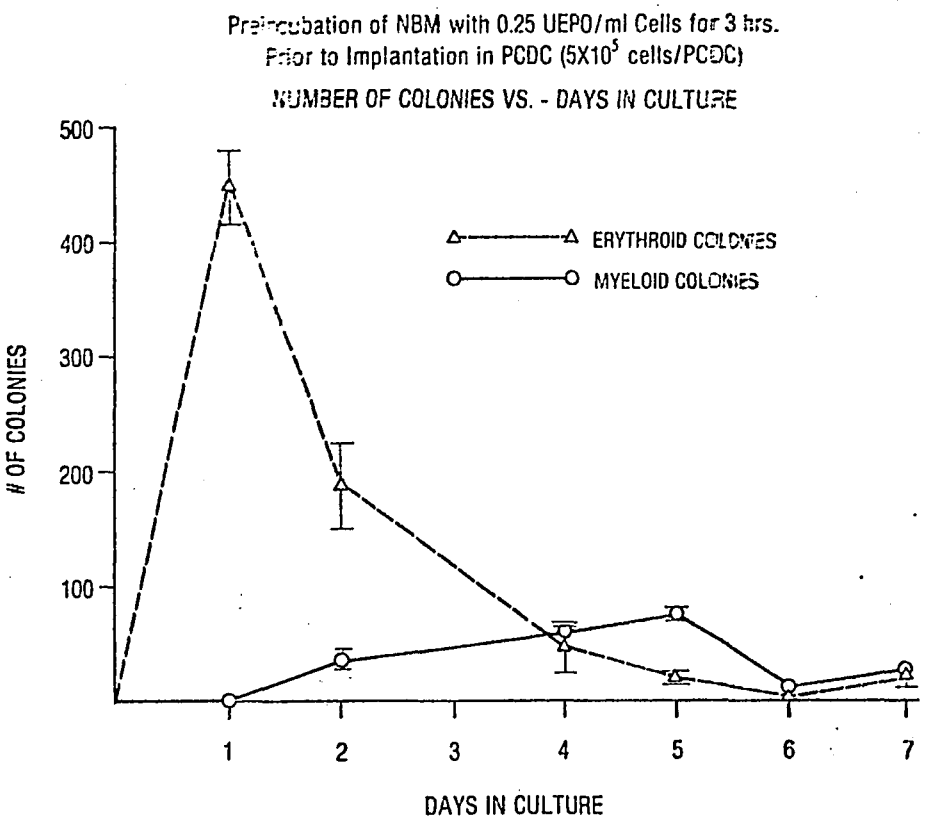

Preincubation of NBM with 0.25 UEPO/ml Cells for 3 hrs.
Prior to Implantation in PCDC (5X10^5 cells/PCDC)
NUMBER OF COLONIES VS. - DAYS IN CULTURE
OF COLONIES
500
400
300
200
100
ERYTHROID COLONIES
MYELOID COLONIES
1
2
3
4
5
6
7
DAYS IN CULTURE

FIGURE #9:

Myeloid colony formation of normal bone marrow cells in PCDC cultures implanted into leukemic hosts on days 0, 4 and 6 after i.v. induction of the Shay chloroleukemia. Each point represents myeloid colonies as percent of control of 2 to 4 chambers harvested from a single leukemic rat. The bars represent the mean percent of control of all rats harvested on a particular day of leukemia.

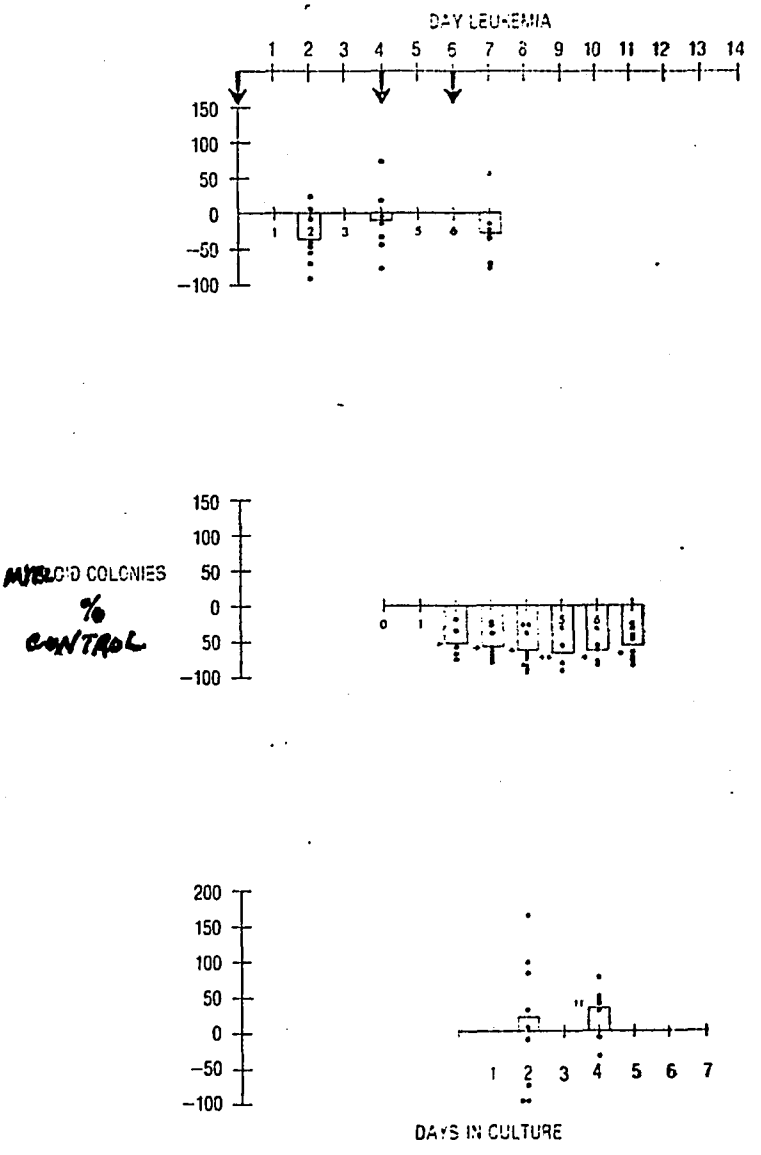

DAY LEUKEMIA
1 2 3 4 5 6 7 8 9 10 11 12 13 14
150
100
50
0
−50
−100
MYELOID COLONIES
%
CONTROL
150
100
50
0
50
−100
200
150
100
50
0
−50
−100
1 2 3 4 5 6 7
DAYS IN CULTURE

FIGURE #10:

Erythroid colony formation of normal bone marrow cells in PCDC cultures implanted into leukemic hosts on days 0, 4 and 6 after i.v. induction of the Shay chloroleukemia. Each point represents erythroid colonies as percent of control of 2 to 4 chambers harvested from a single leukemic rat. The bars represent the mean percent of control of all rats harvested on a particular day of leukemia.

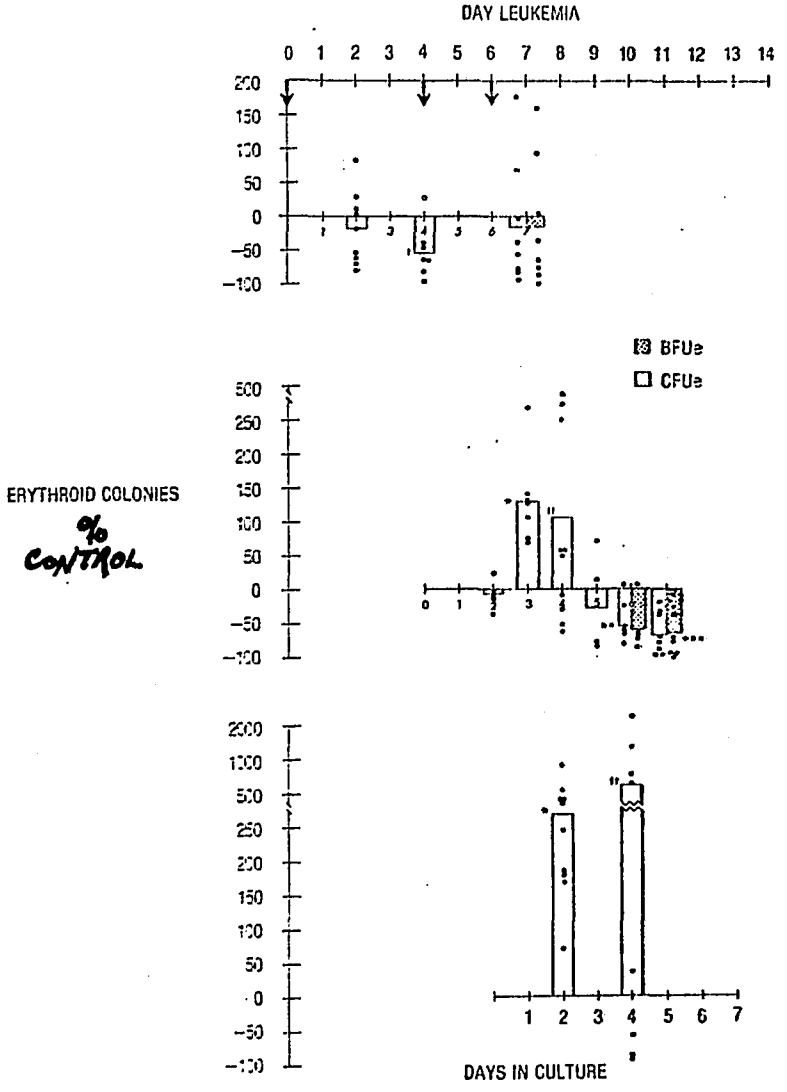

DAY LEUKEMIA
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
200
150
100
50
0
−50
−100
BFUe
CFUe
ERYTHROID COLONIES
% CONTROL
500
250
200
150
100
50
0
−50
−100
2000
1000
500
250
200
150
100
50
0
−50
−100
1 2 3 4 5 6 7
DAYS IN CULTURE

FIGURE #11:

Relationship between the myeloid colonies as percent of control and the percent medullary leukemic myeloblasts. Each point represents data evaluated from a single leukemic animal.

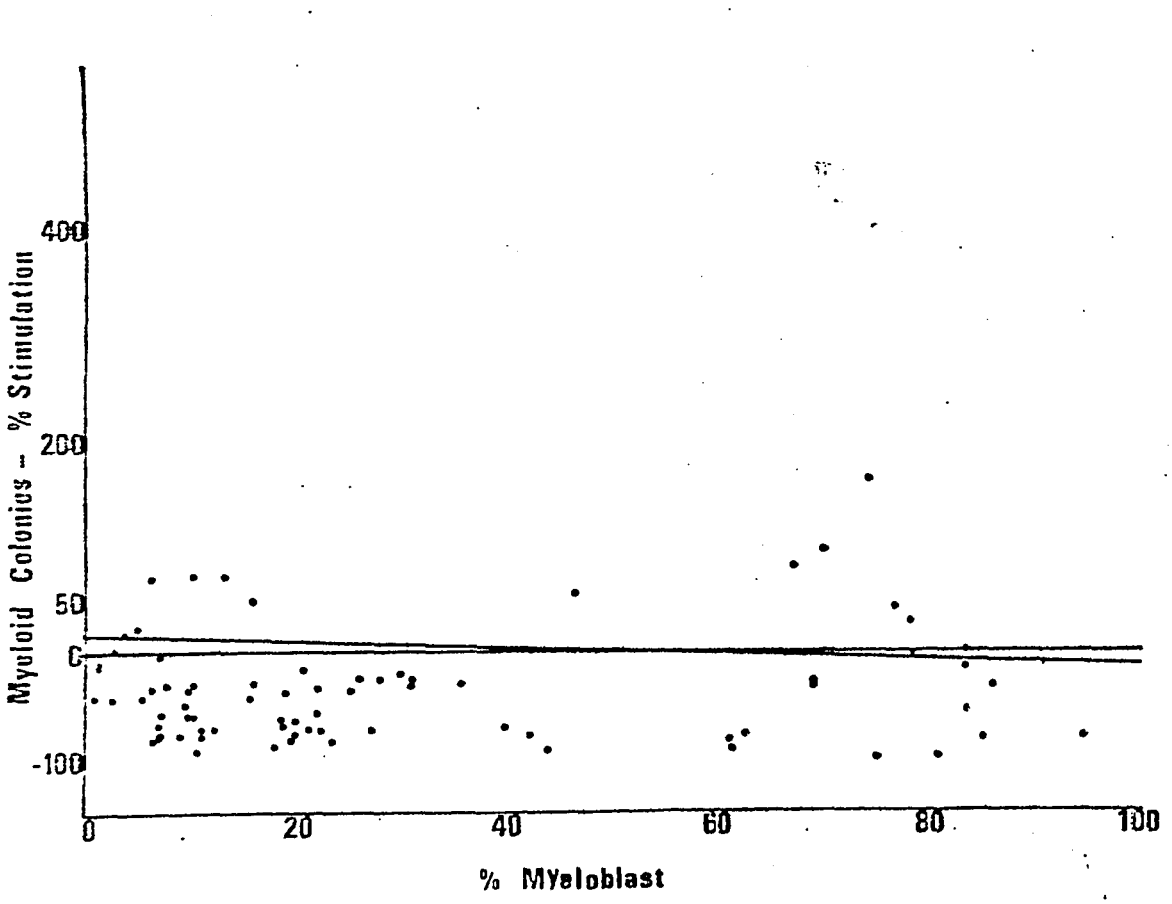

Myeloid Colonies - % Stimulation
400
200
50
0
-100
0
20
40
60
80
100
% MYeloblast

FIGURE #12:

Relationship between the erythroid colonies as percent of control and the percent medullary leukemic myeloblasts. Each point represents data evaluated from a single leukemic animal.

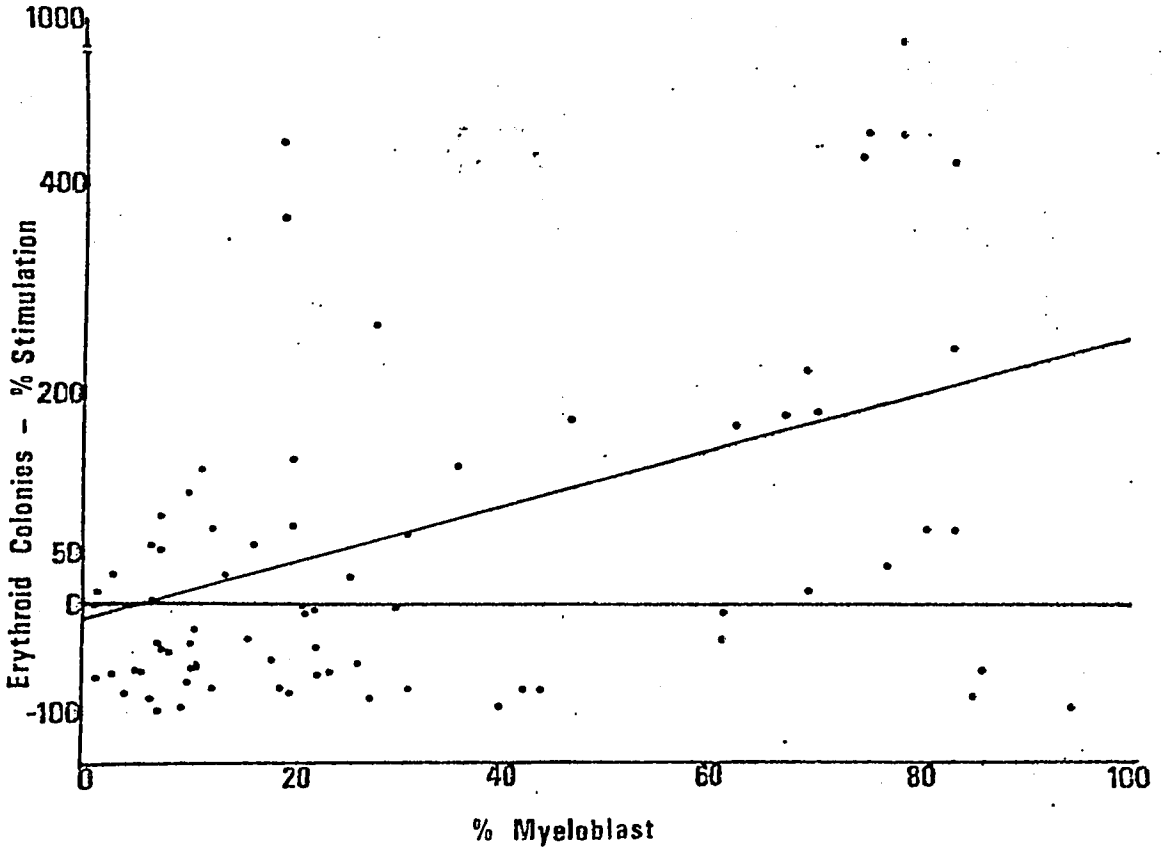

1000
400
200
50
0
-100
Erythroid Colonies - % Stimulation
0
20
40
60
80
100
% Myeloblast

FIGURE #13:

Relationship between the myeloid colonies as percent of control and the percent medullary benzidine positive cells . Each point represents data evaluated from a single leukemic animal.

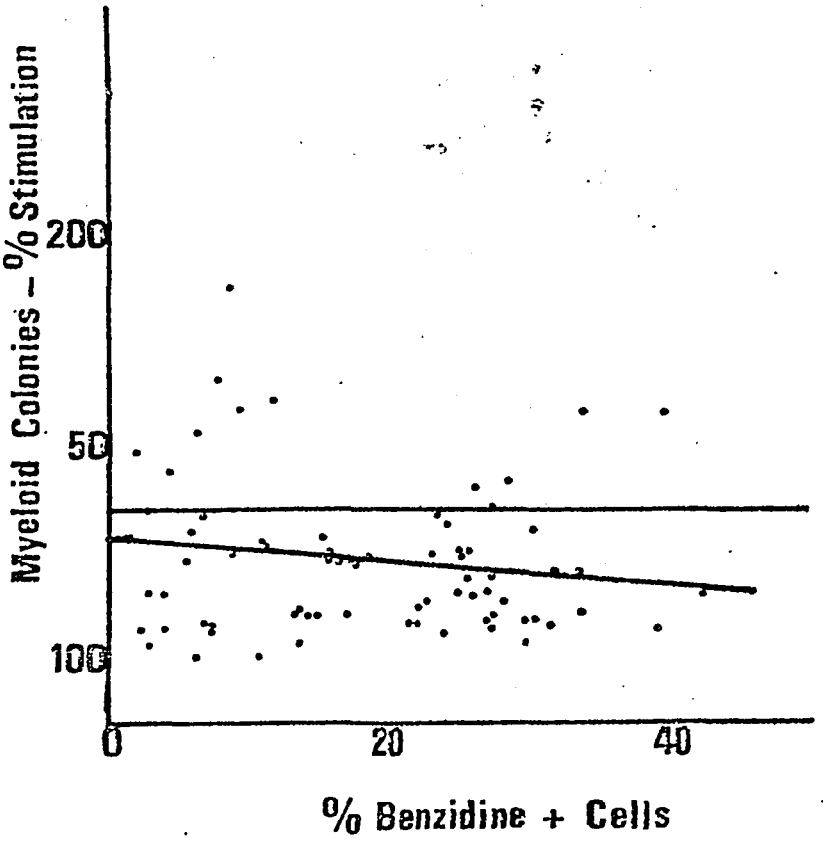

Myeloid Colonies – % Stimulation
200
50
100
0
20
40
% Benzidine + Cells

FIGURE #14:

Relationship between the erythroid colonies as percent of control and the percent medullary benzidine positive cells. Each point represents data evaluated from a single leukemic animal.

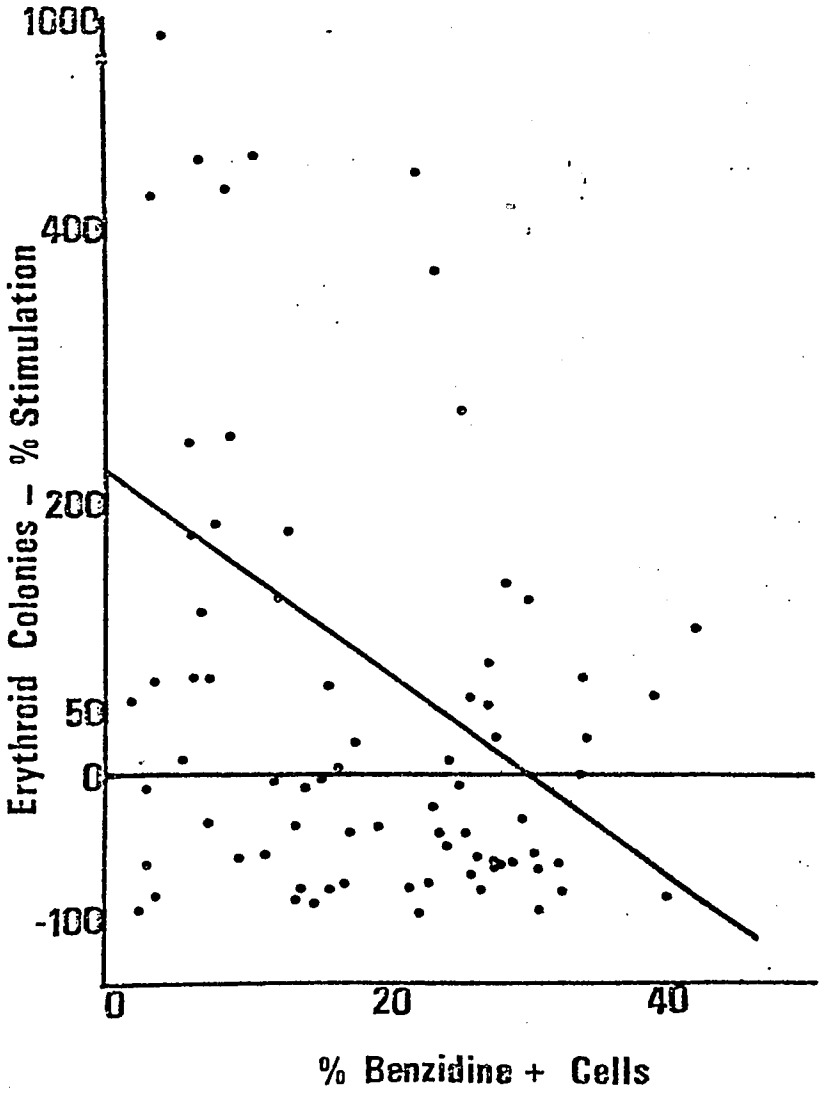
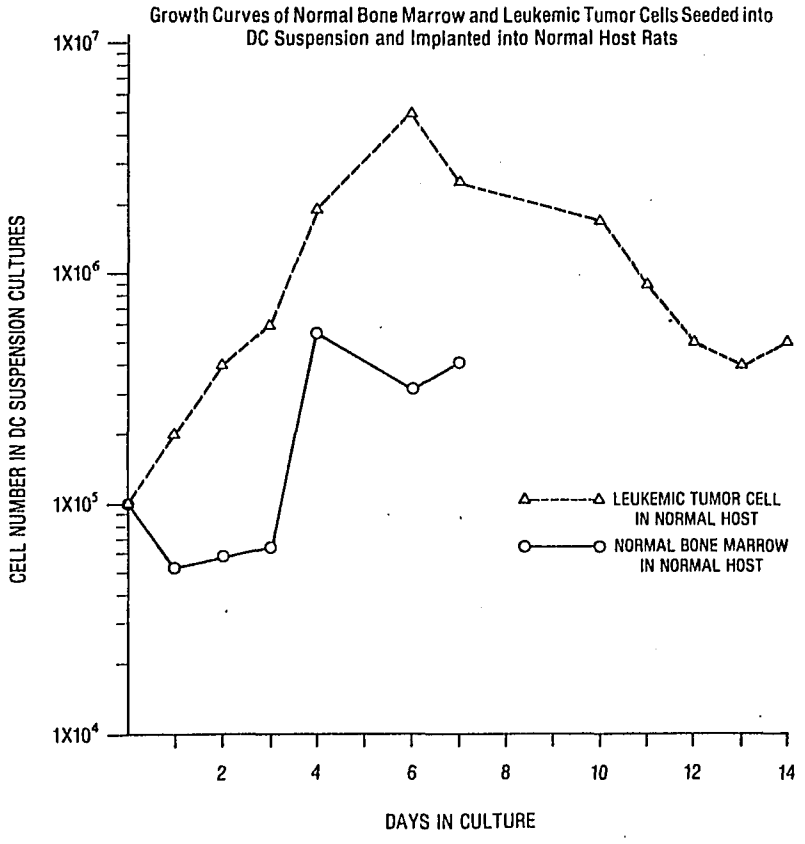

1000
400
200
50
0
-100
Erythroid Colonies - % Stimulation
0
20
40
% Benzidine + Cells

FIGURE #15:

Growth curves of normal bone marrow and leukemic tumor cells seeded into diffusion chamber suspension cultures and implanted into normal host rats. Chamber cellularity is evaluated over a 14 day time course.

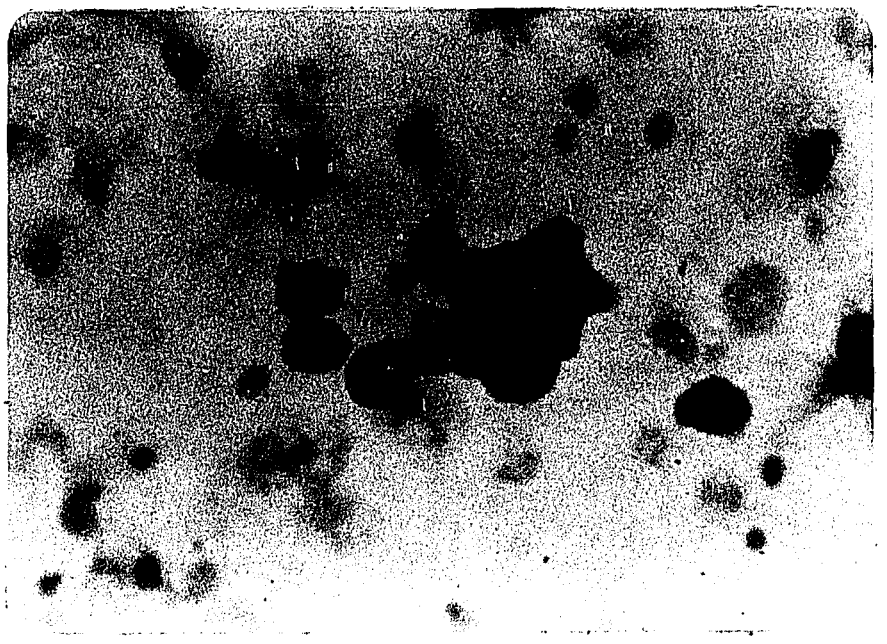

Growth Curves of Normal Bone Marrow and Leukemic Tumor Cells Seeded into DC Suspension and Implanted into Normal Host Rats
1X10^7
1X10^6
1X10^5
1X10^4
CELL NUMBER IN DC SUSPENSION CULTURES
2
4
6
8
10
12
14
DAYS IN CULTURE
LEUKEMIC TUMOR CELL IN NORMAL HOST
NORMAL BONE MARROW IN NORMAL HOST

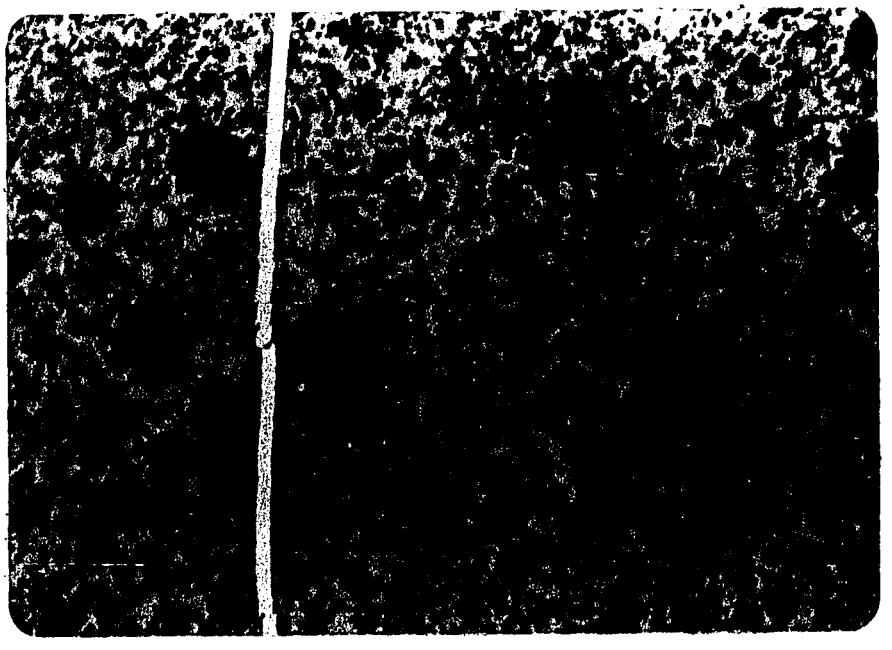
PHOTOPLATE #1

Early Granulocytic Colony Consisting of Myeloblast-Promyelocyte Cells. Magnification: x 1000

PHOTOPLATE #2

Day 4 Granulocytic Colonies Ranging in Size and Degree of Cell Maturation. Magnification: x 160



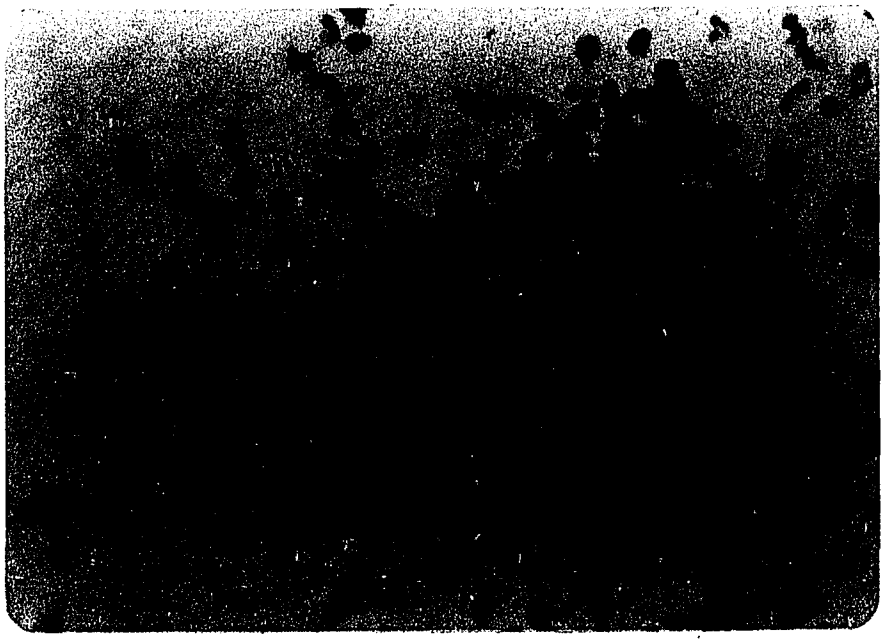


PHOTOPLATE #3

Day 4 Granulocytic Colony Consisting of Myelocyte-Metamyelocyte Cells. Magnification: x 1000

PHOTOPLATE #4

Granulocytic Colonies in the Metamyelocyte-Mature Neutrophil Stage Observed in the Process of Breaking up on Day 5 of Culture. Magnification: x 400

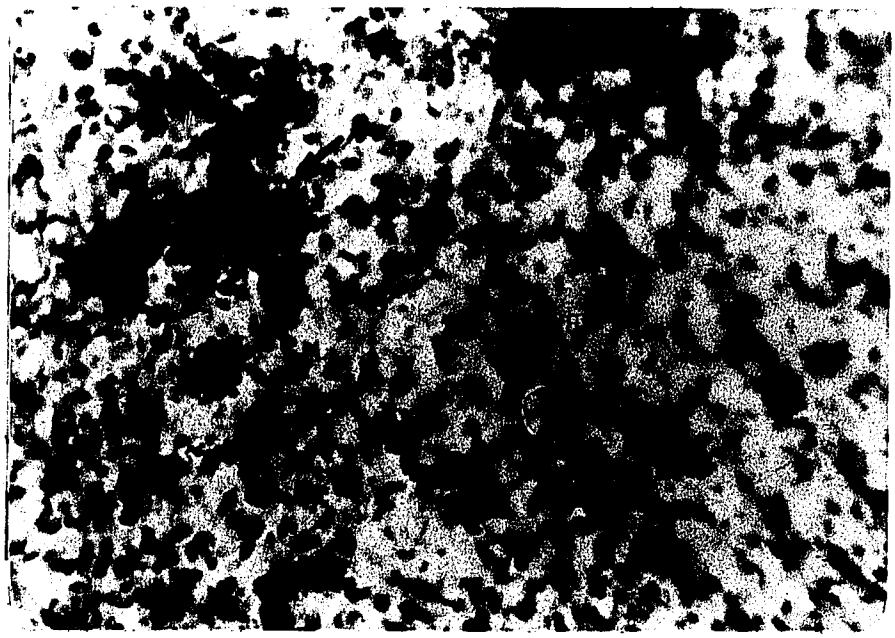


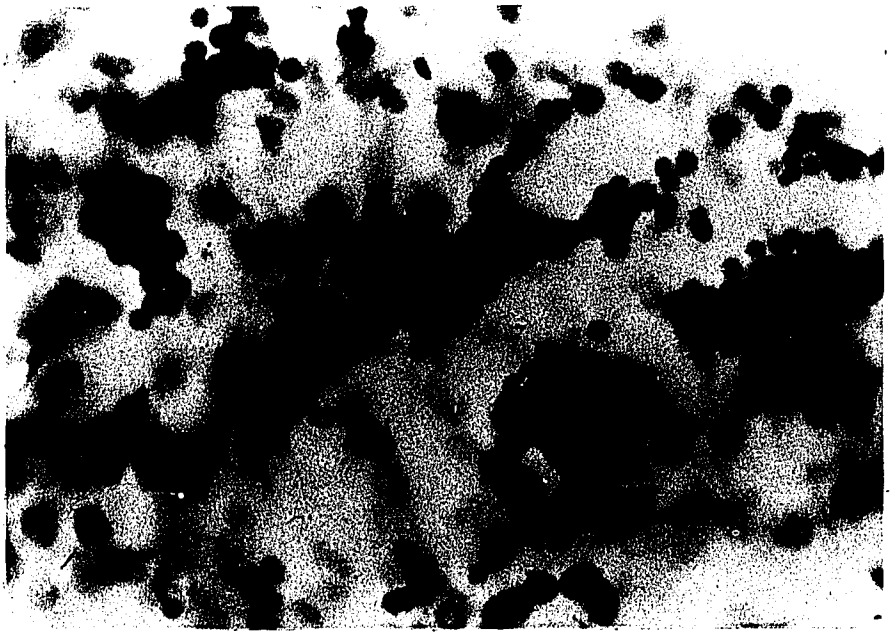
PHOTOPLATE #5

Erythrocytic Bursts: Large Discrete Aggregates Observed on Day 7 of Culture Consisting of 6 to 8 identifiable Erythroid Colonies. Magnification: x 160

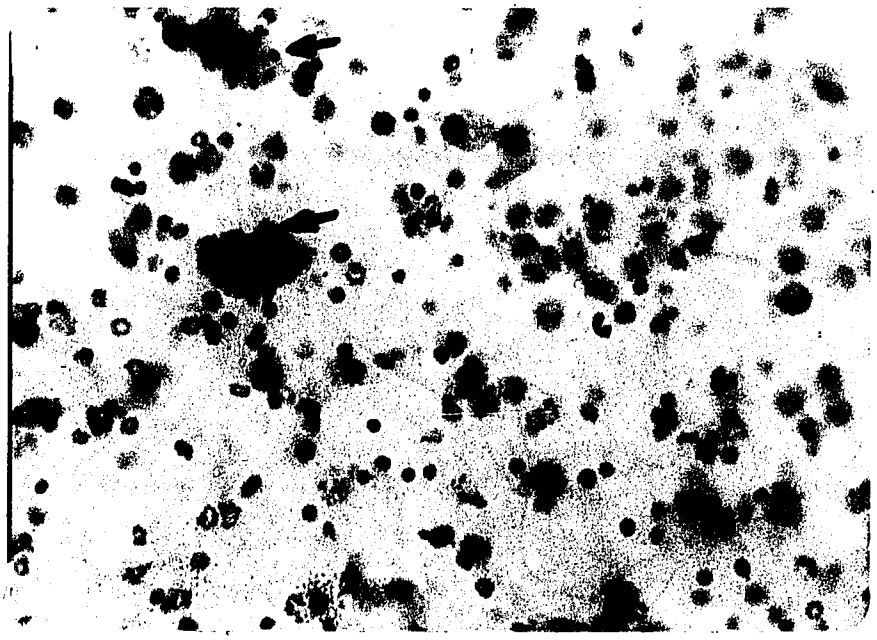
PHOTOPLATE #6

Erythrocytic Burst. Magnification: x 400.





Erythroid Colonies Observed on Day 2 of Culture.
Magnification: x 400



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APPENDIX A

BENZIDINE/WRIGHT'S-GIEMSA STAINING PROCEDURE FOR BONE MARROW SMEARS:

1. Slides are placed in a staining rack and immersed in a staining jar containing 1% 3,3'-dimethoxybenzidine (aged 2 to 4 days) for 2 minutes.

2. Rack and slides are drained on toweling ansd quickly immersed in 7½% H_2O_2 for 1 minute.

3. Rack and Slides are drained on toweling and rinsed in distilled water for 1 minute.

4. Slides are individually drained and placed in a horizontal position on rack.

5. Undiluted Wright's stain, sufficient to cover the slide uniformly, is added for 1 minute.

6. Approximately 2½ ml. of phosphate buffer (Giordano) pH 6.4-6.5 is added to the slide containing Wright's stain and is mixed by blowing on the slide. The diluted stain remains on the slide for 3 minutes.

7. While in a horizontal position, the diluted Wright's stain is rinsed off with tap water. The horizontal position prevents the precipitation of mettalic elements on the smear.

8. Excess water is drained from the slide; slides are again placed in a horizontal position and flooded with freshly prepared Giemsa stain (1/10 dilution of stock in distilled water) for 3¼ minutes.

9. Stain is rinsed as in step 7 above.

APPENDIX B

BENZIDINE/HEMATOXYLIN STAINING PROCEDURE FOR PLASMA CLOT DIFFUSION CHAMBER PREPARATIONS:

1. Plasma clot diffusion chamber preparations are stained by adding the following stains directly onto the clot:

A.	1% Benzidine	2 minutes
B.	7½% H_2O_2	1 minute
C.	Water	1 minute
D.	Hematoxylin	2 minutes
E.	Rinse in running tap water	several minutes
F.	1% Ammonia water	10 seconds

APPENDIX C

MEDIA FOR IN VITRO CULTURE:

1.	Methylcellulose	50 ml.
2.	McCoy's 5A media	36 ml.
3.	Essential Amino Acis	0.8 ml.
4.	Non-Essential Amino Acids	0.4 ml.
5.	Glutamine	0.4 ml.
6.	Serine	0.04 ml.
7.	Vitamines	0.4 ml.
8.	Asparginine (10mg/ml.)	0.16 ml.
9.	Sodium Pyruvate	1.0 ml.
10.	Sodium Bicarbonate (7.5%)	1.0 ml.
11.	Fetal Calf Serum	10.0 ml.
12.	Antibiotics	1.0 ml.

PREPARATION OF METHYLCELLULOSE FOR IN VITRO CULTURE:

1. To 125 ml. of freshly autoclaved distilled water (still boiling), 4g of methylcellulose powder is added.
2. The methylcellulose solution is allowed to cool so that the solution reaches room temperature.
3. 100 ml. of McCoy's 5A (2x) media is added to the methylcellulose solution.
4. The resulting methylcellulose media is stirred for 48 hours, frozen and then thawed to remove granularity.