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AN ANIMAL MODEL OF ERYTHROBLASTIC LEUKEMIA IN THE RAT

City University of New York PH.D. 1979

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AN ANIMAL MODEL OF ERYTHROBLASTIC LEUKEMIA IN THE RAT

by

ETA RENA BACON

A dissertation submitted to the Graduate Faculty of Biology in partial fulfillment of the requirements for the degree of Doctor of Biology, City University of New York.

1979

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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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ABSTRACT

This study presents a new animal model for erythroblastic stem cell leukemia. Young adult rats injected intravenously with $5x10^6$ tumor cells developed erythroblastic leukemia (EBL) with tumor cells infiltrating the bone marrow, spleen, and liver. The pathogenesis was monitored by examining peripheral blood changes with disease progression. Levels of circulating reticulocytes dropped sharply by day 6 post-i.v., indicating a corresponding drop in erythroid production. Hemolytic anemia was also symptomatic of the disease, with 64% of the animals demonstrating a sharp decrease in hemtocrit levels by day 8 post-i.v. Peripheral white blood cell counts along with differentials indicated that granulopoiesis was stimulated. Levels of white blood cells were greater than $40,000/mm^3$, while blasts in the circulation did not exceed 16%. Examination of the cellular composition of the hematopoietic organs showed no significant changes in the early phase of the disease. A marked decrease of nucleated erythroid cells in the late phase was evident. The stem cell compartments assayed for colony formation on plasma clots, or methyl cellulose in vitro, were also affected. Bone marrow erythroid progenitors (CFU-E) were sharply repressed, whereas granulocytic precursors (CFU-C) levels were increased. The spleen, which is normally not hematopoietic, demonstrated in many cases compensatory hematopoiesis; CFU-Es were found at days 4-5, while CFU-Cs were found at days 7-8. It was proposed that erythroid inhibition may be due to the direct action of an EBL cell product. CFU-Es from normal bone marrow, grown in the presence of 20% EBL conditioned media were repressed by 65%; a myeloid tumor (Shay)

conditioned media was capable of repressing CFU-E growth by only 25%.

In conclusion, the results of the present study suggest a specific pattern to the pathogenesis of the EBL which may best be described as specific repression of normal erythroid differentiation concomitant with progressive increase in leukemic cell growth. Furthermore, the EBL may also serve as an effective model for DiGuglielmo's syndrome, a human leukemia, which has also been characterized by ineffective erythropoiesis.

Acknowledgements

It is with great pleasure that I express my thanks and deep appreciation to my sponsor Dr. Eugene S. Handler. The intellectual stimulation he provided, his generous sharing of insight, ready availibilty whenever advice was sought, his gentleness and patience were an immeasureable contribution to my intellectual growth and to the successful completion of my graduate career. I also wish to express my gratitude to Dr. Evelyn E. Handler whose concern and encouragement at the most trying times were particularly meaningful.

My thanks to Ms. Betty Semel, our excellent technical assistant for her invaluable helpfullness, support, and friendship. My thanks also to my fellow graduate student Joan Glick Bieler, with whom I shared the joys and frustrations of research and scholarship, and whose friendship will remain invaluable.

My indebtedness to my family for their loving concern, understanding, and constant readiness to help. I particularly want to mention my father, Rabbi Bezalel Orlanski, of blessed memory, who taught me how to think and how to ask questions and whose vision of the person I could develop into, will always be my constant inspiration.

Finally, my special thanks to my husband, Joshua, whose sensitivity, encouragement, understanding, unselfishness and love has enriched my life, and given impetus to my work.

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AN ANIMAL MODEL OF ERYTHROBLASTIC LEUKEMIA IN THE RAT

INTRODUCTION

A. Erythroleukemia

The concept that the erythorid compartment of the bone marrow could proliferate abnormally and develop into a leukemic disease was first described by DiGuglielmo in 1917. Erythroleukemia also referred to as DiGuglielmo's syndrome, has been defined by Dameshek (1969) as a "self-perpetuating myeloproliferative disorder of undetermined origin". Typically, erythroblastic hyperplasia of the bone marrow, consisting of megaloblastic and normoblastic cells is seen, and in some instances, abnormal multinucleate erythroblasts are found in the bone marrow and in the circulation. The development of anemia, which almost always occurs, is striking in light of the fact that the bone marrow abounds with immature erythroblasts. These blasts, however, demonstrate various deficiencies in heme synthesis, iron metabolism, and other enzymatic abnormalities (Dameshek, 1969). It is currently believed that the genesis of DiGuglielmo's syndrome, as with other leukemic diseases, is due primarily to the inability of the blast to differentiate into a mature functioning cell, rather than to the expanded proliferation of blasts (Gunz, 1973).

Thus, Quiesser et al. (1975) investigated the cell cycle of erythroblasts in erythroleukemia by examining labelling indices of the hematopoietic cells of the bone marrow. A comparison of the percent of erythroid precursors in the S-phase of the cell cycle (assayed by ^{3}H-thymidine incorporation) to the amount of DNA present in the precursor cells (assayed by cytophotometry of Feulgen staining) showed a marked decrease in the labelling index from the basophilic erythroblast to the polychromatophilic erythroblast. A considerable proportion of unlabelled diploid cells in the early polychromatophilic erythroblast compartment was also evident, indicating that these cells were not in cycle. These studies show that levels of early erythroblast proliferation are substantially lower than normal, while more mature erythroid precursors (polychromatophilic erythroblasts) are blocked in G_2, as evident by a striking elevation of DNA content in these cells.

With the knowledge that both proliferation and maturation of erythropoietic progenitors is dependent on the hormone erythropoietin (epo), Adamson and Finch (1970) studies levels of erythropoietin in patients with DiGuglielmo's syndrome, to determine whether the failure of red blood cell precursors to mature could be ascribed to hormonal deficiencies. They reported that as with patients who are anemic from causes other than leukemia (e.g. blood loss, vitamin deficiency, etc.), levels of erythropoietin were highly elevated. Furthermore, erythropoietin production was responsive to levels of erythrocytes in

the peripheral blood evident by the drop in epo levels after whole blood transfusion. Perhaps even more interesting was the finding that changes in the circulating levels of epo affected erythroid blast proliferation in the bone marrow. After transfusion, levels of marrow erythroid precursors, iron turnover, and levels of reticulocytes all decreased. In addition, levels of abnormal multinucleate erythroid precursors characteristic of erythroleukemia also decreased. These findings were interpreted to show that: i) the erythroid hyperplasia of DiGuglielmo's syndrome is to some extent responsive to physiologic control by the hormone epo; ii) the erythroleukemia may well be self-perpetuating; the failure of the erythroid cells to mature into functional red blood cells results in increased epo production (the normal response to erythroid deficiency). The hormone then further stimulates erythroid blast proliferation, but due to the intrinsic defect in the nature of the leukemic blast, cannot push the cells toward maturity to subsequently curtail hormone production. Thus, the progression of the leukemic disease involves two basic deficiencies, the failure of the abnormal cell to develop into functionally differentiated cells, and the inability of normal precursors to respond to physiological control mechanisms such as hormonal regulation of erythropoiesis.

B. Animal Models of Erythroleukemia: Friend and Rauscher Leukemias

The nature of these deficiencies has been investigated in detail by using animal models as an analog to human leukemias. In particular, the studies on animal models of erythroleukemia have concentrated on defining

the specific properties of the transformed leukemic cell which prohibit normal differentiation and maintain the cell in the blast state, with the ultimate goal of elucidating the control mechanisms involved in the expression of differentiation. For example, Friend (1957) described a mouse leukemia which was characterized as an erythroblastosis accompanied by anemia. Infiltration of the spleen, liver and bone marrow by leukemic cells was evident at the terminal stages. Fragments of these organs implanted subcutaneously resulted in tumor development at the site of the transplant. The cells resembled reticular cells and the tumor appeared devoid of erythroid elements. However, maturation could be induced by the adoptive transfer of Friend cells into lethally irradiated mice. In this case, both non-differentiated neoplastic cells and erythroid precursors, e.g. proerythroblasts and normoblasts formed colonies on the surface of the spleen, and the tumor origin of these colonies was clearly demonstrable by the presence of tumor antigen on the surface of these cells (Rossi and Friend, 1970; Friend et al. 1974)

In describing the control process of Friend virus infection, it became clear that Friend virus actually comprised a complex of viruses. Mirand (1968) while working with Friend virus infected spleen filtrates, discovered a strain of virus which has precisely the opposite effect of DiGuglielmo's syndrome. Mirand designated the virus originally reported by Friend, which produces lymphocytosis, erythroblastosis and anemia as FV-A, and the second strain which produces polycythemia along with granulocytosis and reticulum cell proliferation as FV-P. These responses were separated biologically by infecting pregnant mice with FV-P. These ani-

mals developed polycythemia, while the offspring developed anemia. Passage of filtrate from leukemic offspring no longer gave rise to the polycythemic response, indicating loss of the FV-P virus. FV-P which does not cross the placental barrier, initiated erythropoiesis in hypertransfused or exhypoxic animals, mimicking the action of erythropoietin. Stimulation of the erythroid compartment by injection of epo prior to virus infection, enhanced the effectiveness of FV-P virus and resulted in an earlier development of disease symptoms, i.e. splenomegaly, polycythemia, etc. These studies suggested the hypothesis that the target cell for FV-P virus was also the erythropoietin responsive cell (ERC), so that stimulation of the ERC compartment increased the number of targets for FV-P infection. Thus, infection of the same target cell population by either strain of virus appears to have completely opposing effects of the developmental capacity of the erythroid cells. FV-A infection blocks complete expression of erythroid characteristics and anemia develops, while FV-P derepresses the control mechanisms to allow unrestricted erythroid cell expression and proliferation, independent of physiologic control and polycythemia develops.

While the in vivo experiments described above have shown that Friend cells maintain their capacity for differentiation, in vitro studies have attempted to delineate the cellular site where induction for differentiation occurs. Friend cells have been successfully maintained in culture and various clones of FV-A cells have been induced to differentiate into recognizable erythroid precursors through the normoblast stage (Friend et al. 1971). Differentiation of erythroblasts and hemoglobin synthesis has been obtained by culturing cells in a variety of polar and non-polar

compounds e.g., DMSO, butyric acid, ethylene glycol, etc. (Leder and Leder, 1975, Preisler and Lyman, 1975, Tanaka et al. 1975). While the mechanism of action of these inducers is still not clear, many hypotheses have been proposed. Due to the cryoprotective nature of many of the inducing compounds, it has been suggested that the site of action is at the level of the cellular membrane. They postulate that the cellular membrane may have a primary role in the ability of the cell to differentiate, perhaps by facilitating changes in ion flow, or nutrient incorporation into the cell (Preisler and Lyman, 1975). However, Travers (1974) has proposed that the inducers may act at the level of the DNA by opening up the promoter region, which then allows transcription of the differentiative information. It may well be that in transformed leukemic cells, transcription of normal erythrocytic genetic information is blocked by the virus and chemical inducers may interfere with this inhibition to allow the derepression of gene expression. Further support for this hypothesis comes from work by Alter and Goff (1976) who reported that FV-A infected cells grown in the presence of various chemical inducers express different species of hemoglobin upon induction.

Another murine erythroleukemia which has been studied extensively is induced by Rauscher leukemia virus (RLV). Infection with RLV typically results in ineffective erythropoiesis, erythroblastosis in spleen followed by anemia at the terminal stages (Rauscher, 1962). Both Friend and Rauscher diseases are similar and while many studies have been undertaken to define differences in the two systems, the approach taken in these investigations has basically been analogous. For example, Nooter and Ghio (1975) reported that bone marrow cells from RLV infected animals grown

in vitro on methylcellulose developed erythroid colonies in the absence of exogenous erythropoietin. Furthermore, normal bone marrow cells incubated in vitro with RLV could be transformed and would subsequently grow into erythroid colonies in the absence of epo. This effect could be erradicated by neutralizing the virus with anti-RLV antiserum (Nooter and Bentvelzen, 1976). Similar findings by Axelrad and his colleagues showed the growth of FV-P transformed cells on plasma clots also independent of epo stimulation (Liao and Axelrad, 1975, Clarke et al. 1975). FV-A infected cells could also be induced to differentiate into erythroid colonies after exposure to DMSO and developed erythropoietin responsiveness (Goldstein et al. 1974).

In summary, the studies presented above utilizing both viral models for the erythroleukemic disease have essentially focused on elucidating mechanisms of developmental regulation at the cellular level. The investigations have also shown that in vivo manipulations such as plethora induction in exhypoxic animals, or anemia induction by phenylhydrazine treatment or repeated bleedings, influence both the susceptibility of the host to viral infections and the time course of the disease development (Siedel, 1976; Weitz-Hamburger et al. 1975). While these studies may have been designed to demonstrate in vivo factors which may affect the differentiative capacity of virus infected cells, it has also been determined that the in vivo manipulations are, in fact, affecting the size of the target cell pool. Moreover, increases in numbers of leukemic cells have been found to be primarily due to ongoing viral infection of cells during disease development (Tambourin and Wendling, 1971). While the utilization of these leukemic models has enabled the examination of the nature of

leukemic cells, differentiation restriction, cell cycle parameters of infection etc., the horizontal infection of the hematopoietic stem cell compartments by virus introduces a new complicating factor generally not analogous to the human disease. Thus, while there are instances reported in the literature which indicate a viral etiology for human leukemias, e.g., the involvement of Epstein Barr virus in Burkitt's lymphoma (Fenner and White, 1970), there is strong evidence supported by chromosomal studies to indicate a clonal origin for AML, CML, and erythroleukemia (Rundles, 1973). Another major issue which the viral model leaves unresolved is why the expression of "normal" hemic elements is restricted by leukemic cells, and what is the mechanism of this restriction. The purpose of the present series of studies is to directly address these issues by using another class of animal model for erythroleukemia. Here a limited number of leukemic cells are introduced into normal animals (rats). This allows investigation of the dynamic process of leukemogenesis as well as the interactions between leukemic and normal hematopoietic cells. Furthermore, utilization of the rat as host for erythroleukemic disease eliminates the complication of "horizontal" infection of hematopoietic cells within the host, reported to occur in Friend and Rauscher infections. This is due to the fact that the rat does not readily shed virus even when c-type particles are evident as found in Shay chloroleukemic infection (Chen et al. 1972). Whereas transplantable models for myeloid and lymphoid leukemias have been available for study e.g., Shay chloroleukemia, L5222, L1210, a transplantable model for DiGuglielmo's syndrome until now has not been available.

C. Background of the Erythroblastic Leukemia (EBL)

The erythroblastic stem cell leukemia was originally induced by Huggins and Sugiyama (1966) who administered pulse doses of 7,12, dimethylbenz(a)anthracene (DMBA) to young adult rats for 100 days. Subsequently, 80% of the animals developed leukemias of diffuse hepatic origin. The cells which predominated were large dense basophilic blasts with prominent nucleoli. These large cells invaded both the hepatic sinusoids and the red pulp of the spleen, whereas the integrity of the lymph follicles was preserved until the terminal stages of the disease. Sugiyama (1971) reported that the carcinogen caused chromosomal aberrations in 36% of the cases studied. Bone marrow cell chromosomes were studied under the following conditions, DMBA treatment, plus anemia (induced by bleeding), DMBA and polycythemia (induced by packed red blood cell injection) with and without exogenous erythropoietin administration. Sugiyama (1971) reported that the induction of erythropoiesis by either removing mature red blood cells, or exogenous epo administration enhanced the vulnerability of the cells to chromosome damage by the carcinogen, indicating that the target cell for the carcinogen action was the erythropoietin responsive cell. Thus, when numbers of ERC increased, the numbers of targets for DMBA action also increased. Bird et al. (1972) induced erythroleukemia with pulse doses of 7,8,12 trimethylbenz(a)anthracene (TMBA) later described by Huggins to be a less toxic and therefore more potent carcinogen. The induced erythroleukemia was studied extensively in this report. Advanced leukemia was characterized by a marked reduction in body weight, and by severe anemia. A single feeding of the carcinogen decreased levels of erythropoiesis. After repeated

feedings severe hypoplasia of all hematopoietic elements of the bone marrow was reported. These bone marrow changes coincided with the appearance of erythroblastic stem cells (of unknown origin) in the spleen. Small irregular clusters of leukemic cells were also evident in the bone marrow early in disease development. As the leukemia advanced, the leukemic foci coalesced until ultimately all of the hematopoietic marrow was replaced by leukemic cells. Thus, the carcinogen acted initially to depress normal hematopoiesis in addition to causing the neoplastic transformation of erythroblastic stem cells. The application of carcinogen in order to induce disease may be very meaningful in studying the leukemic process, especially as it becomes increasingly apparent that a great majority of all cancers are due to the carcinogenic action of environmental pollutants (Cairns, 1975). However, there are drawbacks. Not all animals respond to the carcinogen by developing erythroleukemia. In some cases mammary tumors as well as other leukemias were manifested (Huggins, 1966). In addition, pulse doses of carcinogen need to be administered for 50 days before the erythroleukemia may appear. Therefore, a major development in the study of erythroleukemia occurred with the establishment of an erythroblastic stem cell line in vivo. Wise (1974) reported that it was possible to maintain a line of TMBA transformed cell in vivo when the cells were injected intraperitoneally into neonatal rat pups. The cells homed in the liver where they proliferated rapidly, and sequential transplantation of tumor cells from the liver could be readily performed.

In the present series of studies we report that these erythroblastic cells when injected intravenously homed to the hematopoietic organs, i.e.

bone marrow and spleen as well as to the liver, and induced an erythroleukemia. Specifically, the injection of $5x10^6$ EBL cells i.v., into young adult rats (100-120 g) induces a disease which mimics DiGuglielmo's syndrome. The use of young adult rats for subjects as opposed to neonates facilitated detailing the pathogenesis of the erythroleukemia. Hematologic parameters, such as levels of peripheral white blood cells, hematocrits, reticulocyte counts, and peripheral blood differentials were readily monitored. In addition, the functionally defined erythroid and myeloid committed stem cells were examined in this study. In so doing, we have attempted to develop a new animal model of erythroleukemia where the effect of erythroleukemic development on normal hematopoiesis can be assessed, and the interactions of normal and leukemic cells both *in vivo* and *in vitro* can be studied.

MATERIALS AND METHODS

Experimental Plan

The general experimental procedure involved three sets of experiments. In the first series of experiments, leukemia was induced in experimental animals by i.v. injection of $5x10^6$ erythroblastic leukemic stem cells (EBL), which had originally been grown and maintained in neonatal rat pups. At various days post-injection, peripheral blood was drawn from the caudal vein and routine hematological assays were performed, namely, peripheral white blood cell counts, reticulocyte counts, hematocrits and peripheral blood differentials. At the terminal stage of the disease the animals were sacrificed, and the bone marrow, spleen, and liver were removed for *in vitro* assay of both the granulocyte progenitor cell (CFU-C)

and the erythrocytic progenitor cell (CFU-E) in these hematopoietic organs.

In the second series of experiments, the CFU-C and CFU-E were assayed during the middle stages of the disease. In these animals the hematologic analysis was eliminated, because it was found that the peripheral blood picture does not significantly change until the terminal stages of the disease. At the same time that the bone marrow and spleen were removed for growth in vitro, smears were made from these tissues and from the liver in order to follow the progress of the leukemic development.

The third series of experiments investigated the effect of various kinds of conditioned media on normal bone marrow CFU-C and CFU-E growth in vitro. These included media conditioned by normal bone marrow, liver and mature neutrophil cells and two types of leukemic cell conditioned media, one conditioned by EBL cells and the other conditioned by Shay cells (a myelocytic leukemia).

Animals

Randomly bred, hooded Long-Evans rats were used in all experiments. A breeding colony was maintained to provide rat pups for EBL tumor transplant. The experimental animals were California born (Simonsen Labs) males. Animals that provided sera for colony stimulation activity were either California born or Hunter College born males. The animals were housed in stainless steel cages (open bottom for experimental, and closed flat bottom for rat pups) in an air conditioned room and provided with unlimited food and water.

EBL Tumor Induction

The cells were maintained in vivo in neonatal pups. Frozen EBL cells originally obtained from Dr. W.C. Wise at the Medical University of South Carolina (Charleston, South Carolina), were injected intraperitoneally in 1-4 day old pups and grew successfully into hepatic tumors. The life span of the animals injected with $4x10^6$ cells was about 14-17 days. When hepatic enlargement was observed (usually by day 10), the animal was sacrificed by cervical dislocation and the body surface was washed down with 70% ethanol. Under sterile conditions, the abdomen was opened and the entire liver was removed into a 60mm petri dish and bathed with pyrogen-free sterile saline (Abbot Labs, North Chicago, Ill.). The liver was then minced with two scalpels into small pieces. The cells and saline were aspirated up into a 10 ml syringe with an attached stainless steel needle and expelled about four times. Since the leukemic liver is quite friable, this procedure is sufficient to obtain a cellular suspension. The cellular suspension was then filtered twice through a glass wool to remove debris and non-disassociated clumps. Cellular concentration was determined and $4x10^6$ cells were then injected intraperitoneally into 2-4 day old neonatal rat pups with a 1 ml syringe, 26 gauge, ½ inch needle. All the animals in the litter were injected, and the pups were returned to their mother. When cells were removed for storing in liquid nitrogen, the same procedure described above was used with the following mofification: The cells were removed in NCTC-109 (Microbiological Labs, Bethesda, Md) and 20% fetal calf serum (Grand Island Biologicals, Grand Island, N.Y.). The cells were frozen at various concentrations ranging from $2-20x10^6$ cells per vial. Dimethylsulfoxide (DMSO) was added to each vial, 0.2 ml of 20% DMSO was added to 1 ml of cells. When the cells were removed from the liquid nitrogen for growth in vivo,

they were rapidly thawed in a shaking warm water bath (37°C). One ml of NCTC-109 was added to the contents of each vial, and the cells were spun down in a refrigerated centrifuge for 5 minutes at 1,000 rpms. The cells were then resuspended in fresh NCTC-109 and injected i.p. into rat pups.

Shay Tumor Maintenance

The tumor cells were grown in California born rats that weighed approximately 50 g on injection. The cells were injected subcutaneously at various doses, ranging from 25-50x10^6 cells. After the tumor was well developed, but before the skin was broken through (approximately 10 days post-injection), the animal was lethally anesthetized, and the tumor was excised in the following manner: The animal was first submerged in a wescodyne solution (3 oz. are diluted with 5 gallons of tap water, West Chemical Products, Long Island City, N.Y.) to surface disinfect and then pinned to a cork board. A cut was made distally to the tumor, i.e. on the opposite side of the tumor location and then with a forceps, the skin was pulled over to reveal the tumor and pinned down. With a small scissors, incisions were made in the face of the tumor. About 15 solid spherical sections approximately 3/4 cm in diameter were picked up with a small forceps and placed in a homogenizing flask. The flask was then filled with sterile saline, up to the neck, and a ground teflon homogenizer was inserted and pushed down gently a few times to disrupt the tumor. The suspension was then filtered twice through glass wool to remove debris and large clumps. An aliquot was removed for counting in a hemacytometer to determine the cellular concentration. These cells could then be injected subcutaneously to grow into a solid tumor, injected intravenously

to produce the leukemia, or grown in vitro for conditioned media.

Leukemia Induction

Leukemia was induced by the intravenous injection of tumor cells. Young adult male rats, weighing approximately 100-120 g were injected with either Shay or EBL cells. The animals were anesthetized with ether and the jugular vein was surgically exposed. $.5x10^6$ tumor cells were injected into the jugular using a 26 gauge one half inch needle on a 1 ml syringe. The incision was closed with autoclips.

Peripheral Blood Analysis

The development of the EBL leukemia was monitored with routine hematological technique on various days post-injection. The procedure was as follows: The animals were first weighed and then wrapped loosely in gauze to facilitate handling. The tails were snipped to obtain the blood samples. The first few drops of blood were discarded. The next few drops were drawn up into a white blood cell pipet and diluted with acetic acid-methylene blue for hemacytometer counting after all the samples were collected. The next few drops were drawn into a heparinized capillary tube for hematocrit (hct) determination. The samples were collected, spun in a hematocrit centrifuge for 5 minutes and the hct values were read from a Clay-Adams chart. The next few drops of blood were drawn into another capillary tube prefilled with new methylene blue stain for reticulocyte staining (see appendix 1a for staining procedure). Peripheral blood smears were prepared with the next few drops. The slides were dried quickly under cold air, fixed with methanol, and subsequentially stained with Wrights-Giemsa. Reticulocyte stained slides were examined at

approximately 1200x under oil. Approximately 1000 red blood cells were counted per slide. Peripheral blood smears were counted at 400x and about 500 nucleated cells were counted per slide.

Preparation of Tissue for Growth In Vitro

The animals were lethally anesthetized with ether and washed with a wescodyne solution. The organs were removed under sterile conditions.

Bone marrow cells were obtained in the following manner: An incision was made on the ventral surface of the leg, the muscle was exposed, and the femur was dislocated from its socket and pushed out through the muscle. Using forceps, the femur was raised and the surrounding tissue and tendons were scraped away with a Jaeger Keratome (Roboz Institute, Washington, D.C.). The bone was cut with a bone cutter as close to the base as possible. The bone marrow was flushed out of the bone with a 3 ml syringe 21 gauge 1½" needle, filled with growth medium into a sterile plastic test tube. The bone marrow samples were kept on ice to prevent clumping. Bone marrow plugs were disrupted in one of two ways. For CFU-C plating, the plug was first gently disrupted by aspirating the cells up in a sterile 10 ml syringe with a long stainless steel needle, and then expelling the cells. This procedure was repeated a few times until the cells were dispersed. The cells were then aspirated up and passed through a sterilized nitex filter. The filter consists of a small stainless steel grid and a small nitex disc (53u pore size mesh, Tobler and Traber, Elmsford, N.Y.), held in place with two teflon washers in a cap and base assembly. The entire unit is attached to a long stainless steel needle. This apparatus serves to remove cellular

debris from the sample, and remove non-disassociated clumps. An aliquot was removed with a sterile wintrobe tube for cell count determination by hemacytometer counting. For CFU-E plating the above procedure may be used, or the marrow plug may be more simply dispersed by gently pipetting up and down with a sterile serological pipet.

Spleen cells were obtained in the following manner: The abdomen was opened by making one large central incision from the posterior to the anterior end of the abdomen. At the base of the original incision two diagonal incisions were made, one on each side going up. The spleen was exposed with a small pair of forceps, cleaned from the surrounding fat and mesentary, and removed with a small pair of scissors into a pre-weighed 60 mm petri dish. The spleen was then weighed and bathed in medium. After all the samples were collected, each spleen was cut in half and flushed with media (in which the spleen was bathed) with a 3 ml syringe 21 gauge 1½ inch needle. When the cells were collected for colony growth, each spleen half was flushed three times, when more cells were needed, i.e. for conditioning media, the spleens were flushed until they became translucent. Once collected, the cells were kept in a test tube on ice.

The liver was the last organ removed due to extensive hemorrhaging in the abdominal cavity which occurs when the hepatic portal system is cut. The entire liver was removed and placed in a sterile pre-weighed 100 mm petri dish, weighed, and then bathed in growth medium. When all the samples were collected, a small piece of liver was removed into a clean petri dish and flushed with medium in the same manner as the spleen. The cells were collected into a test tube and kept on ice. Both spleen and liver cell counts were determined by counting an aliquot in the hemacytometer.

CFU-C Assay

Sera Preparation: The following procedure was used to obtain sera for colony stimulating activity (CSA). Male rats (250g) were etherzied lightly. An incision from the lower abdomen to the rib cage was made and the aorta was exposed. The blood was drawn with a 21 gauge 1 inch needle attached to a 20 ml glass syringe, from the point where the aorta branches into the arteries, which lead into the legs. The blood was expelled into centrifuge tubes and allowed to stand at room temperature to clot. Blood from at least 3 animals was pooled and then centrifuged at 4°C for 1 hour at 12, 100g. The serum was aspirated with a Pasteur pipet and frozen in small aliquots. Before use, the serum was filtered through a millipore filter (pore size 0.20 μ) to sterilize.

CFU-C Plating: Prior to plating 0.2 ml of rat sera, as a source of colony stimulating activity (CSA), was pipetted into each plate before the cells and media were added. Twenty-five ml of media was prepared for each sample, 12.5 ml of methyl cellulose containing McCoy's 5A, previously prepared as in appendix 2a, was mixed with 12.5 ml of McCoy's 5a supplemented with essential and non-essential amino acids, vitamins, sodium pyruvate, and 20% fetal calf serum (prepared as per appendix 2b). To this mixture 0.83 ml$\times 10^6$ cells were added for a final concentration of 10^5 cells per 3 ml. The media with the cells were delivered into sterile disposable 35 mm x 15 mm Falcon plastic petri plates with a sterile disposable 10 ml pipet, 3 ml per dish. Six plates were prepared for each sample, thus the pipet was only filled twice per sample and was then discarded. The plates were gently rotated to evenly distribute the cell mixture and the rat

serum. The cells were grown in a 37°C humidified incubator at 7½% CO_2 for 7 days. The colonies were scored on the eighth day with the aid of an inverted microscope and counted at 78x. Twenty or more cells constitute a colony, 8-15 cells constitute a cluster.

CFU-E Assay

CFU-E Plating: Early in the day, a mixture of beef embryo extract, heat inactivated fetal calf serum, bovine serum albumin, NCTC 109, 1-asparagine, and erythropoietin was prepared (in control groups the epo volume was replaced by NCTC 109) as per directions in appendix 3a. The media was distributed into individual small sterile test tubes and kept at 4°C until plating. The cells were collected in HMEM media previously prepared as per instructions in appendix 3b. The cells were spun down in a refrigerated table top centrifuge at full speed for 10 minutes. The collection media was then decanted and the cell pellet was resuspended in fresh media to a concentration of $5x10^6$ cells per ml. The appropriate cell sample was added to each small test tube, 0.1 ml of cells to each test tube. Citrated plasma (Grand Island Biological, Grand Island, N.Y.) was added to each test tube, 0.1 ml, and the contents of the test tube was quickly and gently mixed by pipetting up and down, and then delivered to each microtiter well, 0.1 ml per well. Each well now contained $5x10^4$ cells. Six clots were prepared from each sample. (The microtiter wells were not u.v. irradiated, but care was taken to prepare the culture dishes under sterile conditions, and there did not appear to be any contamination problem.) An open 35mm x 10mm petri dish filled with sterile distilled water was placed along with the microtiter wells in a large closed petri dish (100 mm) to increase

the humidity within the dish and thereby facilitate colony growth. The cells were grown for 2 days at 37°C in a humidified incubator at 5% CO_2.

Fixing, Staining and Scoring Clots: The clots were rimmed with a microstainless steel spatula and turned out onto a clean slide, 3 clots per slide. A strip of Whatman #1 filter paper 3/4"x2" was placed over the clots. This served to remove excess liquid. When the clots appeared to be somewhat flattened, another strip of filter paper was placed over the present one. Gentle pressure was applied as the paper was lowered, to further flatten the clots. The top piece of paper was removed, and a few drops of gluteraldehyde fixative (prepared as per instructions in appendix 3c) was placed over each clot and allowed to fix for 6 minutes. If the filter paper appeared to have dried, more drops of gluteraldehyde were applied during this interval. At the end of the time period the filter paper was removed (Care must be taken not to allow the clots to stick to the surface of the filter paper . A dissecting needle may be used to loosen the clots from the paper if necessary.) The slides with the affixed clots were placed in a staining rack and gently lowered into a staining dish filled with distilled water and allowed to rinse for 8 minutes. The slides were then removed and dried under cold air. The slides were stained with benzidine and counterstained with hematoxylin following the procedure in appendix 3c. The slides were dried, and coverslips were mounted with Permount (Fisher Scientific Co., N.J.). To facilitate counting the colonies in the clot, a grid of cellulose acetate scored in 1 mm squares was placed over the slide. Eight or more benzidine positive cells constitute a colony, 4-7 constitute a cluster.

Conditioned Media

Tumor cell suspensions were obtained in the manner used to collect the cells for in vivo transplant (see pp 13).

Normal liver cells were obtained from 10 day old rat pups to serve as controls for the EBL cells. The normal livers were removed and prepared for culture in the same manner as the EBL tissue (see pp 13).

Mature neutrophils were collected in the following manner: Adult male rats weighing approximately 200 g were injected intraperitoneally with 0.1 ml of endotoxin (Salomonella typhosa, Disco, Detroit, Mich.) diluted at 5 mg/ml. Three hours later lavage was performed in the following manner: The animals was anesthetized in ether. Approximately 10 ml of sterile saline was then injected into the peritoneal cavity. The animal was slowly rotated and the cells within the peritoneal cavity were withdrawn. The cells were centrifuged in the cold at 1000 rpms for 7 minutes, the saline was decanted, and the cells resuspended in a small volume of growth media. A small aliquot was removed to determine the cell concentration. The procedure could be repeated in a few hours to obtain more cells.

Conditioning the Medium: The cells were all grown at a concentration of $5x10^6$ cells per ml, in McCoy's 5A prepared as per appendix 4. The cells were grown at 37^oC in a humidifed incubator at 5% CO_2. After either 24 or 48 hours, the cells were removed and the contents of each flask was centrifuged in a refrigerated Sorval centrifuge at 5000 rpms for 20 minutes. The supernatant (which is the conditioned media) was decanted, filtered through a 0.20 μ nalge filter and frozen in small aliquots until use. The effect of the conditioned media was assayed by substituting either a portion of the NCTC 109 in the CFU-E culture system or the McCoy's 5A in the CFU-C

culture system.

Culture Smears

Bone marrow smears were prepared in the following manner: The femur was removed as previously described and was split with the aid of a scalpel or strong scissors. A fine paintbrush, pre-dipped in fetal calf serum until saturated, was gently brushed across the exposed marrow and then stroked onto a pre-cleaned slide. The slides were rapidly air dried and fixed with methanol. The slides were subsequently stained with benzidine and counterstained with Wright's-Giemsa as in appendix 5.

Spleen and liver smears were either prepared in a manner smilar to that used for preparing bone marrow smears, or were impressed on slides. For paintbrush smears, a small piece of the organ was excised, a paintbrush dipped in fetal calf serum was brushed across the freshly cut surface of the tissue and applied to a precleaned slide. For impression slides, a small slice of tissue held with forceps was first rinsed either in media or saline, and then directly applied to the slide very quickly. In both cases the slides were rapidly air dried and then fixed with methanol for subsequent staining as above.

Histology

Bone marrow plugs, spleen and liver slices were fixed in buffered formalin. The tissue was embedded in paraffin, and sliced in 7-10 μ sections. Slides were stained with hematoxin and eosin.

RESULTS

In the initial studies, rats were injected with either $5x10^6$, $10x10^6$ or $20x10^6$ EBL cells/100g body weight. In subsequent studies $5x10^6$ EBL cells/100g body weight were routinely administered since it was found that this dose allowed both leukemic cell proliferation and animal survival of up to 15 days post-injection. However, in most cases during the early studies, it was observed that the animals died within 9-10 days of extensive internal hemmorhaging. Therefore, hematologic parameters were followed up until this time, and the animals were then sacrificed for either histological studies or in vitro investigations.

I. Hematologic Parameters

A total of 79 animals were i.v. injected with leukemic cells and an additional 35 animals were monitored as controls. Animal weight, hematocrit, reticulocyte count, peripheral white blood cell count determinations and peripheral blood differentials were performed during the development of the disease. The EBL injected animals were subdivided into four categories on the basis of changes found in these four peripheral hematological measures. Forty-nine animals were classified in group A, as a result of hematological abnormalities in at least two of the parameters measured, and 10 animals were classified in group B_1 because of hematological abnormalities in only one parameter. Group B_2 (11 animals) showed no peripheral blood abnormalities, but upon further investigation showed organ involvement in the disease. Group C (11 animals) showed no peripheral blood or hematopoietic tissue involvement and were assumed for this study to be non-leukemic.

A. Animal Weight: All the animals, both EBL injected and control showed steady weight gain throughout the experiment. The mean weight gain for the most strongly leukemic animals (Group A) for 8 days was 42g $\pm$ 12, for the control group $\bar{x}$ = 48 ($\pm$ 9), the difference in weight gain was not significant.

B. Hematocrit: The development of the disease was often accompanied by anemia at the terminal stages. The frequency distribution of animals as a function of hematocrit levels is presented in Table 1. In group A 30 animals showed the anemic response. Five animals showed hematocrit levels below 30, 9 animals 30-35, 16 animals 36-39, and 17 animals 40 and greater. In group B_1 only 2 out of 10 animals developed anemia (By definition, the hemtalogic parameters were all normal for groups B_2 and C). In order to follow the anemic development with time, the mean values for the daily hematocrits were plotted for the animals in group A which developed anemia (Figure 1). In some cases on the first or second day after injection of the tumor cells, there was a sharp drop in hematocrit. Since this drop was also apparent in some control animals, it is likely that it is not a response to the injection of the leukemic cells per se, but is due to the trauma of the various procedures which are performed on the animals during the first few days of the experiment. Hematocrit levels for the control animals recovered after the second day, while in the leukemics the hematocrit started to drop significantly by day 7. At this time the mean hematocrit was 38 with the lowest value at 31. Hematocrit levels continued to drop reaching a mean of 31 by day 9, with the lowest value being at 15. It should be noted, however, that averaging the daily hematocrit masks the fact that the animals did not develop anemia at the same rate, that is,

in some cases the anemia developed sooner than in other cases. Many of the animals rebounded from the initial post-injection drop with a slight elevation and then the hematocrit levels dropped quite sharply. Thus, some animals were quite anemic while others were experiencing the post-injection rebound. When these values were averaged together, the effects tended to mutually cancel each other, yielding a smooth curve. The elevated standard deviation of the mean hematocrits at the later stage is a clear indication of the disparity of the rates in the individual animals.

The serum from the severely anemic animals was very yellow, indicating a high bilirubin content in the blood. This suggests definite liver dysfunction with disease progression. The hematocrit may serve as a strong indicator of degree of leukemic infiltration in the hematopoietic organs. Hematocrit was strongly correlated to levels of bone marrow blasts, $r = -0.67$ ($p < .001$) i.e., as the blast levels increased in the bone marrow, levels of circulating red blood cells dropped. Similarly, the correlation between hematocrit and liver weight was $r = -0.71$ ($p < .001$). The corresponding conditions in the control group showed no such correlation ($r = -0.08$, and -0.13 respectively). A listwise multiple regression performed with hematocrit as the dependent variable and bone marrow, spleen, and liver blasts and benzidine positive cells (B+) as the independent variables, indicated that the factors which contributed the greatest to changes in the level of hematocrit, are the levels of bone marrow blasts followed by liver blasts, spleen blasts, and spleen benzidine positive cells respectively, $R = 0.68$, $r^2 = 0.46$ ($p < .01$). It is interesting to note that levels of spleen B+ are more strongly correlated to hematocrit levels, than are bone marrow B+.

C. Reticulocytes: Concomitant with the drop in mature circulating red blood cells was a drop in levels of circulating reticulocytes. In group A, 28 animals developed a reticulocyte drop. Table 2 shows the frequency distribution of animals as a function of reticulocyte levels. Twenty-three animals had reticulocyte (retic) levels below 5%, 15 animals had retic levels between 5-10%, and 3 animals showed levels above 10%. In group B_1, 4 animals showed retic levels below 5%, 2 had retic levels between 5-10% and 2 had retic levels greater than 10%. Figure 2 shows changes in retic levels with time for the animals in group A where reticulocytopenia developed. It can be seen that retic levels dropped initially at day 1, remained relatively constant for the next 4 days and then declined gradually starting at day 5 post-injection. Thus, the overall drop in retic levels preceded the hematocrit drop by about 2 days. In the control group there was a strong negative correlation between hct and retic at days 7, 8 $r = -0.76$ ($p < .001$), while the leukemic animals showed a weak positive correlation, $r = 0.22$ ($p < .08$). Thus, in the normal animals reductions in hct levels, which may be due to various factors, were associated with accelerated release of immature red blood cells into the circulation, resulting in an increase in retic levels. In the leukemic animal, the drop in hematocrit could not be compensated by input from the hematopoietic systems, since there was a concomitant drop in erythroid precursors (benzidine positive cells) in the hematopoietic organs. The correlation of retic levels with benzidine positive cells in the spleen and bone marrow was $r = 0.65$ ($p < .001$) and $r = 0.61$ ($p < .001$) respectively. Thus, when erythroid precursors decreased in the hematopoietic organs, levels of retics were also reduced. On the other hand, there was no significant correlation in the

control groups between benzidine positive cells in the bone marrow and spleen, and retic levels in the circulation. A multiple regression performed with retics as the dependent variable, showed that the greatest contributing factor to changes in retic levels inthe leukemic animal was levels of spleen benzidine positive cells, followed by bone marrow B+, liver and spleen blast levels, respectively ($R = 0.77$, $r^2 = .60$ ($p < .01$). Thus, in the leukemic animal the drop in levels of circulating reticulocytes was strongly associated with both the drop in erythroid precursors and also with the degree of leukemic development.

D. Peripheral Nucleated Blood Cell Counts: A dramatic rise in circulating nucleated cells (CNC) was observed. While the cells were primarily nucleated leukocytes, i.e. granuolocytes, lymphocytes and monocytes, due to the presence of erythroid blasts within this population, the term circulating nucleated cells (CNC) can be considered more accurate. Table 3 presents a frequency distribution of animals as a function of CNC levels. In group A, 38 animals showed a significant increase in levels of circulating CNCs, while 12 animals did not. The distribution was as follows: greater than $30x10^3/mm^3$, 21 animals; $20\text{-}30x10^3/mm^3$, 17 animals; $10\text{-}20x10^3/mm^3$, 9 animals; below $10x10^3/mm^3$, 3 animals. The greatest number of nucleated cells observed was $77x10^3/mm^3$. In groups B_1 through C, no animals showed CNC counts greater than $20x10^3/mm^3$. In group B_1, 5 animals had CNC counts higher than $10x10^3/mm^3$, and 5 animals had CNC counts below $10x10^3/mm^3$. Figure 3 shows the time course for changes in peripheral CNC levels for the animals in group A that developed leukocytosis. The curve shows a rapid increase by day 5, with the number of circulating CNCs doubling for many cases in a 24-hour period. Levels of circulating CNCs were strongly

correlated to levels of spleen blasts, $r = 0.76$ ($p < .001$), liver blasts $r = 0.56$ ($p < .002$), bone marrow blasts $r = 0.60$ ($p < .001$), liver weight $r = 0.66$ ($p < .001$), and to spleen weight, $r = 0.62$ ($p < .1$)(the low level of significance is due to the low number of cases for this correlation). A multiple regression performed with circulating nucleated cells as the dependent variable, and spleen, liver and bone marrow blasts, as the independent variables yielded an $R = 0.86$, $r^2 = 0.74$ ($p < .01$). This means that approximately 75% of the variability in circulating nucleated cell levels can be explained by the variations in levels of blast cells in the hematopoietic organs.

Differentials performed on peripheral blood smears showed wide variations in levels of neutrophils during the development of the EBL. The animals which had elevated numbers of circulating nucleated cells also were found to have slightly elevated neutrophil levels (a few cases showed elevated neutrophils by day 3 post-injection; this may be ascribed to the actual stress of the injection and/or sampling). In order for the percentage of neutrophils to have remained constant as the CNC levels increased, the absolute number of neutrophils must also have increased. This would indicate increased granulocyte release and activity in the granulopoietic system.

Blast cells were found in peripheral blood circulation at levels as high as 16% blasts at the the terminal EBL stages. In group A, 29 animals showed significant levels of circulating blasts (1% blasts was chosen as the critical point since these levels were not reached by control animals). Eight animals had 0-0.5% blasts; 6 animals had 0.5-0.9% blasts; 16 animals had from 1.0-3.0% blasts; and 13 animals had greater than 3% blasts. Although

some animals showed circulating blasts at day 1 after injection, significant levels of circulating blasts were not reached until day 7, $\bar{x}$ = 2.2%. In some cases, levels of blasts increased dramatically from day 6, rising in one animal from 0.3 to 4.7% in a 24-hour period. The mean blast count at day 6 was 0.8%.

Levels of blasts in the peripheral blood showed the strongest correlation to levels of spleen blasts, $r = 0.65$ ($p < .001$) and a somewhat lower correlation to bone marrow blasts, $r = 0.40$ ($p < .02$). While there was no significant correlation to liver blasts, $r = 0.28$ ($p < .15$), peripheral blast levels were significantly correlated to liver weight, $r = 0.44$ ($p < .02$). The multiple correlation coefficient of peripheral blood blasts with these three independent variables yielded a multiple $R = 0.72$, $r^2 = 0.53$ ($p < .05$) showing that changes in spleen, liver and bone marrow blasts may account for 53% of the variation in circulating blast levels.

II. A. Organ Involvement

As indicated previously, the peripheral blood parameters serve to some extent, as indicators of the degree of organ involvement in the disease, while also being symptomatic of disease progression. The hematopoietic organs were therefore examined at two intervals post-injection, at 4-5 days (early to mid-leukemic stage) and 7-8 days post-i.v. (late leukemic phase). Dramatic changes in the appearance of the liver and spleen were visible at the later stages. During EBL development, the liver enlarged in size from a mean weight of 10.1 g to a mean of 23.1 g, with some cases reaching as high as 33 g. The liver became granular, and mottled in appearance; whereas, a smooth red appearance is characterstic of a healthy liver. Similar

changes occurred in the spleen. The mean weight for control spleens was 0.6 g, the mean for EBL spleen was 1.9 g with a high recorded at 2.3 g. The gross appearance of the spleen changed, becoming lighter in color, grainy in appearance and growing longer and broader. The bone marrow also changed in appearance with the marrow plug becoming lighter in color. The split femur had a whiteish appearance indicating large areas of fat accumulation and/or decreased circulation. Although total marrow cellularity was not assessed, there were strong indications of a dramatic decrease in marrow cellularity. This was evident in the diminished number of cells which could be obtained from the femurs of leukemic animals for subsequent assessment of in vitro growth.

B. Histology

Histological sections prepared from normal or leukemic bone marrow, spleen, and liver showed further changes occurring during EBL development. Incursions of tumor cells into normal cellular foci were apparent in all the hematopoietic organs. This is most dramatically seen in the liver, where the regular acidophilic cords of cells are disrupted by basophilic tumor cells (See plate 1). In the spleen, tumor cell proliferation was found in the red pulp, whereas the white pulp did not appear to be affected (see plate 2). There were instances in the bone marrow where tumor cells filled the hematopoietic space and normal hemopoietic foci were not evident (see plate 3). The cellular morphology in the hematopoietic organs was more easily demonstrated by examining smears prepared from the hematopoietic organs. As can be seen in plate 4 the EBL blast cell for the most part resembles primitive erythroblasts. The large basophilic nucleus containing 2 or more nucleoli is surrounded by a rim of

densely stained cytoplasm. Therefore, both normal and tumor blasts are included in the values of blast levels in the hematopoietic organs discussed below.

Changes in the hematopoietic organs were not readily apparent at 4,5 days post-injection; however, closer inspection e.g., organ smears with differential counts, revealed that changes did occur in the cellular make-up of these organs, even at the early stage. Cellular composition of the hematopoietic organs in the non-injected controls animals was also examined (see figure 4). In normal animals the bone marrow contained about 2% blasts (number of controls for this gp is 14). Frequency distribution analysis of these controls showed 85% had blast counts 0-2%, and 11% had blast counts between 3-5% and one animal had a blast count of 8%. Levels of blasts in control spleens showed a similar distribution. Eighty-eight percent of the control animals had spleen blast counts between 0-2%, and 12% had a spleen blast count from 3-5%. Liver blasts in the controls were never found to be greater than 2%. As can be seen from figure 4, the distribution of blast cells in the leukemic animal is clearly different from the controls. In the early stages of EBL development (4,5 days post-i.v.), 57% of the animals showed elevated levels of blasts in the bone marrow (above 2% blasts), and 35% showed elevated levels of blasts in the liver. Spleen blast levels, however, were not significantly different from control. At 7,8 days post-i.v., the frequency distribution of blasts in the bone marrow in the EBL animals differs dramatically from control, 78% of the animals had blast counts above 2%. Indeed in 20% of the animals, blast counts higher than 20% were found, and in some cases, there was greater than 50% blasts in the bone marrow. In the spleen and liver, blast pro-

liferation was even more apparent. Eight-nine percent of the animals had spleen blast levels greater than 5%, and 77% had liver blasts greater than 5%. Although levels of tumor cells in the bone marrow were elevated, extreme high levels on the order of 50% blasts occurred most frequently in the livers of the injected animals.

Levels of benzidine positive cells in the hematopoietic organs also changed dramatically with disease progression (figure 5). As discussed previously, the hematologic parameters such as reticulocyte levels began to show changes at about 5 days post-injection. Regression analysis showed changes in levels of circulating retics to be most strongly associated with changes in levels of spleen and marrow benzidine positive cells ($R = 0.75$, $r^2 = 0.56$, $p < 0.01$). Thus, a need to examine the cellular composition of the hematopoietic organs also at the early stages of the disease, i.e., days 4,5 post-i.v., was indicated. In the control group, the frequency distribution of animals as a function of B+ cells in the bone marrow and spleen is a bell curve, with the peak for bone marrow at 20-25% and for spleen at 16-20% B+ cells. No animal showed B+ cells in the bone marrow below 10%, and only 7% of the animals had B+ cells below 15%. Similarly, only 10% of the 4,5 day EBL animals had bone marrow B+ cell levels below 15%, indicating no significant drop in levels of erythroid precursors in the bone marrow early in disease development. Thirty-six percent of control animals had B+ cell levels in the spleen below 16%, while 26% of the 4,5 day EBLs had B+ cell counts below 16% (a 10% difference from control). It would, therefore, appear that the greater than 50% drop in levels of circulating retics was not directly caused by changes in levels of erythroid precursors in the

hematopoietic organs occurring at the early stage of the disease but was due to other factors to be discussed later. Examination of the cellular make-up of these organs at 7,8 days post-i.v. indicates dramatic alterations in levels of B+ cells. In the bone marrow, the greatest number of animals (31%) have B+ cell levels below 5%, while virtually no control or 4,5 day EBL animals had such low levels of B+ cells. The dramatic reduction of B+ cells in 7,8 day EBLs, indicates that the proliferative potential of the erythroid precursor cells present at 4,5 days post-i.v. was impaired. A similar shift occurred in levels of spleen B+ cells. At 7,8 days post-i.v., 65% of the animals had spleen B+ cells below 11%, 25% of 4,5 day EBL and 16% of the controls showed these low B+ cell levels. Thus, at the later stage of the disease there was a dramatic shift in levels in B+ cells in both the bone marrow and spleen. The high correlation between circulating retic levels and B+ cells in the bone marrow and spleen, at the later phase of EBL development, indicates that both may be repressed by a common factor. The evidence does not argue for a causal relationship.

At 7,8 days post-i.v., levels of B+ cells in the bone marrow correlated with levels of bone marrow blasts, $r = -0.57$, $r^2 = .33$ ($p < .001$). Changes in bone marrow B+ cells were strongly inversely associated with spleen and liver weight, $r = -0.88$ ($p < 0.003$) and $r = -0.72$ ($p < 0.001$) respectively, and to levels of spleen and liver blasts $r = -0.62$ ($p < 0.001$) and $r = -0.44$ ($p < 0.03$). Multiple regression analysis with B+ cells in the bone marrow as the dependent variable, and spleen weight and blasts as the independent variables showed R to equal 0.99 ($p < 0.05$). It would, therefore, appear that while levels of tumor cells increased in the other

hematopoietic organs, levels of B+ cells in the bone marrow were concurrently affected. The sharp decline in B+ cells in the bone marrow cannot be simply attributed to proportional changes, i.e. as more blasts are counted, less B+ cells are included in the final value. It is apparent from a scan of a bone marrow smear that the cellular content of the bone marrow was altered. Foci of developing erythroid cells could no longer be found in many cases. If the decline in levels of B+ cells was simply proportional, examination of bone marrow smears without quantitation would show that there were B+ cells, but that they were present in smaller proportions. Furthermore, fully 65% of the animals had B+ cells in the bone marrow below 5%, whereas only 35% of the animals had blast counts above 5%; thus, a much larger proportion of animals showed depressed B+ cells in the marrow, than showed elevated blast counts.

In the spleen, the decline of B+ cells even at the early stage shows the strongest correlation to increases in liver blasts, $r = -0.52$ ($p < 0.005$) and to liver weight, $r = -0.64$ ($p < 0.001$), and showed no significant correlation to levels of spleen blasts $r = 0.002$ ($p < 0.99$). This is not surprising since spleen blasts did not significantly differ from control in this group of rats. In the spleen, levels of erythroid precursors are affected without indications of direct involvement with tumor blasts. In the late leukemics, changes in spleen B+ cells were strongly correlated to liver and spleen weight, $r = -0.77$ ($p<0.01$). Multiple regression analysis with levels of spleen B+ cells as the dependent variable and spleen weight, liver B+ cells and blasts as the independent variables, yielded a multiple $R = 0.92$, $r^2 = 0.85$ ($p<0.05$). Thus, greater than 85% of the variability in levels of spleen B+ cells could be associated with changes in these parameters.

Very low levels of B+ cells are normally found in the liver. In the early EBL phase there were animals whose livers contained greater than 5% B+ cells which was not seen in control livers. As the disease progressed, less than 2% B+ cells were found in EBL livers (see Figure 5). The incidence of liver B+ cells shows a negative correlation with levels of bone marrow blasts ($r = -0.36$, $p < 0.06$), suggesting a pattern of reduction in liver B+ cells as blasts increase in the bone marrow. Liver B+ cells were also negatively correlated to spleen blast levels ($r = -0.37$, $p < 0.07$). Thus, it appears that early in disease development B+ cells are found in the liver. As the disease progresses, levels of B+ drop while blast levels increase.

III. In Vitro Determinations

A. Colony Growth

Examination of the functional activity of hematopoietic precursors of the hematopoietic organs from the leukemic animal may serve two functions. Determinations may be made of the component factors in the hematologic changes that occur in the leukemic animal e.g., changes in retic levels may be explained by looking at the colony forming ability of the erythroid precursors from the bone marrow (CFU-E) as opposed to looking at levels of B+ cells in the bone marrow. One may also look for parameters that may serve as predictors, e.g. changes may occur in the growth potential of erythroid precursors in the early stages of the disease, before external manifestations are evident.

The CFU-E and CFU-Cs were examined from the bone marrow and spleen of the leukemic animals at 4,5 days post-i.v., and at 7,8 days post-i.v.,

the liver was examined for colonies only from the 7,8 day group. The data can be analyzed along two dimensions, the first is to look at changes in growth pattern apparent at the two different intervals, 4,5 day vs. 7,8 day as a function of time. However, in some cases, the post-injection time may be misleading because an individual animal at day 7 may show a degree of leukemic infiltration comparable to the mean value seen at days 4,5. This suggests that the degree of leukemic development, expressed as percent blasts or liver or spleen weight is a second dimension which may be a more generally accurage indicator. The results, however, indicate that both measures are important when analyzing colony growth from leukemic animals.

B. Erythropoiesis In Vitro

Bone Marrow CFU-E: The erythroid colonies were grown as described in the Methods section and were assessed after 2 days in culture. Aggregates of 8 or more benzidine positive nucleated cells were scored as a colony, 4-7 B+ cells were scored as a cluster. Erythroid colonies for the most part were found to contain 32 or more nucleated B+ cells. The 4,5 day series (Group I) and the 7,8 day series (Group II) were done in different seasons and therefore as a result of changes in environmental conditions, and changes in lot numbers of many of the components of the growth media, the data for the controls is not grouped. The mean number of colonies for control Group I was 84.2 colonies and for Group II was 61.3 colonies, indicating that the former, indeed, grew under more favorable conditions. Variations in levels of colonies within the control groups were not significantly cor-

related to levels of bone marrow B+ cells or blasts (see Table 5).

At 4,5 days post-i.v., the variations in levels of CFU-E are most strongly correlated to levels of bone marrow and spleen B+ cells, $r = 0.41 (p < 0.03)$ and $r = 0.45$ $(p < 0.01)$ respectively. Thus, less than approximately 20% of the variations of these values may be attributed to changes in levels of B+ cells in the bone marrow and spleen. The spleen B+ cells which probably do not have a direct effect on levels of bone marrow CFU-E may serve as an indicator of the state of erythroid precursors in the total organism. There was no significant correlation between colony growth and levels of bone marrow blasts $r = -0.17$ $(p < 0.18)$. A multiple regression analysis with CFU-E levels as the dependent variable, and bone marrow, spleen, and liver B+ cells as the independent variables, yielded a multiple $R = 0.55$, $r^2 = 0.31$ $(p < 0.05)$. This showed that all the independent variables in the equation account for only 31% of the variation in bone marrow CFU-E levels, during the early phase.

At 7,8 days post-i.v., there is a negative correlation between bone marrow blasts and marrow CFU-E, $r = -0.35$ $(p < 0.03)$. The relationship between these two parameters is non-linear, and this may account for the low coefficient of correlation. In Figure 6 levels of bone marrow CFU-E are assessed against bone marrow blast levels. Here the animals were divided into groups determined by percent bone marrow blasts. In animals with 0-2% or 3-5% blasts, bone marrow CFU-E at lower than control levels were found. Bone marrow from animals with 6-10% blasts grew colonies at higher than control levels, and in animals with blasts counts above 10%, levels of erythroid colonies were all very much lower than control. The results

indicate an abnormal population of animals, evident in defective CFU-E, among the group of animals with low blast levels. One possible cause of the very low blast counts in this group of animals may be the depressed level of total bone marrow cellularity evident many times in terminal stages of the disease. A multiple regression analysis performed with CFU-E from the bone marrow as the dependent variable, and liver B+ cells liver blasts, spleen B+ cells, spleen blasts, bone marrow B+ cells, bone marrow blasts, and liver weight as the independent variables, yielded a multiple $R = 0.81$, $r^2 = 0.66$ ($p < 0.01$). Thus, while each of the individual factors in themselves are not highly correlated with bone marrow colonies, the combination of factors associated with tumor development accounted for greater than 65% of the changes in colony growth levels. A stepwise analysis showed that the most strongly correlated variables to changes in bone marrow colony levels, were levels of B+ cells in the liver, and liver blasts. Thus, it is apparent that while in the early stages of EBL development, the factors which are most strongly associated with bone marrow CFU-E growth are levels of erythroid precursors in the bone marrow and spleen, at the later stages, however, changes in colony growth are most strongly associated with pathologic changes in the liver.

Bone Marrow Clusters: Erythroid clusters (ECL) were scored both in the early and late phase of EBL. The correlation between levels of clusters and colonies was very high during the initial stages of the disease, $r = 0.95$ ($p < .001$). There was no increase in cluster formation in cases with depressed levels of colonies. At the later phase, the correlation

between ECL and CFU-E was still high, $r = 0.81$, $p < 001$.

Spleen CFU-E:

Levels of erythroid colonies in the spleens of control animals varied. In Group II control, mean levels of spleen CFU-E were of 1.95 colonies per $5x10^4$ cells plated. However, the first control group yielded much higher levels of erythroid colonies, $\bar{x} = 18.5$ CFU-E per $5x10^4$ cells plated. It is interesting to note that the overall colony forming ability was elevated in this group. Here the coefficient of correlation between spleen granulocytic colonies and spleen CFU-E was $r = 0.82$ ($p < 0.004$), whereas there was no significant correlation in the other control group. In Figure 7 levels of spleen colonies are shown for various levels of blasts in the spleen. In the early leukemic group there were elevated levels of spleen CFU-E with increasing blast levels. Some cases showed extremely high CFU-E levels, with one animal showing a mean of 60 colonies per $5x10^4$ spleen cells. In the late leukemic group there were no significant numbers of erythroid spleen colonies, whereas granulocytic growth was evident.

In the early leukemic phase there was a positive correlation between spleen B+ cells and spleen CFU-E, $r = 0.55$ ($p < 0.001$), indicating that in a significant proportion of the cases, the levels of spleen colonies increased as levels of spleen B+ cells increased (see Figure 8). Physiologically, the increase in B+ cells reflects an increase in CFU-E in the spleen. Thus, levels of B+ cells in the spleen may serve as an indicator of progenitor cell activity. The correlation for the same two variables in the control animals and animals in the later leukemic phase is not significant

$r = 0.29$ ($p < 0.36$) and $r = 0.29$ ($p < 0.14$) respectively. The results indicate the presence of a transient compensatory erythropoiesis occurring in the spleen in the early stages of leuekmia. Spleen B+ cells at this time are negatively correlated with liver weight, and with liver blasts, $r = -0.63$ ($p < 0.001$) and $r = -0.51$ ($p < 0.005$). Thus, the following picture emerges; early in EBL development, i.e. while liver blasts and liver weight are low, levels of B+ cells in the spleen do not differ significantly from control, but the spleen shows increased CFU-Es when the cells are grown in vitro. As the disease progresses and levels of liver blasts increase, the CFU-Es in the spleen decrease, as do numbers of B+ cells in this organ. This is in direct contrast with the early phase of the disease (days 4,5) when increases in blasts levels in the spleen are associated with an increase in CFU-E levels (see Figure 7). However, this effect is transitory since at days 7,8 post-i.v., no significant numbers of CFU-E could be demonstrated in the spleens, regardless of blast levels.

Spleen Clusters: Spleen cluster growth closely followed the pattern of colony growth, at both the early and late phase. The coefficient of correlation between colonies and clusters was $r = 0.80$, $p < .001$ in the early-mid leukemics, and $r = 0.83$, $p < .001$ in the late leukemics. Levels of clusters were strongly associated with levels of benzidine positive cells in the positive cells in the spleen. At days 4,5 post-i.v., the coefficient of correlation between spleen erythroid clusters (ECL) and spleen B+ cells was $r = 0.46$, $p < .006$. At days 7,8 post-i.v., the correlation between spleen ECL and spleen B+ cells was $r = 0.71$, $p < .00001$. On the other hand, colony (CFU-E) growth levels from the 7,8 day spleen are not this strongly associated with levels of spleen B+ cells. (The correlation between spleen

CFU-E and spleen B+ cells, in 7,8 day EBL animals was $r = 0.29$, $p < .07$.) Thus, it would appear that the decrease of spleen cluster growth that occurs later in the disease is due to lower levels of recognizable erythroid precursors present in the spleen. In the 7,8 day EBL animals, the correlation between levels of spleen erythroid clusters and spleen weight was $r = -0.78$ $p < .02$, between erythroid clusters and liver blasts, the correlation was $r = -0.55$ $p < .003$. Thus, levels of clusters decreased, as levels of liver tumor cells increased. The correlation between spleen erythroid cluster and spleen blasts was not significant $r = -0.25$, $p < .44$.

Liver CFU-E:

No CFU-E grew from liver cells taken from 7,8 day EBL animals regardless of blast levels. Control liver cells did not grow CFU-E in vitro.

C. Granulopoiesis In Vitro

Bone Marrow CFU-C:

The CFU-C were grown as was described in the Methods section and were examined after 7 days of growth. Colonies were for the most part spherical in shape and grew in tight compact cellular aggregates usually composed of greater than 50 cells. An aggregate of 16 or more cells was scored as a colony, 8-15 cells were scored as a cluster. The mean number of colonies in the first control group was 43.8 per 10^5 cells plated and for the second control group, $40.4/10^5$ cells. In control Group I approximately 40% of the variation in colony numbers can be accounted for by changes in levels of blasts in the bone marrow ($r = 0.62$, $p < 0.05$). This

would indicate that the greater the level of bone marrow blasts, the greater the number of granulocytic colonies in the marrow. It is interesting to note that a significant correlation between bone marrow CFU-C and levels of spleen B+ cells is present in control Group I ($r = 0.61, p < 0.06$), suggesting that in cases where granulopoiesis increased in the bone marrow, erythropoiesis was augmented in the spleen.

In the early stages of leukemia (days 4,5), there is a positive correlation between bone marrow CFU-Cs and bone marrow blasts, ($r = 0.43$, $p < 0.009$). However, the levels of correlation between the bone marrow CFU-C and bone marrow blasts is not as high as in control Group I. In Figure 9 levels of CFU-C are shown for animals grouped according to levels of bone marrow blasts. Levels of bone marrow CFU-C increased with increasing levels of blasts non-linearly. The data indicates a stimulatory effect of EBL infection on CFU-C levels.

At 7,8 days post-i.v., the correlation between bone marrow colonies and blasts in bone marrow and spleen is $r = 0.31$ ($p < 0.06$) and $r = 0.43$ ($p < 0.01$) respectively. As can be seen in Figure 9, increasing levels of bone marrow blasts were associated with increases in CFU-C levels, although the magnitude of the increases was not as high as in the early leukemic animals. Levels of liver blasts, liver weight, or spleen weight were not significantly correlated to bone marrow CFU-C (Table 6). Thus, while bone marrow and spleen blast levels were correlated to colony growth, the levels of tumor cells in the liver did not appear to be directly correlated to colony growth. A question which these data pose is whether high levels of blasts serve as stimulators for granulopoiesis. Does the suppression of

erythropoiesis that occurs with high levels of blasts allow for increasing levels of granulopoiesis, or is the increase in granulopoietic activity a response to certain stimuli which are coincidental to both increases in bone marrow and spleen blasts. When one examines the correlation between CFU-E and CFU-C in the later stage leukemic animals, one finds a small but significant degree of correlation, $r = -0.30$, $p < .05$. This would indicate that the erythroid repression which occurs at this phase may allow for part of the variability in CFU-C levels.(Levels of CFU-E and CFU-C in the early-mide leukemic animals, are not significantly correlated, $r = -0.15$ $p < .21$.) A stepwise multiple regression analysis of bone marrow CFU-C at days 4,5 post-i.v., as the dependent variable, and levels of bone marrow blasts and B+ cells and weight as the independent variables yielded a multiple $R=0.60$, $r^2= 0.36$, $p < .05$. The two greatest contributing factors to the regression equation were levels of bone marrow blasts, and liver weight. Stepwise multiple regression performed with CFU-C from the day 7,8 leukemic as the dependent variable, and with all the variables mentioned above as the independent variables, yielded a multiple $R= 0.70$, $r^2= 0.49$ $p < .05$. The greatest contributing variables to this regression equation, were levels of spleen and liver blasts. (Regression performed with spleen weight and liver weight as the independent variables, and CFU-C at 7,8 days post-i.v. as the dependent variable, yielded a multiple $R= 0.88$, $r^2= 0.77$, $p < .05$.) Levels of spleen blasts were positively correlated to bone marrow CFU-C, while levels of liver blasts were negatively correlated to colony growth. This would indicate that the conditions in the animal most favorable to bone marrow granulocytic colony growth, were those where spleen blast increases precede liver blast increases. In other words, the data seems to indicate that

if tumor blasts seed almost exclusively in the liver, this does not affect BM-CFU-C in the way as an increase in blasts in both the spleen and liver.

Bone Marrow Granulocytic Clusters:

Granulocytic clusters (GCL) in the early phase were most strongly associated with levels of liver blasts $r = 0.33$ $p < .04$. The correlation between GCL and bone marrow blasts was not significant $r = -0.24$ $p < .11$ (Colony, CFU-C, growth at this point was correlated with levels of bone marrow blasts $r = 0.43$ $p < .009$). In the later phase, i.e. days 7,8 post-i.v., levels of granulocytic clusters were associated with levels of bone marrow blasts, $r = 0.33$ $p < .05$ (the correlation between CFU-C and blasts was $r = 0.32$ $p < .06$). At this point, growth of clusters closely followed colony growth, the correlation between these two variables was $r = 0.90$ $p < .001$. Thus, in the later phase, levels of GCL increased when levels of colonies also increased.

Spleen CFU-C:

Overall levels of spleen CFU-Cs were very low for both the control ($\bar{x}=2.9\pm1.9$) and the EBL animals. There was however occasional growth of significant numbers of colonies from the EBL spleens. At 4,5 days post-i.v. the spleens from animals with splenic blast counts below 2% showed CFU-C growth (3 cases showed colonies, the mean number of colonies from these cases was 7,9 and 9 colonies per 10^5 cells). In the 7,8 day post-i.v. animals, colonies grew from spleens with greater blast counts. The correlation between spleen blasts and spleen CFU-C was $r = 0.33$, $p < .06$. In these cases, 5 animals that had greater than 20% blasts in the spleen

had significant levels of colonies (2 cases actually had means of 32 and 34 colonies/10^5 cells)(see Figure 10). No control animals had such high levels of spleen colonies. Multiple regression performed with spleen colonies as the dependent variable, and levels of spleen blasts, bone marrow blasts, liver blasts, bone marrow, spleen and liver B+ cells, and liver weight as the independent variables, yielded a multiple R = 0.82, r^2=0.67 $p < .05$. Thus, splenic granulopoiesis appears to be strongly correlated to variables associated with the advanced stage of leukemic development. In addition, the correlation between levels of spleen CFU-C and levels of bone marrow CFU-C is $r = 0.52$, $p < .002$. The correlation between these two variables in the early stage of leukemic is not significant $r= -0.10$ $p < .29$. Thus, in the 7,8 day EBL animals, levels of spleen CFU-C will increase in many cases as levels of bone marrow CFU-C increase (see Figure 11). Thus, the pattern of splenic granulopoiesis differs from that of erythropoiesis. In the initial stages of the disease, while levels of bone marrow CFU-E are decreasing with increasing blast levels, spleen CFU-E increase with increasing spleen blast levels. At the terminal stages, levels of CFU-E in the bone marrow and spleen decline sharply, while levels of blast cells increase in both the bone marrow and spleen. On the other hand, CFU-C growth increases steadily with increasing levels of blast development. It would, therefore, seem that initially the spleen is involved in compensatory erythropoiesis, and then switches to granulopoiesis at the later phase of the disease. Both the element of time post-injection, and levels of blasts are factorial in determining when the switch over from erythropoietic to granulopoietic activity will occur.

Spleen Granulocytic Clusters:

Early in EBL development, the correlation between spleen GCL and spleen colonies was $r = 0.75$, $p < .001$. There was no significant correlation between levels of spleen clusters at this time to either levels of spleen blasts, bone marrow, or liver blasts (see Table 7). The correlation between spleen GCL and colonies at the later phase was $r = 0.93$, $p < .001$. The correlation between spleen GCL and spleen and liver blasts was low, but significant, $r = 0.29$, $p < .08$, and $r = -0.31$, $p < .07$ respectively. A stepwise multiple regression performed with levels of spleen granulocytic clusters as the independent variable, and levels of spleen blasts, bone marrow blasts, liver blasts and liver weight as the independent variables yielded a multiple $R = 0.73$, $p < .05$. Thus, levels of spleen clusters as well as levels of spleen CFU-C were affected by the degree of blast proliferation.

Liver CFU-C:

The liver did not show any CFU-C activity in any animals (control or leukemics) regardless of blast levels.

IV. Conditioned Medium

Conditioned media was prepared from EBL, Shay, normal liver, and granulocytes as described in the methods section. Colonies were grown from pooled bone marrow cells harvested from 3 normal young adult male rats in each experiment (each value is a mean of 5 or 6 samples).

EBL CM:

EBL media, conditioned for 24 hours, was examined for its effect at two concentrations, on both erythroid and granulocytic colonies (see Figure 12). CFU-C growth was strongly inhibited when grown in either 10 or 20% EBL conditioned media. At 10% concentration EBL CM, inhibition of CFU-C growth was 73-76% (a mean 25 colonies/10^5 bone marrow cells vs. 145 colonies in control) and in 20% EBL conditioned media, inhibition was 100%. In vitro CFU-E growth was more resistant to inhibition, and the effect on CFU-E growth was not as pronounced. When CFU-Es were grown in 10% EBL conditioned medium, virtually no inhibition was observed (104 colonies/$5x10^4$ bone marrow cells vs. 105 CFU-E in control). In 20% EBL conditioned media, 20-22% inhibition was observed ($\bar{x}$=32 colonies/$5x10^4$ bone marrow cells plated).

When EBL cells were allowed to condition media for 48 hours, a much stronger effect was found. CFU-C growth in 10% or 20% of this conditioned media was strongly inhibited. At 10% EBL CM, inhibition of CFU-Cs was 56% ($\bar{x}$ of 47 colonies/10^5 cells to 104 colonies in control), when grown in 20% EBL CM inhibition was virtually total, 99% inhibition. CFU-E growth was not inhibited when grown in 10% EBL media conditioned for 48 hours. CFU-Es when grown in 20% of concentrations of this media were inhibited 54% ($\bar{x}$ of 40 colonies/$5x10^4$ cells to 85 colonies in control).

When EBL cells were freeze-thawed after 48 hours conditioning time, CFU-E growth inhibition was apparent even when grown in a 10% solution; colony growth was inhibited 27% ($\bar{x}$ of 50 colonies/$5x10^4$ cells to 70 colonies

in control). When grown in 20% of the freeze-thawed EBL CM, CFU-E inhibition was 87% ($\bar{x}$ of 9.2 colonies/$5x10^4$ cells). This EBL CM totally inhibited CFU-C growth at both 10-20% concentrations.

Shay CM:

Shay conditioned medium was also examined for its effect on *in vitro* colony growth. Twenty-four hour Shay CM was inhibitory to CFU-C growth (see Figure 13). In 10% CM colony inhibition was 16% ($\bar{x}$ 122 colonies/10^5 cells vs. 146 colonies in control) and in 20% CM growth inhibition was 76% ($\bar{x}$= 35 colonies/10^5cells) (a greater than four-fold increase in inhibition with only a doubling of CM concentration). Twenty-four hour Shay CM had little effect on CFU-E growth at either 10- or 20%; at 10% CM there was no inhibition, at 20% inhibition was 5% (the 24-hour EBL CM at the same concentration inhibited CFU-E growth, 22%).

Forty-eight hour Shay CM showed strong inhibitory effects of CFU-Cs at both concentrations. When grown in 10% CM, CFU-C inhibition was 49% (54 colonies/10^5 cells vs. 104 for control) and in 20% CM inhibition was 99%. Again, the effect of Shay CM on CFU-E growth was not pronounced, at 10% CM, CFU-E growth was stimulated, at 20% CM a 27% reduction was observed. (63 colonies/10^5 cells vs. 85 for controls). (The EBL 48-hour CM at this concentration inhibited CFU-E growth 64%).

Freeze-thawed Shay CM inhibited 76% of CFU-C growth at 10% CM, ($\bar{x}$ = 15 colonies/10^5 cells vs. 63 colonies for control), and at 20% CM, CFU-C growth was 99% inhibited. CFU-E growth was only minimally inhibited by this CM preparation. In 10% CM, CFU-E growth was inhibited 3.2% and

in 20% CM growth was inhibited 26% (51 colonies vs. 70 colonies for control. EBL freeze-thawed media inihibited 27% of CFU-E growth in 10% concentrations, and inhibited 87% of CFU-E growth in 20% concentrations.)

Thus, the inhibitory pattern of EBL CM and Shay CM differs. EBL CM acted to inhibit both CFU-C and CFU-E growth effectively, while Shay CM was more effective inhibiting CFU-C than CFU-E growth. As can be seen from Figure 12, the effect of Shay CM on CFU-C growth, at the lower concentrations, is time dependent. The longer the conditioning time, the greater the inhibitory effect. EBL CM at 20% concentration has a similar effect on CFU-E growth. The level of inhibition of CFU-E growth almost doubles after an additional 24-hour conditioning time.

Granulocyte CM

Peritoneal exudate cells obtained by peritoneal lavage 4 hours following endotoxin administration were allowed to condition media for 24 hours. (This population of cells includes greater than 80% neutrophils as well as a small percentage of mononuclear elements.) The inhibitory effect on CFU-E and CFU-C growth by this media was examined. CFU-Cs in 10% granulocyte conditioned medium (GCM) were inhibited 20% ($\bar{x}$ of 117 colonies/10^5cells, 145 CFU-C in control), in 20% GCM CFU-C growth was inhibited 93% ($\bar{x}$of 10 colonies/10^5 cells). CFU-E growth was also inhibited by the GCM preparation at both concentrations. CFU-Es grown in 10% GCM were inhibited 32% ($\bar{x}$ of 72 CFU-Es/5x10^4 cells vs. 105 in control); when grown in 20% GCM growth was inhibited 46% ($\bar{x}$ of 57 CFU-Es/5x10^4 cells. At the lower concentration, GCM was more inhibitory to CFU-E growth than to CFU-C growth. At the higher concentration CFU-C growth was maximally inhibited.

Neonatal Liver CM:

The liver from 10-day neonate rat pups was allowed to condition media for 48 hours. CFU-C growth was stimulated in the presence of 10% CM; colony levels were 128% of control (133 colonies/10^5 cells vs. 104 for controls). Colony growth in the presence of 20% CM was not affected. The effect of liver CM on CFU-E growth was slight. When grown in the presence of 10% CM, CFU-E growth was 91% of control (77 colonies/$5x10^4$cells vs. 85.3 for control) and in 20% CM, CFU-E growth was 123% of control (105 colonies/$5x10^4$ cells).

Cluster Growth:

The effects of the various conditioned media on erythroid and granuloid cluster formation *in vitro* was also assessed.

EBL CM:

EBL CM conditioned for 24 hours had a definite stimulatory effect on erythroid cluster formation (see Figure 14). When grown in 10% EBL CM, erythroid cluster formation (ECL) was 159-167% of control (67 clusters/$5x10^4$ cells vs. 40 for controls). In 20% EBL CM ECL growth was 120% of control (60 clusters/$5x10^4$ cells). Forty-eight hour EBL CM at concentrations of 10%, maintained a stimulatory effect on ECL growth, levels of 143% of control were reached (69 clusters/$5x10^4$ cells vs. 48 for controls). At 20% CM there was, in fact, an 8% drop in ECL levels (44 clusters/$5x10^4$ cells). Freeze-thawed EBL CM had no stimulatory effect on ECL growth at 10% CM (44 clusters/$5x10^4$ cells vs. 44 for control), and inhibited ECL growth 66% in 20% concentrations (19 clusters/$5x10^4$ cells). Thus, it

appears that the level of ECL stimulation decreases with increasing concentrations of CM. This may be due to increasing levels of inhibitor in the CM preparations.

Granulocyte cluster (GCL) growth was susceptible to EBL CM inhibition even at the lowest concentrations (see Figure 14). GCLs grown in 10% EBL media conditioned for 24 hours were inhibited 64-76% (31-42 clusters/10^5 cells vs. 92 for controls). When GCLs were grown in 20% of this CM, inhibition was 91-97% (2-8 clusters/10^5 cells). Forty-eight hour EBL CM inhibited GCL growth 33% at concentrations of 10% (47 clusters/10^5 cells vs. 71 for controls), and inhibited GCL growth 95% at concentrations of 20% (3.2 clusters/10^5 cells). Freeze-thawed EBL preparation inhibited GCL growth 95% at 10% concentration (1.5 clusters/10^5 cells vs. 34 in controls) and 100% at 20% concentration.

Shay CM:

Twenty-four hour Shay CM stimulated ECL growth (see Figure 15). When grown in 10% Shay CM : ECL grew 201% of control (80.5 clusters/$5x10^4$ cells vs. 40 for control), and in 20% CM ECL growth was 215% of control (86.2 clusters/$5x10^4$ cells). The stimulatory effect of Shay CM diminished with conditioning time. Forty-eight hour Shay CM, at 10% concentrations showed minimal stimulation; ECL growth was 16% above control (56 clusters/$5x10^4$ cells vs. 48 for control), while at CM concentrations of 20%, ECL growth was 40% above control (68 clusters/$5x10^4$ cells). Thus, with doubling the CM levels ECL growth was also doubled. Freeze-thawed Shay CM stimulated ECL growth, at both 10% and 20% concentrations. When ECLs were grown in 10% CM, levels of ECL were 142% of control (62 clusters/$5x10^4$ cells vs. 44

for controls); in 20% CM ECL levels were 111% of control (49 clusters/ $5x10^4$ cells). The stimulatory effect is diminished with increasing concentrations.

Growth of granulocytic clusters (GCL) was stimulated in low concentrations of Shay CM (see Figure 15). A 10% concentration, of 24-hour Shay CM stimulated GCL levels to 146% of control (134 clusters/10^5 cells vs. 92 for control). However, when the cells were grown in 20% of this CM preparation, GCL levels were inhibited 47% (42 clusters/10^5 cells). Shay media conditioned for 48 hours inhibited GCL growth at both 10 and 20% concentrations. When grown in 10% of this CM, GCL levels were inhibited 22% (56 clusters/10^5 cells vs. 71 for control); in 20% CM, GCL growth was 100% inhibited. Freeze-thawed Shay CM was also inhibitory. In 10% CM, cluster growth was inhibited 60%, 14 clusters/10^5 cells vs. 33 clusters in controls. In 20% inhibition was virtually total.

Leukocyte conditioned media (GCM) had no inhibitory effect on ECL growth at either 10% or 20% concentrations. In fact, ECL growth was increased to 133-135% of control. This is an important point because it shows that the overall effect of GCM on erythroid growth is not inhibitory. While colony growth may be repressed, cluster growth is elevated. The total number of colonies and clusters does not differ significantly from control. On the other hand, GCM inhibited GCL growth. GCLs grown in 10% GCM were 13% inhibited (80.4 clusters/10^5 cells vs. 92 clusters in controls); GCLs grown in 20% GCM were inhibited 75% (32 clusters/10^5 cells).

Neonatal liver CM stimulated the growth of erythroid clusters *in vitro*. When grown in 10% CM erythroid clusters were 108% of control (52

clusters/$5x10^4$ cells vs. 48 in controls), and 20% CM erythroid clusters were 143% of control (69 clusters/$5x10^4$ cells). Granulocytic cluster formation was not enhanced. In 10% CM GCL was 100% of control (72 clusters/10^5 cells vs. 71 in controls, and in 20% CM cluster formation was 88% of control (63 clusters/10^5 cells).

EPO Independent Growth:

Very low levels of clusters and colonies grew in the absence of epo. Even in cultures where erythroid cluster formation was substantially stimulated as in the presence of Shay and EBL CMs, significant levels of epo independent growth were not found ($\bar{x}$ of 4 clusters/$5x10^4$ cells vs. 2 clusters in controls). Thus, the conditioned media acted to augment cluster formation in the presence of epo but did not eliminate the need for the hormone for cluster or colony growth.

To summarize, ECL growth was not inhibited by any Shay CM preparations, but higher concentrations of EBL CM did inhibit ECL growth. ECLs were more resistant to inhibition than CFU-Es. In many cases there was ECL stimulation, where CFU-E growth was not affected, e.g. 24-hour Shay CM at 10% concentration, allowed CFU-E growth to be 108% of control and ECL growth to be 201% of control; 24-hour EBL CM at 10% concentration, allowed CFU-E growth to be 100% of control, while ECLs were 167% of control. These results would indicate that here the CM has a direct stimulatory effect on ECLs, and that the increased levels of clusters are not due to depressed levels of colonies. GCL were much more susceptible to inhibition by both

the Shay and EBL CM. While 10% Shay media conditioned for 24 hours did stimulate GCL growth, all other preparations were inhibitory to GCL growth. In all cases, cluster formation was more resistant to CM inhibition than was colony formation. It may be that increased levels of granulocytic clusters occur at the expense of colonies, i.e. clusters are aborted colonies. Because all cases of increased granulocytic clusters occurred where there were depressed levels of colonies, the origins of the GCL cannot be determined.

DISCUSSION

These studies report on an animal model of leukemia which is characterized in part by ineffective erythropoiesis. Specifically, there is a reduction in erythropoiesis accompanied by hemolytic anemia concurrent with increased levels of granulopoiesis. This model is, therefore, similar to DiGuglielmo's syndrome. Dameshek (1969) reported abnormalities in heme synthesis, ^{59}Fe incorporation, ALA-dehydrogenase levels occur during this disease. Thus, while erythroblasts abound in the marrow, there is also severe anemia, indicating a lesion in the maturation of erythroid precursors to functional red blood cells. The advantages of the animal model presented here include: 1) rapid time course of disease development (7-8 days); 2) high infection levels (close to 80% of animals injected with a low dose of EBL cells develop the disease and 3) use of an animal model, i.e. the rat, whose hematopoietic system more closely resembles that of a human. In both organisms the spleen is not normally the site of erythrocytic or granulocytic production.

I. Tumor Cell Proliferation

The course of the disease is farily predictable. Close to 80% of the animals injected with a low dose of EBL cells ($5x10^6$ cells per 100g body weight) developed the disease. The EBL cells grew in the bone marrow, spleen and liver with the spleen and liver mass doubling or even tripling with disease progression. Histological sections prepared of spleen and liver showed massive infiltration of tumor cells. This change was dramatically demonstrated in the liver where liver cords composed of regular rows of acidophilic cells were replaced by irregularly spaced basophilic tumor cells. In the bone marrow, blasts replaced normal cellular elements and reached levels of greater than 70% of the total cellular content.

At the terminal stages of disease progression, i.e. days 7,8,9 tumor cells appeared in the peripheral circulation. In some cases levels of blasts were greater than 10%. However, this does not begin to approach levels of blasts in the circulation of animals infected either with the myeloid leukemias Shay chloroleukemia or L5222 (Handler and Handler, 1970, Hoelzer and Harriss, 1973). In many cases of EBL, while levels of blast cells in the hematopoietic organs were very high, circulating blasts remained low. For example, animal IX-9s liver contained 73% blasts, while only 1% of the total nucleated cells in the peripheral blood were blasts. Similarly, animal XIX-6 had blast levels in the bone marrow of 65%, liver 62%, spleen 26%, while in the peripheral blood blasts were only 1.4%. Thus, the data suggests that the integrity of the vasculature in the hematopoietic organs is generally maintained during disease development. While levels

of correlation between liver blasts and peripheral blood blasts show a trend toward significance ($p < 0.15$), the correlation coefficient is quite low ($r=0.28$). However, spleen blasts and peripheral blood blasts are strongly correlated ($r=0.66$, $p < 0.001$). This would indicate that the degree of splenic blast infiltration is closely associated with levels of blasts in the circulation. One could postulate that the blasts are entering the circulation from the spleen. Electron microscopic examination of rat marrow during Shay leukemic development indicated that leukemic cells in the peripheral blood originated from the marrow, evident by the presence of myeloblasts in the vascular sinuses (Chen et al. 1972). The destruction of the adventitial layer of the sinus appeared to be instrumental for sinus deterioration. Similar studies performed in the EBL model would help explain both the origin of blasts in the circulation and their relatively low numbers, in this disease.

II. Erythropoiesis

The effect of EBL tumor cell infiltration on normal red blood cell production was investigated. Erythropoiesis was assessed in three parameters: a) both mature and immature RBCs in the circulation were assessed from daily drawing of blood samples and from measuring hematocrit and reticulocyte levels; b) levels of erythroid precursors were assessed by differential staining of hematopoietic organ smears and counting numbers of benzidine positive (hemoglobin containing) nucleated cells; c) growth of erythroid precursors was assayed *in vitro* on plasma clot cultures.

A. Peripheral Blood Determinations

Approximately 66% of the infected animals showed reduced levels of circulating RBCs by day 9 post-injection. Hematocrit (hct) levels for the most part were inversely correlated to blast levels in the bone marrow ($r=-0.67, p< 0.001$). Although at first glance it may appear that the decline in levels of circulating RBCs is due to diminished production of RBCs when the hematopoietic foci are invaded by leukemic cells, upon further inspection, this supposition is not supported. The normal life span of a RBC in rats is approximately 55 days (the range is 47-60 days depending on the strain, Harriss, 1974). A shut down in production would account for a daily 2% drop (Hoelzer and Harriss, 1973). When examining hct changes during a 9-day EBL course, one finds a hct drop of approximately 12% (1.3% per day). However, this analysis is highly misleading because the hct remains basically stable for 7 days and then drops sharply. The initial fluctuations which occur at days 1,2,3, post-injection, occur also within control groups and have been ascribed to the effects of initial animal manipulations, e.g. tail snipping. The rapid hct decline subsequent to day 6 shown in Figure 1 is more remarkable when one examines individual cases. As mentioned in the results section, due to the variability in the rate of anemia development in the different animals, the curve is an approximation of the trend but not an actual reflection of the magnitude of the changes. Thus, animal II-5 shows a hct level at day 7 of 40, day 8 hct is 32 and day 9 hct level is 15. This sharp drop in RBC levels indicates that the EBL disease is accompanied by hemolysis. In other types of leukemia as well, a hemolytic effect due to leukemic infection has been reported

(Hoelzer and Harriss, 1973; Harriss, 1974; Handler and Handler, 1970). The hemolytic effect may not be due to direct interactions of the tumor cells, or their products with the RBCs, but may actually be a side effect of accompanying bacterial infection which often results in hemolytic anemia (Beutler, 1973). While there is no direct evidence for bacterial infection in the EBL animals, the high incidence of neutrophilia which accompanies the disease as well as the high incidence of diarrhea in these animals may be indicative of bacterial infection.

While hemolysis may account for the anemia at the later stages of EBL, there are strong indications for a depression in erythropoiesis. This is suggested by the decrease in reticulocytes that accompanies disease development. For example, animal II-5 discussed earlier, showed reticulocyte (retic) levels at day 7 of 16%, day 8 retics were 6%, and day 9 retics were 6% (note the sharp drop in retics from days 7 to 8). In this case, at the terminal stages of EBL, the levels of circulating RBCs were depressed, while in addition, the proportion of incoming RBCs into the circulating pool was also depressed. This clearly indicates that homeostasis of erythroid production was disrupted. The animals could not compensate for decreased levels of circulating RBCs which suggests impairment of erythroid production. (The drop in retic levels was not always accompanied by a drop in hct as in the case presented above. In many cases, retic levels declined 2 days prior to hct decline. This indicates a drop in erythroid production and precludes the possibility of increased RBC production as a response to the hemolytic effect described earlier.)

The maturation time from the erythroid precursor to the circulating reticulocyte is 3-5 days (Harriss, 1974, Erslev, 1973). A decline in levels

of retics at day 7 would suggest either a drop in erythroid production at days 2-4 post-i.v., or a defect either in the release of reticulocytes into the circulation or reticulocyte sequestration. However, studies by Dornfest et al. (1971) of reticulocyte sequestration in normal, anemic, and leukemic (Shay) rats, indicated that in the case of the leukemic animals which were concurrently anemic, there was a decrease in spleen sequestration of reticulocytes. With the increased blast levels found in EBL spleens, it is unlikely that the spleen would increase its surveillance of reticulocyte levels in the circulation.

B. Organ Smears

Examination of the cellular content of the hematopoietic organs, namely the spleen, bone marrow, and liver, at days 4-5 post-injection, showed no dramatic changes in levels of recognizable erythroid precursors (benzidine positive cells) in the hematopoietic organs (Figure 5). At this stage levels of benzidine positive (B+) cells in the leukemic aniamls did not differ significantly from control. However, as is evident from Figure 5, there was a marked reduction in levels of B+ cells at days 7-8 post-i.v., indicating that non-differentiated erythroid stem cells which develop into recognizable erythroid progenitor cells are deficient in either their actual number, or in their ability to replicate in vivo early in disease development. There is evidence of similar erythroid repression in the other leukemic models. Hoelzer and Harriss (1973) investigated changes which occur in the marrow of animals infected with L5222 leukemia. They examined changes in levels of large basophilic normoblasts, small basophilic normo-

blasts, polychromatophilic and acidophilic normoblasts (the latter two are non-dividing stages). They reported that with disease development, levels of early erythroid precursors declined, while levels of orthochromatophilic normoblasts increased during leukemogenesis. A marked decrease in levels of ^{59}Fe incorporation in the hematopoietic organs was apparent at days 5, post-injection (Hoelzer et al. 1973) due to a sharp reduction in erythropoiesis. Shay chloroleukemia is also accompanied by erythroid repression. Handler and Handler (1972) reported decreased ^{59}Fe incorporation into heme in the marrow cells from animals injected with Shay cells. Animals whose marrow contained as little as 10% blasts showed dramatic alterations in heme synthesis. Thus, in these two models of myelogenous leukemia, erythroid involvement is apparent and is expressed as severe erythroid repression.

The erythroleukemia models previously presented in the literature are the Friend and Rauscher leukemias (FV and RLV respectively). As was discussed earlier, Friend and Rauscher are both very complex diseases that have various pathogenic pathways. Both involve more than one virus in disease development, and different strains of virus yield different responses. In both Friend and Rauscher leukemias, anemic response strains have been studied. The lesion is apparently a maturation defect in the virally transformed cells which can, however, be overcome *in vitro* by the action of chemical inducers such as dimethylsulfoxide (DMSO) butyric acid, etc. (Preisler et al. 1975, Leder and Leder, 1975). Non-defined environmental factors have also been shown to influence Friend cell differentiation *in vivo*. Thus, the transplantation of non-differentiated Friend cells into

a lethally irradiated mouse will result in the maturation of the virally transformed cells into recognizable erythroid cells which still show the presence of virus (Rossi and Friend, 1970). It appears, however, that not all leukemic cells may be responsive to the differentiation stimuli. In vitro studies with FV-A clones have found both inducible and non-inducible clones (Orkin et al. 1975). Weitz-Hamburger et al. (1975) reported that after a double challenge of animals with RLV and phenylhydrazine, levels of circulating retics initially increased to 40% of the circulating RBC population, then subsequently declined when new cells could no longer be recruited into the differentiation pathway. In another study, animals were infected with the RLV-A variant of RLV. Morse et al. (1978) reported that these animals developed severe anemia and reticulocytopenia. Incorporation of ^{59}Fe into heme was delayed relative to control despite massive uptake of iron by both the liver and spleen. It was also demonstrated that the RBC half life was considerably shortened after RLV-A infection, resulting in anemia. Due to the ineffective erythropoiesis characteristic of the disease, no compensatory increase in erythroid production occurred. Thus, the common factor in these animal models and the EBL described in this study is the maturation block of functional RBC in vivo in the absence of inducers.

Studies of erythropoiesis in erythroleukemic patients have involved analyzing bone marrow cellular content for erythroblasts (Karle et al. 1974), autoradiography and cytophotometry (Mitrou et al. 1975; Quiesser et al. 1975; Suda et al. 1978). These workers found that accompanying the anemia and reticulocytopenia which occurs in the disease, there is

also a change in erythroid precursors in the bone marrow. Mitrou et al. (1975) reported that the erythroblasts from erythroleukemic (EL) patients had a lower labelling index. The percent basophilic erythroblasts and early polychromatophilic normoblasts in S phase was decreased, and the percent of cells in G_1 was increased. Quiesser et al. (1975) reported that while the time in S for basophilic erythroblasts and early polychromatophilic normoblasts was normal (i.e. no prolonged S phase) a large percentage of basophilic and polychromatophilic erythroblasts were unlabelled. These cells could either be in G_0 or G_1, but most probably the ineffective erythropoiesis was due to the cells being out of cycle.

It would be of interest to determine whether cell cycle parameters are modified after EBL infection. Use of this model would permit not only the analysis of these changes, but would also allow the determination of where and when these changes occurred. In other words, because one can easily discriminate between normal erythroid elements and the transplanted EBL cells, one could follow the effect of the blasts on the various erythroid compartments and determine which compartments are affected by EBL blasts, and if a specific level of blasts need be present before normal erythropoiesis is affected.

C. Assessment of the Functional Capacity of Erythroid Precursors In Vitro:

We have demonstrated that erythroid cell proliferation was effected in vivo, as shown by the reduced levels of reticulocytes in the circulation and reduced levels of recognizable erythroid precursors in the hema-

topoietic organs at days 7,8, post-injection. Since the maturation time from the proerythroblast to normoblast is 2-3 days, the absence of normoblasts at day 7 indicates either a lack of pronormoblasts at days 4,5, or impaired maturation *in vivo* in the leukemic animal. In order to resolve this issue, functional assays of hematopoietic stem cells were performed *in vitro* at days 4,5, and days 7,8, post-injection.

The CFU-E assay as developed by Axelrad and his colleagues (1974) assesses levels of committed erythroid progenitors. These cells, which are not recognizable erythroid cells, respond to erythropoietin *in vitro* and develop into colonies of 16-32 hemoglobin containing (benzidine-positive) cells after 48 hours in culture. The clonal origin of these colonies was first shown by Cormack (1976) who followed the development of a single cell into a colony using time-lapse cinema-photography. Prchal et al. (1977) further demonstrated the clonal origin of erythroid colonies. Using female subjects heterozygous for glucose 6-phosphate dehydrogenase as a source of erythroid progenitors, Prchal et al. reported that each individual colony expressed only one type of isoenzyme, thereby showing the unicellular origin of the colony. Thus, determination of the number of erythroid colonies developing *in vitro* is a direct functional assay of the number of erythroid precursors present in the hematopoietic organs.

In order to monitor CFU-E levels, cells were flushed from the marrow, spleen and liver, washed once, and then plated as described in the methods section. Cells were grown both with and without erythropoietin in order to detect colony growth independent of epo. After 48 hours incubation

at 37°C, colonies varying from 8-32 cells (3-5 divisions) were scored. Levels of erythroid clusters of 4-7 cells(2+ divisions) were counted separately. Clusters scored after 48 hours incubation may be the progeny of either a very early progenitor which forms early recognizable erythroid cells after the division of non-recognizable cells, or, on the other hand, a later progenitor cell with limited replicative potential. Since erythroid cells diminish in size with increased maturation and replication, the size of the cells within the cluster may serve to indicate the developmental stage of the cluster forming cell. It was noted that cells within the clusters appeared for the most part smaller in size than the cells which composed the colonies, thereby suggesting that these cluster forming cells were the progeny of a more mature cell.

The results showed that numbers of bone marrow (BM) CFU-E per $5x10^4$ cells were strongly depressed with EBL disease progression. Since the total marrow cellularity also appeared to be depressed as a function of disease development, the decline in numbers of CFU-E was not simply due to dilution of erythroid precursors within the marrow population, when levels of tumor cells increased in the marrow. It is also important to point out that the decline in CFU-E was apparent even in bone marrow samples with less than 10% blasts. Both levels of colonies and clusters were depressed in the severely leukemic animals. This would argue against the hypothesis that the clusters that were present were the result of a deficient replication potential in the CFU-E compartment, i.e. instead of going through 4 divisions in 48 hours, only 2 divisions occurred. If this had been the case, then the cells within the EBL clusters would

have been markedly larger than the cells which formed the control clusters, which was not the case. All the clusters appeared to be composed from the same size cells, indicating that the CFU-E inhibition which occurred in the EBL did not result from a lengthening of the cell cycle of the progenitor cells. Thus, the data indicated that there was an actual drop in numbers of precursor cells and not a defect in their replicative ability in vitro. This was substantiated by observed changes occurring within the hematopoietic organs. Whereas, levels of B+ cells were not affected by days 4,5 post-i.v., recognizable erythroid precursors virtually disappeared at days 7,8. Since levels of B+ cells were not affected at days 4,5, even where levels of tumor cells were increased, and levels of colonies were depressed, it would appear that a cell more primitive than the recognizable erythroid precursor was affected by the tumor cells. The drop in B+ cell levels at days 7,8 post-i.v., was due to a "shut-down" of the line that feeds into the maturing erythroid compartment. The erythroid cluster forming cell, which most probably arises from a B+ cell, was shown to be more resistant to EBL inhibition. While these cells may not be affected in their replicative ability in the presence of tumor cells, the available numbers of ECL forming cells diminished with increasing EBL progression.

The symptoms of RLV disease are similar to EBL in that ineffective erythropoiesis evident in the low hct levels and reticulocytopenia occurs with disease progression. However, an important difference is that RLV infected animals are still responsive to epo stimulation (Weitz- Hamburger et al. 1975). While RLV acts to stimulate the ERC, the infected compartment can further respond to exogenous epo administration by increasing

^{59}Fe uptake and overcoming the reticulocytopenia. These erythroid cells, however, do not mature into functional long-lived RBCs and retain metabolic deficiencies (Seidel, 1976). Further understanding of RLV action may come from *in vitro* studies. Nooter and Ghio (1975) reported that bone marrow cells from RLV infected animals grew into colonies in the absence of exogenous epo, thereby demonstrating the "epo-like" action of the virus on the ERC. However, Opitz et al. (1977) found no significant levels of epo independent colonies after RLV infection. In fact, the morphological appearance of the CFU-E from both the bone marrow and spleen remained normal even late after infection. A significant difference between the CFU-E from normal and RLV infected animals may well be in the failure of the CFU-E to disintegrate on the plate after more than 48 hours in culture. Opitz et al. as well as Nooter and Ghio both reported that the colonies appeared unchanged even after 5 days in culture. The disintegration of the colonies on the plates is due to the enucleation and dispersion of erythroblasts as they mautre (Axelrad et al. 1974). Thus, the lesion in RLV infected animals may prove to be in the maturation of the poly- or orthochromatophilic erythroblast into a functional RBC both *in vivo* and *in vitro*. (It would be of interest to determine whether the cells can respond to higher levels of epo by accelerating their maturation process *in vitro*.)

The two strains of Friend virus previously described give rise to two different responses. Infecting mice with the Mirand strain (FV-P) results in severe polycythemia, where the cells are epo independent. Upon infec-

tion of normal bone marrow cells with virus *in vitro*, epo independent colonies will grow (Clarke et al. 1975). The number of colonies is proportional to the virus titer used. Epo independence was further demonstrated by Liao and Axelrad (1975) when they reported that anti-epo antiserum did not affect levels of these CFU-E, whereas anti-virus antiserum drastically reduced colony levels. The growth of colonies from FV-P infected mice was also studied by Opitz et al. (1978). They reported that the shift to epo independence is not gradual. The epo independent CFU-E arise from a new and distinct population of cells which appears approximately 7 days after virus infection. These CFU-E are also different in that the maximal size which they achieve after 72 hours incubation is 2-3x greater than the maximal size attained by normal CFU-E at 48 hours. Thus, the FV-P infected CFU-E shows better proliferation potential than control and is also most likely a more immature cell. The growth of FV-P colonies very closely resembles growth of colonies from patients with polycythemia vera (Prachl et al. 1974) in that along with epo independence, the cells retain their ability to mature into functional RBCs.

In the anemic strain FV-A, maturation into functional RBCs is blocked, and in addition, the cells are not responsive to epo stimulation. However, after exposure of FV-A cells to inducing agents such as DMSO, *in vitro*, colonies develop both with and without exogenous epo (Goldstein et al. 1974). In the absence of preincubation of cell in DMSO, colonies do not develop. Thus, the inducing agent may act on the FV-A infected cells in a manner similar to epo to stimulate maturation into erythroid cells. Growth of CFU-E from FV-A infected animals has not been studied.

In light of the fact that FV-A cells injected into lethally irradiated hosts do form erythroid colonies, it may be that changes in growth potential occur under various environmental conditions. However, in vivo epo levels do not appear to affect FV-A cells,since they colonize equally well the spleens of pre-irradiated polycythemic mice, which normal cells would not do (Rossi and Friend, 1970).

In summary, the rat EBL model cannot, in fact, be closely compared to the other erythroleukemia animal models. While RLV cells do not have the ability to fully mature into functional RBCs, they still maintain epo responsiveness. FV-P infected cells are capable of maturing into functional RBCs and need no exogenous epo. The growth of FV-A cells may closely resemble the growth of EBL cells, in that neither one can develop into recognizable erythroid cells (benzidine-positive) in either the presence or absence of epo. However, the growth of "normal" non-infected hematopoietic cells cannot be assayed in the viral erythroleukemia models and, thus, the interactions of normal and leukemic populations cannot be investigated with these models. In order to understand the human disease, both facets need to be studied, the failure of the erythroleukemic blasts to develop normally, and the inability of "normal" cells to respond to physiologic demands for more mature cells.

Hoffman et al. (1975) studied erythroleukemic CFU-E from patients in remission and in relapse. They reported the EL BM cells from patients in relapse did not form CFU-E regardless of levels of epo added to the culture, whereas cells from an EL patient in remission did respond to epo and developed CFU-Es. Thus, it appeared from this report that the

"normal" hemic elements were capable of responding to epo when levels of tumor cells were low; whereas leukemic cells could not (see Koeffler et al. 1978). The removal of leukemic cell populations in vitro by various cell separation techniques available such as velocity sedimentation or density gradients would determine whether they colony forming ability of normal cells could be restored with the elimination of the tumor cell populations, or whether cells developing in the presence of the tumor are irreversibly affected.

A more primitive precursor cell than the CFU-E has been characterized in both murine and human systems (Axelrad et al. 1974; Clarke and Housman, 1977). This cell which is responsive to much higher levels of erythropoietin, is assayed for growth after 7-14 days in culture, when large bursts of colonies predominate. This burst forming cell (BFU-E) is more resistant to perturbations than the CFU-E. Axelrad et al. (1974) reported that levels of BFU-Es are independent of endogenous epo levels, since hypertransfusion of mice did not affect BFU-E levels in the femur. Hara and Ogawa (1977) reported that perturbations of the erythroid compartment (by bleeding, epo injections, etc.) resulted in an increase in peripheral blood BFU-Es and spleen BFU-Es, and a decrease in femoral BFU-Es. This indicated a migratory response rather than a replicative response was effected by the manipulations of the erythron. This may be of interest when considering that BFU-Es were found in the peripheral blood of a patient with erythroleukemia and were shown to be responsive to epo stimulation in vitro (D. Housman , personal communication). However, Nathan et al. (1978) have postulated intrinsic differences in peripheral blood

vs. bone marrow BFU-E. They have found that the incidence of Hbf synthesized by peripheral blood BFU-E is much greater than in marrow BFU-E. They contend that peripheral blood BFU-E are stress cells which can be induced to differentiate under certain environmental conditions, such as lymphoid cell stimulation, but that their growth in vitro does not necessarily reflect the physiologic state. However, since the BFU-E represent a more primitive stem cell, which is closer to the pluripotent stem cell, it would be of great interest to determine how this compartment is affected in the erythroleukemic disease.

D. Compensatory Erythropoiesis

Another response to the stress of leukemic cell infiltration and the shut down of erythropoiesis which occurs in the bone marrow would be either the recruitment of normally quiescent cells into the erythropoietic compartment, or the migration of erythroid precursors to another hematopoietic organ. During the development of the EBL disease, erythropoiesis was found to occur in the rat spleen, an organ which is not normally involved in hematopoiesis. CFU-E were found in the spleen early during EBL development, i.e. days 4,5 post-injection, where blast levels were increasing. The erythropoietic phase was transitory, since CFU-E virtually disappeared from the spleen at days 7,8. Erythroid clusters (ECL) were evident at the later phase and were strongly correlated to levels of B+ cells in the spleen at the time of plating ($r=0.71, p < 0.001$). It is likely that the B+ cells in the spleen noted at days 7,8 are the progeny of CFU-E assayed at days 4,5. These cells may have the capacity to give rise to clusters of 4-7 cells (2-3 divisions) when removed into an

in vitro system.

The erythroleukemia models Friend and Rauscher both utilize a mouse system, therefore, one cannot assay for compensatory erythropoiesis in the spleen, since the mouse spleen is normally involved in hematopoiesis, and the spleen itself is a primary target for infection. Compensatory hematopoiesis has been found to occur in other leukemic models, e.g. Shay chloroleukemia. Handler and Handler (1972) reported that heme synthesis was stimulated in the spleens of leukemic rats; whereas, heme synthesis was repressed in the bone marrow of these animals. Furthermore, levels of CFU-E found in the spleens of Shay infected animals were very high and exceeded control levels for bone marrow CFU-E (Handler and Handler, 1976). Bone marrow CFU-E in the Shay animal were found to be depressed with disease progression. Thus, compensatory erythropoiesis continues to occur in the Shay spleen even at the terminal stages of disease progression. As leukemic proliferation in the spleen continues, compensatory erythroid development eventually falters.

Evidence for compensatory hematopoiesis occurring in leukemic patients is scant. Sjorgen (1976) reported that in cases of human CML, increased levels of erythroblasts in the spleen and liver were evident. However, the labelling index of these cells was low, indicating that even here the proliferative activity was impaired, and a maturation arrests at the level of the basophilic erythroblast was also found to occur in the leukemic spleen, as well as in the bone marrow.

As postulated earlier, the increased levels of splenic erythropoiesis reported in the EBL model could be due to either increased migration of

CFU-E (or earlier BFU-E) from the bone marrow to the spleen, or the development of CFU-E within the spleen from a pre-existing population of cells which is normally quiescent. However, Hara and Ogawa (1977) have found that in the mouse, BFU-E but not CFU-E are present in the circulation. They have further shown that the response to manipulations of the erythron is increased migration of BFU-Es from the femur to the spleen. Therefore, it is likely that the CFU-Es found in the EBL spleens are derived from migratory BFU-Es. Splenic erythropoiesis appears to have only a minor effect on maintaining levels of erythropoiesis within the infected animals, and its importance may only be to serve as an indicator of the degree of leukemic involvement.

III. Granulopoiesis

A. Peripheral Blood Determinations

Differential staining of peripheral blood smears indicated that levels of neutrophils (PMNs) within the circulation rose from a mean of 26% at day 0 to a mean of 29.8% at day 9. The PMN levels begin to show an increasing trend by day 7, the point at which hct and retic levels are definitely declining. Although the increases in percent PMN of total level of circulating nucleated cells appears slight, these values are significant since levels of nucleated cells within the circulation also increased during leukemic development. Thus, both the absolute number of PMNs as well as their relative numbers increased during disease progression.

B. Organ Smears

Examination of the bone marrow in EBL animals showed no substantial decrease in myeloid precursors. In control animals levels of promyelocytes

and myeloblasts yielded a mean of 1.5% metamyelocytes, 4.1%. Severely leukemic animals showed promyelocytes and myeloblasts at 0.9% and metameylocytes at 2.5%. These differences cannot be considered significant, when one considers that within the animals' bone marrow, blast levels are increasing and that differential values are relative rather than absolute.

Similar investigations of hematopoietic organ morphology changes with leukemic development have been analyzed in myeloid leukemia models, Shay chloroleukemia and L5222 (Handler and Handler, 1970; Hoelzer and Harriss, 1973). Levels of myeloid cell precursors in the bone marrow are depressed in both cases with disease progression due to depletion of the myeloid precursor pool. Another method for assaying levels of meyloid cells measures levels of leukocytes mobilized in response to endotoxin. This assesses levels of juvenile and mature neutrophils in the bone marrow which can respond to physiologic need, and indirectly assesses levels of neutrophil production. Hander and Varsa-Handler (1966) found that leukocyte mobilization was dramatically depressed at the terminal stages of Shay chloroleukemia. Levels of leukocytes dropped to a mean of $1.8x10^3$ cells/mm^3 from a range of $10\text{-}60x10^3$ cells/mm^3 in control animals.

Peripheral blood determinations have not been extensively utilized to investigate granulopoiesis in the erythroleukemic models. Broxmeyer et al. (1975) investigated changes in circulating granulocyte levels with RLV-A infection. They found no evidence of granulocytic suppression in the infected animals. Leukocyte mobilization in response to endotoxin was not found to be affected in RLV-A disease.

In summary, during EBL disease development as in the other erythroleukemic models, granulopoiesis assessed from peripheral blood and hematopoietic organ differentials did not appear to be repressed in response to leukemic cell proliferation.

C. In Vitro Assessment of Granulopoietic Activity: CFU-C Assay

Perhaps a more definitve assay for granulopoiesis is the CFU-C assay which assesses levels of granulocytic and monocytic progenitors in an in vitro system. The CFU-C assay involves growing cells on semi-solid media such as methylcellulose or agar in the presence of exogenous colony stimulating factor (CSF). The CFU-Cs grow into discrete round aggregates of 50 or more cells after 7 days incubation. The clonal origin of the CFU-C has been shown by Bradley and Metcalf (1966) by linear regression analysis of numbers of colonies to the numbers of cells plated. Further substantiation for the CFU-C clonal origin was provided by Metcalf et al. (1971) and Moore et al. (1972) who followed the development of a colony from a single cell utilizing micromanipulation techniques. Individual colonies picked off the plates and stained were found to contain both maturing granulocytes and monocytes, or only one of the above cell types. Douglas and Pickering (1976) after closely following levels of CFU-C and bone marrow proliferating granulocyte levels, reported that the CFU-C assay was indeed a good reflection of the proliferative state of the bone marrow.

CFU-C levels in EBL injected animals was found to be elevated both in early and late phases of EBL disease progression. From the data presented in Figure 9, it is apparent that levels of CFU-C increased concur-

rently with levels of blasts in both the early and late leukemic stages. The increase in granulocytic proliferation evident from this data coincides with increases in PMN levels in the peripheral blood.

Increased levels of granulopoiesis have also been found in other erythroleukemia models such as Friend and Rauscher. Golde et al. (1976) examined levels of in vitro stem cell growth during Friend virus infection. The growth of the colonies was dependent on the addition of CSA, spontaneous colonies were not observed even at high plating densities. The cloning efficiency of the leukemic spleen cells was found to be 10x that of noninfected cells (40 colonies per $2x10^5$ cells as opposed to 4 colonies per $2x10^5$ cells). When one considers that during Friend virus infection spleen mass increases to 30 times that of control, it appears that leukemic animals may have at least 300x as many CFU-Cs as controls. These CFU-C appear normal in their growth characteristics, but it may be possible that the CFU-C is also a virus infected cell which retains its potential for differentiation under certain environmental conditions. Similarly, levels of CFU-C in both the marrow and spleens of RLV infected mice were found to be greatly increased at the terminal stages of the disease (Iturriza and Seidel, 1974). Koltun et al. (1976) reported that RLV-A infected Balb/c mice showed elevated levels of marrow CFU-C in the early phase of the disease, i.e. while hct levels were still normal. Spleen CFU-C levels were slightly elevated. As the disease progressed and anemia developed (hct at 20%) levels of marrow CFU-C returned to normal, while spleen CFU-C increased. The morphology of these colonies appeared to be normal. In contrast to the erythropoietic system, there was no indication

of granulocytic repression even though there was strong evidence for viral infection in both compartments. (It is of interest to note that while the target cell for virus infection may be the pluripotent stem cell, there is no manifestation of abnormal development per se within the granulocytic compartment, while the erythroid compartment is severely affected. The selective mechanism of this repression is still open for speculation.)

Animal models of myelomonocytic leukemic indicate perturbations in CFU-C growth. Hoelzer et al. (1974) investigated changes in marrow CFU-C and CFU-C during the development of L5222 disease. They reported that levels of CFU-C remained stable for 2 days and then began to decline. By day 5, levels of CFU-C were down to 45% of normal. The morphology of the colonies and their size was not altered in the disease. Upon injection of the colonies into normal animals tumor did not develop, thereby indicating the non-tumor origin of the colonies which do develop from the marrow of infected animals. Levels of CFU-S (which were characterized morphologically as the small lymphocyte population but not functionally defined) did not seem to be affected in L5222. Thus, in this myeloid leukemia model, while the pluripotent stem cell itself does not seem to be affected in the disease, its sequential development to committed granulocytic progenitors (CFU-Cs) is blocked. However, the course of the disease development may be too rapid to demonstrate changes in the CFU-S compartment, since the CFU-S is a very slowly turning over population. Another point that should be considered is that the block in the developmental process of the pluripotent stem cell may be affected by non-specific toxic factors

generated in the leukemic disease.

The pattern of CFU-C growth in Shay leukemia is different. Handler, et al. (1974) reported that initially during leukemia development, levels of CFU-C increased. At the later stages, smaller colonies of 15-20 cells were abundant. In addition, sheets of tumor cells grew over the surface of the plates and appeared distinct from the granulocytic colonies. Plates seeded just with tumor cells grew only sheets of cells, never round cellular aggregates (clusters) found in mixed populations. It was postulated that these clusters which develop from the late leukemic marrow, represent normal CFU-C whose developmental progress is blocked either in a permanent or temporary fashion by the tumor cells. There may simply be a longer lag time before colonies appear after the cells are transferred in vitro before normal growth can resume. Because of the interference of tumor cell overgrowth in mixed culture preparations, utilization of cell separation techniques to isolate and grow CFU-C from affected marrow would help elucidate the selective repression mechanism.

In summary, the erythroleukemias induced by virus in murine models effect increases in the CFU-C compartment, while in the myeloid models, there is either overall repression (as in L5222) where stem cell maturation and differentiation appears to be blocked, or there is initial CFU-C stimulation followed by both erythroid and granulocytic repression. While both EBL and Shay ostensibly show erythroid repression and myeloid stimulation, there are many important differences: 1) The EBL CFU-C do not show an increase in cluster formation. Levels of clusters which are found are very similar to levels of control clusters. There is no preferential

growth of clusters at the expense of colonies. 2) In the EBL disease, the spleen which can initially show compensatory erythropoiesis at the early phase of the disease, shows compensatory granulopoiesis at the later phase, i.e. there is a switch over from erythroid to granulocytic production. In Shay, the spleen continues to show both CFU-E and CFU-C even at the late stage of the disease. Handler and Handler (1976) reported high levels of spleen CFU-E proliferation even where spleen blasts exceeded 48%. 3) Levels of erythropoiesis from EBL BM are more severely repressed, and at a much earlier stage than in Shay, indicating specific repression of the erythroid compartment.

IV. Conditioned Media Experiments

Colony formation *in vitro* by hematopoietic cells can be modulated by the presence of various factors in the growth media. For example, erythroid colony formation is enhanced in the presence of T cells or media conditioned by activated T cells (Nathan et al. 1978). Granulocytic colony formation is dependent on colony stimulating activity (CSA) which can be obtained from media conditioned by monocytes (Chevernick and LoBuglio, 1972). Conversely, growth of hematopoietic cells *in vitro* can be inhibited in the presence of various factors. One class of these inhibitors, known as chalones are cell type but not species specific. These factors are elaborated by mature cells of any given cell line and act through a feedback mechanism to control proliferation of immature cells within that specific line. Thus, for example, a mature epidermal cell produces a specific chalone inhibitory only to epidermal cells (Bullough, 1975).

Various investigators have found evidence of chalone activity in both

erythropoiesis and granulopoiesis. Lindemann (1975, 1976) found that extract from hemolyzed mature red blood cells acted to inhibit the effect of erythropoietin. Mice made polycythemic and then injected with exogenous epo normally responded by increasing ^{59}Fe incorporation in bone marrow and spleen. When the inhibitor was administered shortly before epo injection, there was a 60% reduction in ^{59}Fe incorporation. The effect of the inhibitor was diminished if it was administered 24 hours before or after the epo. Thus, the proliferation of erythroid cells was shown to be specifically inhibited by the erythropoietic chalone.

Similarly, it has been reported by Rytomma and Kiviniemi (1968a) that tumor cells also produce chalones specifically inhibitory to normal cells originating from the same type of tissue. They obtained a chalone by incubating Shay chloroleukemic cells in saline for 1-2 hours and then roughly purifying the glycoprotein of molecular weight 4,000. The action of the chalone was assayed on normal bone marrow cells in culture. These cells were grown by the hanging drop method from a coverslip and the incorporation of ^{3}H-thymidine was investigated by autoradiography in order to assay mitotic rates in the presence of the chalone. Rytomma and Kiviniemi (1968b,c) reported that the rate of both DNA and RNA synthesis in granulocytes was repressed by the chalone, thereby demonstrating the tissue specific nature of the chalone. Paukovitz (1973) utilized another method to demonstrate cell line specificity of the chalone isolated from Shay leukemic cells. Bone marrow cells were centrifuged through a dense Ficoll solution in order to separate the various precursor subpopulations. He found that the fraction where the greatest inhibition of ^{3}H-thymidine

incorporation occurred, consisted of 80% myeloblasts and promyelocytes. This strongly indicated the specific inhibitory action by the Shay tumor product on granulopoiesis.

Another class of inhibitors has been found to affect hematopoiesis. Broxmeyer et al. (1978) reported that lactoferrin derived from mature neutrophils repressed CSA production and release by monocytes, thereby inhibiting granulocytic production. Erythropoiesis was not found to be significantly affected. On the other hand, Herman et al. (1978) found neutrophils to produce a potent inhibitor which affected erythroid but not myeloid colony formation in vitro. They suggest that their neutrophil product may contain distinct substances each affecting hematopoiesis differently, and that variations in media preparation may affect inhibitor activity.

Leukemic cells have also been found to produce a broad spectrum of inhibitors. Quesenberry et al. (1978) found that the murine myeloid leukemia C1498 inhibited the recovery of both the pluripotent stem cell (CFU-S) and the granulocyte progenitor (CFU-C) from normal marrow grown in a double diffusion chamber adjacent to the leukemic cells. Broxmeyer et al. (1978) reported that human leukemic cells of various etiology elaborated an inhibitory factor. This substance was found to be specifically inhibitory to CFU-C growth in vitro, affecting the CFU-C progenitor in S-phase, while the CFU-D (diffusion chamber progenitor cell) was not affected. Included in this study were media conditioned by erythroblasts, lymphoblasts as well as myeloblasts. In all cases, inhibition of granulocytic colony formation in the presence of the media was found. Thus, this leukemic cell inhibitor appears to be different from the cell type

specific chalone discussed by Rytommma and Kiviniemi (1968) who reported that similarly prepared products from other tumors were not found to inhibit myeloid cell proliferation.

In the present series of studies, it was found that during the EBL disease, CFU-E development was markedly repressed, whereas CFU-C growth was enhanced or not affected. This indicated specific inhibition of erythropoiesis. In order to determine whether EBL cells elaborated a substance specifically inhibitory to erythropoiesis, EBL cells, Shay cells as well as normal hematopoietic cells were allowed to condition media for various amounts of time. Normal erythroid and myeloid colony formation in vitro in the presence of these inhibitors was assessed. The results of the conditioned media experiments presented indicated that EBL conditioned media (CM) was in fact more inhibitory to erythroid growth than was Shay CM on the same bone marrow sample. Whereas, EBL CM conditioned for 48 hours at a final concentration of 20% of the growth media inhibited CFU-E growth more than 60%, Shay CM inhibition under the same conditions was only 25%. These missing colonies were not represented in the ECL compartment, i.e. there was no corresponding increase in cluster formation as was seen with peritoneal exudate CM. Cluster formation was also inhibited by the EBL CM at this concentration. At low concentrations of EBL CM, i.e. 24-hour CM at 10%, erythroid cluster formation was stimulated to 160% of control, while there was no decrease in CFU-E levels. Thus, the increase in cluster formation did not occur at the expense of colony growth. There may be multiple factors released by the EBL cells into the media with time that show agonistic effects. Extensive purifi-

cation and isolation of the CM components would be necessary to clarify these findings. Because the media was not enriched (concentrations of all nutrients were the same as control), increased levels of cluster formation could not be ascribed to supplementation of nutritional factors. Shay CM was also found to be stimulatory to erythroid cluster formation,while being inhibitory to CFU-E growth. Two opposing trends were found; first, levels of ECL stimulation increased with increasing concentrations of CM (Figure 15) and levels of ECL stimulation decreased with increases in CM preincubation time. This suggests that the factor which is responsible for the ECL increase is not stable and deteriorates with time.

EBL CM also showed strong inhibitory activity on CFU-C growth in vitro. EBL CM conditioned for 24 hours at 10% concentration, inhibited CFU-C growth 75% and 20% concentrations of this CM inhibited CFU-C growth 100%. This is surprising because in most cases, CFU-C growth from EBL animals was elevated and not repressed. It would, therefore, appear that the in vitro inhibition of CFU-C growth by EBL cell product is distinct from the in vivo situation. In vivo, other factors such as bacterial infection, may allow the initial inhibition of tumor cells to be overridden resulting in increased myelopoiesis. On the other hand, it may well be that the crude media extract contains various substances some analogous to the cell type specific chalone described by Rytomma and Kiviniemi, others analogous to the granulocytic inhibitor described by Broxmeyer, produced by various human leukemic cells, specifically inhibitory to the CFU-C. Another possibility is that the EBL disease is, in fact, a stem cell lesion and that these cells would produce a chalone inhibitory to

both erythroid and myeloid growth in vitro.

Nature of the Erythroleukemic Target Cell

It is now apparent that erythroleukemia in humans (DiGuglielmo's syndrome) is an expression of a stem cell lesion. While initially during disease progression, the erythroid compartment is primarily affected, later on the disease begins to resemble a "pure" granulocytic leukemia (Gunz, 1973), in that it is difficult to determine whether the primitive cells are erythroid or myeloid. It would be of interest to determine whether the EBL model is, in fact, similar to the human disease in that it is also a stem cell disease with primary expression of the lesion in the erythroid line. If, indeed, the EBL cell is that primitive, a chalone product of this tumor should be inhibitory to both erythroid and myeloid cells (as was found in vitro).

The identity of the target cell of the other erythroleukemic models is still unresolved although there is much evidence to support the contention that the pluripotent stem cell is the prime target. For example, when Friend cells are transplanted into lethally irradiated hosts, both erythroid and meyloid cells grow and virus markers are found on both cell types (Rossi and Friend, 1970). The target cell for Rauscher leukemia may also be the pluripotent stem cell. Seidel (1973) reported that when the CFU-S compartment was "knocked-out" by pretreating the animals with Myleran (busulfan), tumor colony forming cells were also eliminated. This was shown by injecting mice with RLV, followed by busulfan treatment. The spleen cells were then injected into lethally irradiated hosts and

then assayed for tumor colony formation on the surface of the spleen. No tumor colonies were found on the surface of the spleens repopulated with busulfan treated RLV-infected spleen cells. Okunewick and Phillips (1973) found that Friend or Rauscher infections affected both the number of CFU-S within the bone marrow and spleen, and the seeding efficiency (f) of secondary transfers of CFU-S from repopulated spleens into another group of irradiated hosts. Furthermore, pre-treatment of bone marrow cells with anti-RLV antisera eliminated the CFU-S (Okunewick et al. 1976). Bergson et al. (1977) followed changes in spleen CFU-S levels with RLV-A development. They found that early in the disease, CFU-S levels were 5x that of control and continued to increase with disease progression achieving levels greater than 700% of control. Despite high levels of CFU-S in their spleens, these animals were, however, severely anemic (hct, 15-20%).

Thus, while these studies clearly indicate that Friend and Rauscher virus infect the pluripotent stem cell (CFU-S), the primary site of infection appears to be the erythroid compartment. For example, hypoxia which stimulates the erythroid compartment increases the susceptibility to virus infection (Pluznick et al. 1966). Low levels of actinomycin-D which are inhibitory to erythroid cells, specifically, inhibit FLV infection (Tambourin and Wendling, 1971). Many more similar examples in the literature indicate that the size and replicative capacity of the erythroid compartment are critical in host susceptibility to virus infection, and time interval before symptoms of infection appear (Steeves, et al. 1968; Stephenson et al. 1972; Tambourin et al. 1973; van Beek et al. 1976). From

these studies one can conclude either that while the viruses infect the pluripotent stem cell an unknown mechanism interferes specifically with erythroid cell expression, or that a wide range of cells, both pluripotent and committed, are the targets for virus infection.

The leukemic model presented in this study requires further analysis before the nature of the target cell can be identified. Whereas, use of morphologic criteria identify the blasts as erythroid, and the selective inhibition of the erythroid compartment with infection, both serve to indicate the erythroid nature of the blast cells. The data from the conditioned media experiments suggests that further investigation is necessary before a definitive statement can be made. While Kluge et al. (1976) have been able to show that various clones from the original EBL cells can be induced to differentiate in vitro in the presence of DMSO into hemoglobin producing cells in an analogous manner to Friend cells, to date, these cells have not been stimulated to differentiate along any other pathway. Theoretically, lethally irradiated mice could be transplanted with EBL cells in order to determine whether both granulocytic as well as erythrocytic cells could be induced to mature in this unique environment. Another possibility would be to grow the EBL cells within diffusion chambers, which allow the diffusion of host factors into the chamber, but prevent the dissemination of tumor within the host. By utilizing this method, Vilpo (1973) was able to demonstrate the maturation of Shay chloroleukemic tumor cells into recognizable granulocytes. The tumor cells matured until the metamyelocyte and meylocyte stages and alkaline phosphatase activity was detected after only 4 days of cell growth within the diffusion chamber.

Differentiation of Shay leukemia cell grown in diffusion chamber has not been obserbed by Handler and coworkers (personal communication). Another growth system utilizing both the diffusion chamber technique and the growth of progenitor cells on plasma clots was developed by Steinberg et al. (1976). Discrete colonies of erythrocytes and granulocytes grew in plasma clot diffusion chambers (PCDC) in vivo. Levels of these colonies were responsive to physiologic control, in that phenylhdrazine treatment with subsequent peripheral blood anemia stimulated the growth of erythroid colonies within the PCDC and endotoxin treatment increased the incidence of granulocyte colonies. Since the growth of normal cells within the PCDC has been shown to be regulated by diffusable factors, it would be of great interest to determine whether growth of tumor cells, namely EBL cells could be affected in this in vivo situation. A precedence for in vivo stimulation of normal differentiation of tumor cells is suggested by the results of Lobue et al. (1974), which showed that phenylhdrazine treatment modified the effect of RVL infection in vivo, and temporarily allowed the maturation block of erythroid cells to be overridden. Immunofluorescence studies indicated that a large proportion of the reticulocytes and mature erythrocytes formed after phenylhydrazine treatment bore viral markers. Thus, the application of the PCDC technique to the study of EBL cells would enable the determination of whether anemic stress in vivo can influence EBL differentiation without exposing the animal to EBL disease which develops rapidly in the young adult rat (7-10 days). It is also of interest that in the study by Lobue et al. (1974) granulocytic precursors along with megakaryocytes and monocytes bore viral markers during

RVL disease. While there are no similar markers for EBL cells, the stem cell nature of these blasts would be demonstrated if these non-differentiated cells could be induced to differentiate along the granulocytic pathway within diffusion chambers in a manner analogous to the Vilpo study. Thus, although the stem cell nature of the EBL cell is still open to speculation, there are many possible means at hand which may be used to approach this problem experimentally.

SUMMARY AND CONCLUSION

In summary, the series of experiments reported in this paper provide the basis for a new animal model of an erythroblastic stem cell leukemia. This EBL disease was initially induced by administering 7,12 dimethylbenz(a)anthracene to young adult rats. We found that a line of these leukemic cells could be maintained when injected into neonatal rat pups intraperitoneally. The EBL cells grew preferentially in the liver. By day 14, when the liver was massively infiltrated with tumor cells, the animal was killed by cervical dislocation, the liver was excised, disrupted and the cells retransplanted into another host. The results of the present study showed that upon intravenous injection of young adult rats, an erythroblastic leukemia developed, with major infiltration by the tumor cells of the bone marrow and spleen as well as the liver. The pathogenesis of the disease was monitored daily by taking hematocrits, reticulocyte and peripheral white blood cell counts and peripheral blood differentials. It was found that along with a decline in erythroid production (indicated by a severe drop in levels of circulating reticulocytes), a large proportion (64%) of the animals developed hemolytic anemia by day 7 post-injection, whereas levels of circulating granulocytes increased. Examination of the hematopoietic organs, i.e. bone marrow and spleen at the early phase (days 4-5 post-injection) did not indicate a significantly different distribution in erythroid precursors. When the bone marrow and spleen were examined at the later phase (days 7-8), a sharp drop in nucleated erythroid

cells was evident, whereas myeloid precursor cells were affected even at the initial phase of the disease. Consequently, the stem cell compartments of the bone marrow, spleen and liver were examined. The erythroid progenitor (CFU-E) was grown on plasma clots in the presence of erythropoietin. The meyloid precursor (CFU-C) was grown on methyl cellulose in the presence of serum colony stimulating factor. Multiple regression analysis showed that the variance in levels of CFU-E could be accounted for primarily by changes in bone marrow blast infiltration and liver mass. Even in the initial phases of the disease, when tumor cell levels were low, 5% CFU-E,levels were depressed. However, levels of meyloid precursors in the bone marrow were not depressed during leukemic development and in many cases, were elevated.

Under normal conditions, the spleen in rats is not hematopoietic but can respond under stress. In many cases of erythroblastic leukemia (EBL) splenic compensatory hematopoiesis was found in the early phase of the disease. When the spleens were assayed at the later phase, compensatory erythropoiesis was replaced by compensatory granulopoiesis.

The specific repression of erythroid progenitor cells may be due to specific inhibition by an EBL cellular product. Many investigators have reported that tumor lines elaborate substances which inhibit normal cellular proliferation. It was found that both 20% Shay and 20% EBL conditioned media totally repressed CFU-C growth from normal bone marrow. However, whereas Shay conditioned medium inhibited CFU-E growth 25%, EBL inhibition was greater than 65%. Thus, EBL effect appears to be more generalized, inhibiting both erythroid and meyloid growth <u>in vitro</u>,

while Shay is more effective to myeloid inhibition. These results suggest that the EBL is pluripotent stem cell in origin. This mimics DiGuglielmo's syndrome which is a stem cell disease and is also characterized by ineffective erythropoiesis. The lack of repression of myeloid cells in EBL animals may be due to the preponderance of stimulatory signals, such as bacterial infection etc., which can overcome the inhibition.

In conclusion, EBL disease is characterized by ineffective erythropoiesis which is evident when examining peripheral blood changes, bone marrow and spleen cellular composition, and in vitro stem cell compartments. The spleen serves as a site for compensatory erythropoiesis at the initial phase of the disease and switches to compensatory granulopoiesis at the later phase. Data from conditioned media experiments showing repression of both erythroid and granulocytic colony growth in vitro, indicate the stem cell nature of the EBL tumor cell.

The experimental model for erythroleukemia presented in this study has been shown to have advantages over the pre-existing erythroleukemia models. The time course of the disease is predictable at a given dosage, greater than 80% of the animals develop the disease and the time course is fairly rapid. Furthermore, utilization of this animal model in future research may provide insight in understanding basic normal as well as abnormal differentiation processes.

TABLE 1

NUMBER OF ANIMALS FALLING INTO DIFFERENT CATEGORIES OF HEMATOCRIT LEVEL AND STAGE OF LEUKEMIC DEVELOPMENT

Hct	A	B_1	B_2	C
<30%	5	0	0	0
30-35%	9	0	0	0
36-39%	16	2	0	0
40%	17	8	9	11

TABLE 2

NUMBER OF ANIMALS FALLING INTO DIFFERENT CATEGORIES OF PERIPHERAL BLOOD RETICULOCYTE LEVELS AND STAGE OF LEUKEMIC DEVELOPMENT

% Retics	A	B_1	B_2	C
5%	23	4	0	0
5-10%	15	2	5	3
< 10%	3	2	0	7

TABLE 3

NUMBER OF ANIMALS FALLING INTO DIFFERENT CATEGORIES OF CIRCULATING NUCLEATED CELL LEVELS AND STAGE OF LEUKEMIC DEVELOPMENT

Peripheral CNC/mm^3	A	B_1	B_2	C
> $30x10^3$	21	0	0	0
$20-30x10^3$	17	0	1	0
$10-20x10^3$	9	5	4	8
$10x10^3$	3	5	4	3

TABLE 4

NUMBER OF ANIMALS FALLING INTO DIFFERENT CATEGORIES OF BLAST LEVELS IN THE PERIPHERAL BLOOD AND STAGE OF LEUKEMIC DEVELOPMENT

% Blasts	A	B_1	B_2	C
> 3%	13	0	0	0
1-3%	16	1	0	0
0.5-0.9%	6	0	1	0
0-0.5%	8	4	1	0
0%	2	5	7	4

TABLE 5

CORRELATION COEFFICIENTS OF BONE MARROW CFU-E LEVELS WITH ERYTHROID PRECURSORS (B+) AND TUMOR BLASTS IN BONE MARROW. ALSO SHOWN ARE THE NUMBER OF CASES (IN PARENTHESES) AND THE SIGNIFICANCE LEVEL OF THE CORRELATION FOR EACH GROUP.

	BM B+	BM Blast	Spl B+	Spl Blast	Liv B+	Liv Blast	Spl wt.	Liv wt.
Contr. I	-0.20	-0.002	0.18	-0.211	-0.14	-0.13	-	0.18
	(14)	(14)	(14)	(14)	(14)	(14)		(14)
	p< 0.47	p < 0.99	p < 0.53	p < 0.47	p < 0.61	p < 0.64		p < 0.51
Contr. II	0.24	-0.42	-0.62	0.66	-0.211	-0.78	-	0.14
	(13)	(13)	(11)	(11)	(6)	(6)		(11)
	p < 0.43	p < 0.15	p < 0.04	p < 0.02	p < 0.69	p< 0.06		p < 0.67
4.5 day EBL	0.42	-0.18	0.45	-0.11	0.03	-0.22	-	-0.26
	(28)	(28)	(28)	(28)	(28)	(28)		(27)
	p < 0.03	p < 0.36	p< 0.01	p < 0.56	p < 0.88	p< 0.25		p < 0.18
7.8 day EBL	0.22	0.25	0.29	-0.36	0.51	-0.48	-0.91	-0.36
	(32)	(32)	(30)	(30)	(28)	(27)	(6)	(28)
	p< 0.22	p < 0.17	p< 0.10	p< 0.05	p < 0.005	p< 0.009	p < 0.01	p< 0.06

TABLE 6

CORRELATION COEFFICIENT OF BONE MARROW CFU-C LEVELS WITH ERYTHROID PRECURSORS (B+) AND TUMOR BLASTS IN BONE MARROW, SPLEEN AND LIVER. ALSO SHOWN ARE THE NUMBER OF CASES (IN PARENTHESES) AND THE SIGNIFICANCE LEVEL OF THE CORRELATION FOR EACH GROUP.

	BM B+	BM Blast	Spl B+	Spl Blast	Liv B+	Liv Blast	Spl wt.	Liv wt.
Contr.I	0.03	0.62	0.61	-0.30	0.53	- 0.05	-	-0.18
	(10)	(10)	(10)	(10)	(5)	(5)		(9)
	p< 0.92	p< 0.05	p< 0.06	p< 0.39	p < 0.35	p< 0.92		p< 0.63
Contr.II	-0.06	0.05	0.14	0.64	0.16	0.009		-0.07
	(14)	(14)	(14)	(14)	(14)	(14)		(14)
	p< 0.82	p < 0.84	p < 0.62	p< 0.01	p < 0.58	p< 0.97		p < 0.79
4.5 day	0.10	0.43	-0.09	0.15	-0.27	0.23		-0.12
	(28)	(28)	(28)	(28)	(28)	(28)		(27)
	p < 0.59	p< 0.02	p < 0.62	p< 0.44	p < 0.16	p< 0.23		p < 0.56
7.8 day	-0.28	0.315	-0.008	0.43	-0.43	-0.14	0.33	0.065
	(25)	(25)	(26)	(26)	(25)	(25)	(6)	(25)
	p< 0.17	p < 0.12	p < 0.97	p< 0.03	p< 0.03	p < 0.51	p<0.52	p< 0.75

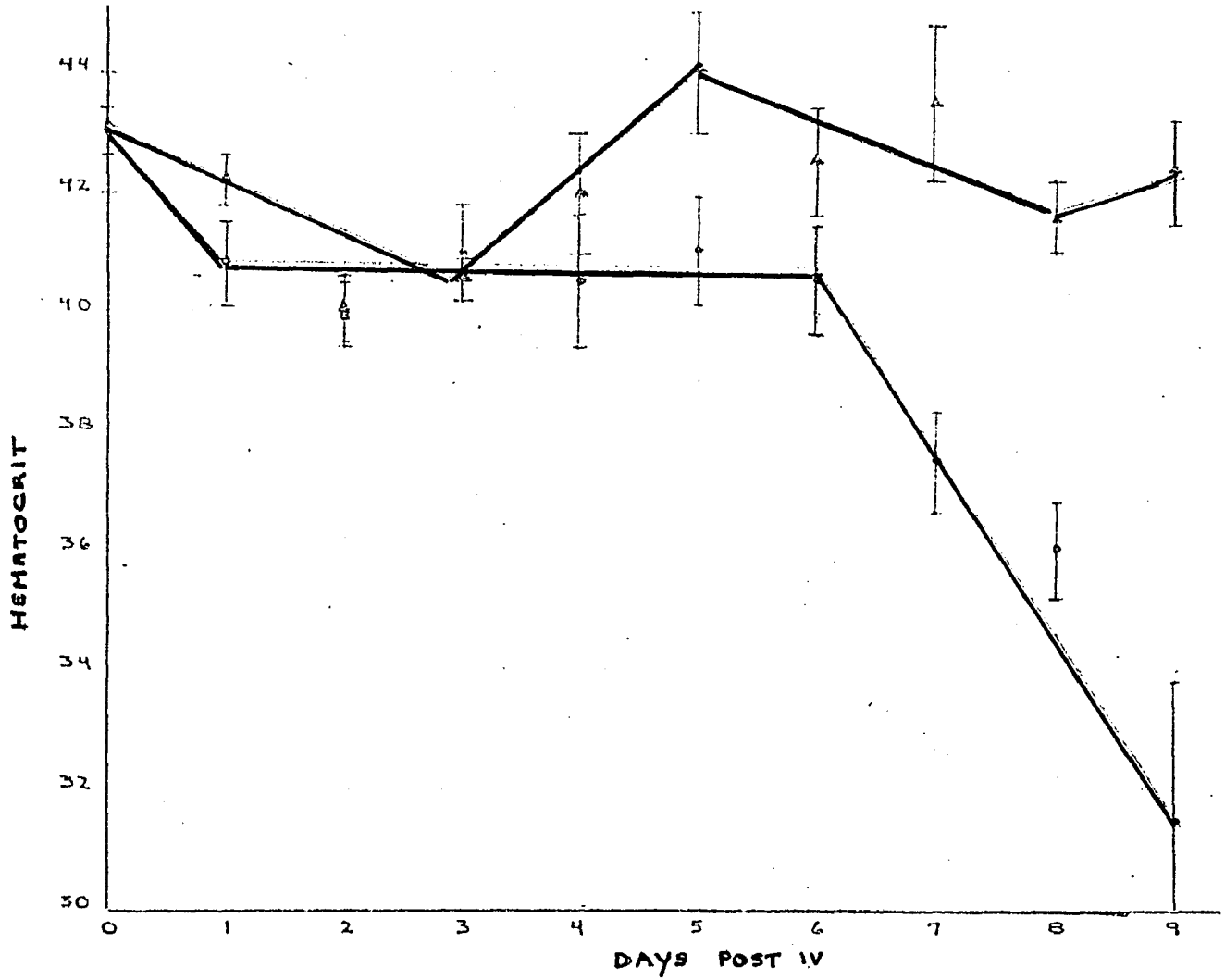
TABLE 7

CORRELATION COEFFICIENTS OF SPLEEN GRANULOCYTE CLUSTER LEVELS WITH ERYTHROID PRECURSORS (B+) AND TUMOR BLASTS IN BONE MARROW, SPLEEN, AND LIVER. ALSO SHOWN ARE THE NUMBER OF CASES IN PARENTHESES AND SIGNIFICANCE LEVEL OF THE CORRELATION FOR EACH GROUP.

	BM B+	BM Blast	Spl B+	Spl Blast	Liv B+	Liv Blast	Spl CFU-C	Spl wt.
Contr. I	-0.13	-0.17	0.003	-0.06	-0.04	-0.11	0.98	-
	(12)	(12)	(12)	(12)	(12)	(12)	(11)	
	$p < 0.67$	$p < 0.59$	$p < 0.99$	$p < 0.85$	$p < 0.89$	$p < 0.73$	$p < 0.001$	
Contr. II	-0.08	-0.43	0.18	-0.29	-0.49	-0.33	0.73	
	(9)	(9)	(9)	(9)	(5)	(5)	(11)	
	$p < 0.83$	$p < 0.24$	$p < 0.64$	$p < 0.44$	$p < 0.39$	$p < 0.57$	$p < 0.009$	
4,5 day EBL	-0.07	0.18	0.07	0.14	-0.21	0.11	0.75	
	(27)	(27)	(27)	(27)	(27)	(27)	(27)	
	$p < 0.70$	$p < 0.36$	$p < 0.69$	$p < 0.48$	$p < 0.27$	$p < 0.56$	$p < 0.001$	
7,8 day EBL	-0.15	-0.15	0.07	0.29	-0.27	-0.31	0.93	-0.55
	(22)	(22)	(23)	(23)	(22)	(22)	(24)	(4)
	$p < 0.49$	$p < 0.50$	$p < 0.74$	$p < 0.18$	$p < 0.22$	$p < 0.16$	$p < 0.001$	$p < 0.41$

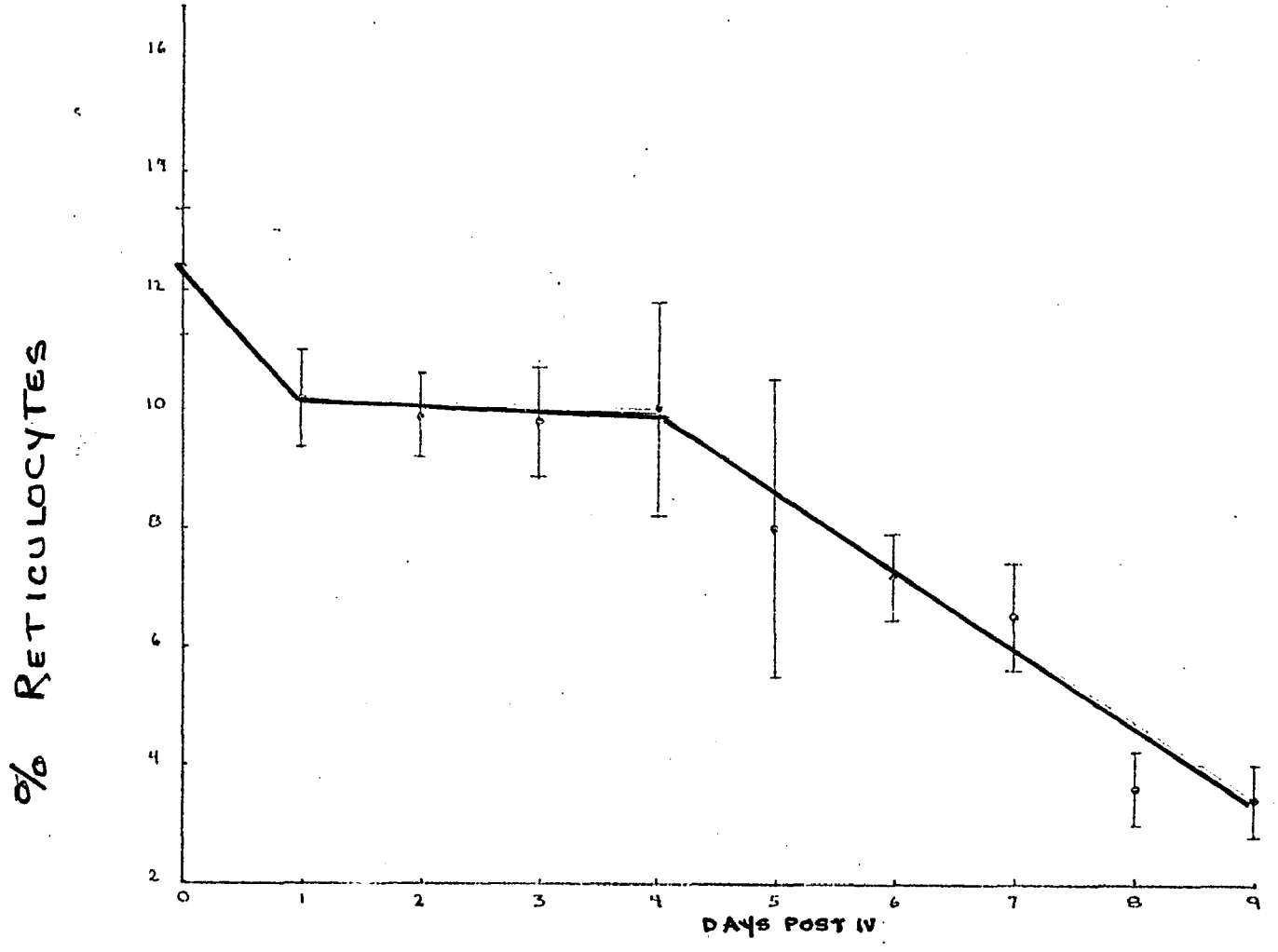
Legend to Figure 1

Daily changes in hematocrit level for control () and EBL injected () animals who showed peripheral blood changes (Group A). The curves shown represent a best visual fit for the individual data points. SEMs are given by the vertical lines.


HEMATOCRIT
44
42
40
38
36
34
32
30
0
1
2
3
4
5
6
7
8
9
DAYS POST I.V.

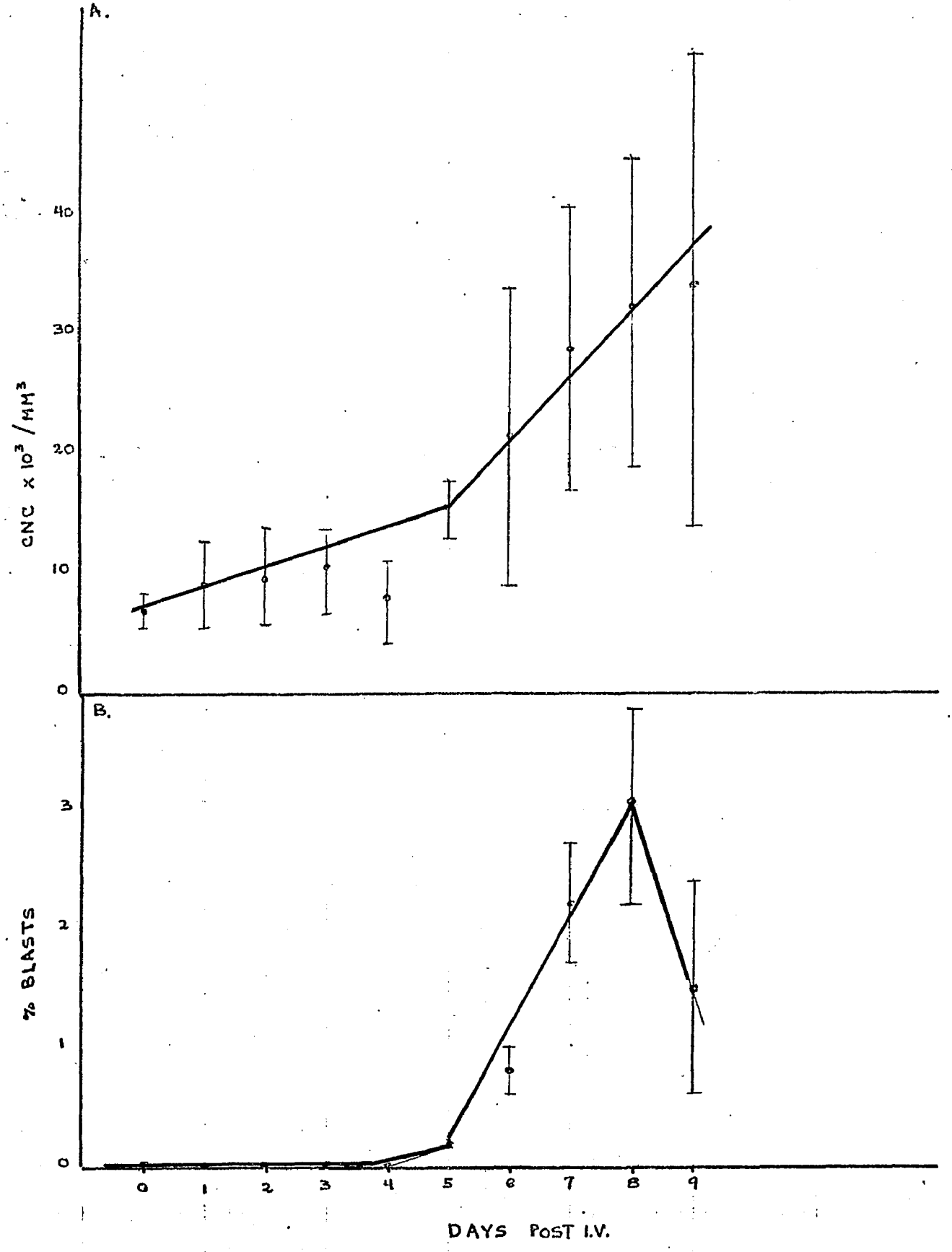
Legend to Figure 2

Daily changes in reticulocyte level for EBL injected animals who showed peripheral blood changes (Group A). The curves shown represent a best visual fit for the individual data points. SEMs are given by the vertical lines.


% RETICULOCYTES
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
DAYS POST IV

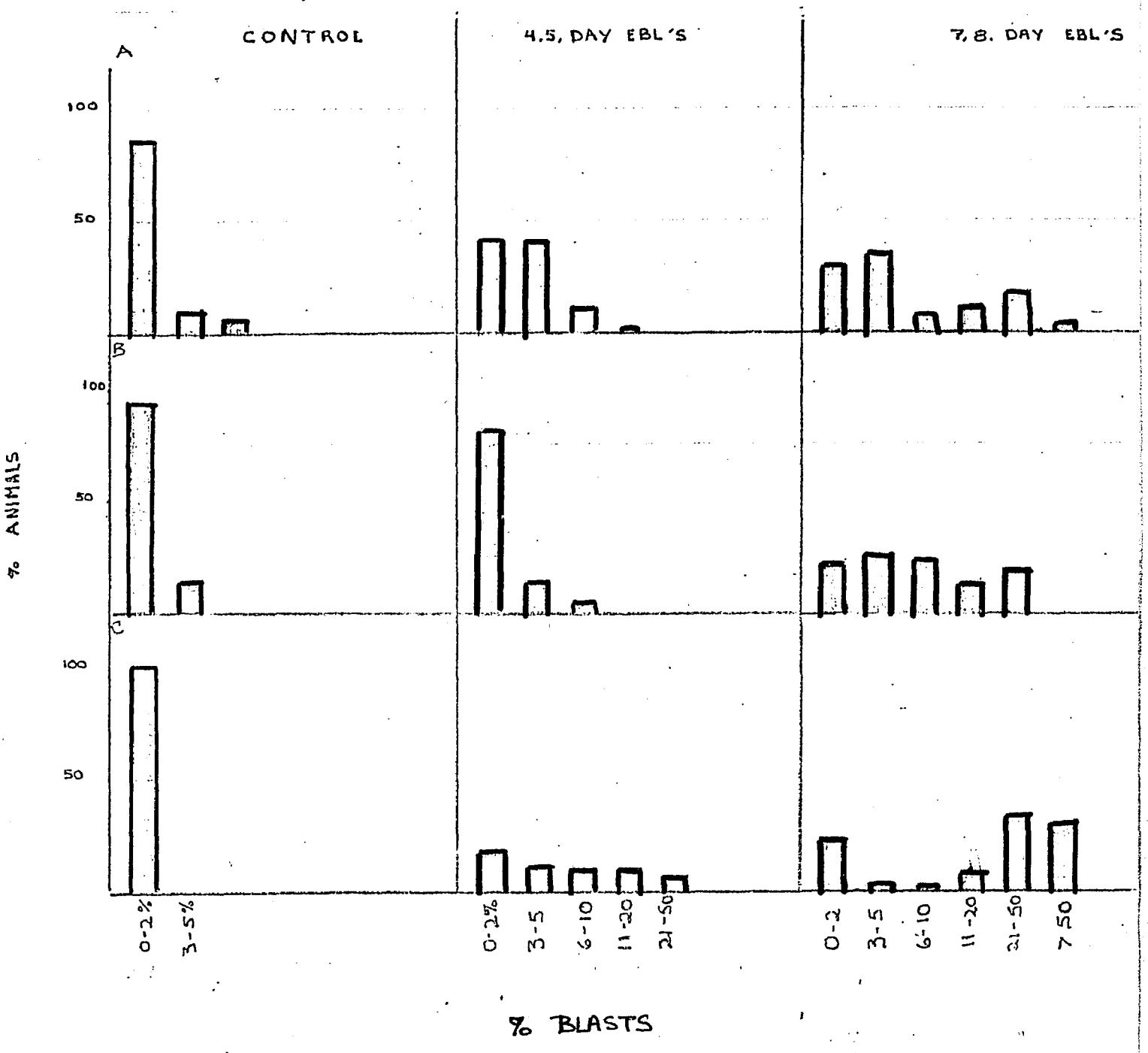
Legend to Figure 3

Daily changes in circulating nucleated cells (a) and peripheral blood blast evels (b) for EBL injected animals who showed peripheral blood changes (Group A). The curves shown represent a best visual fit for the individual data points; SEMs are given by the vertical lines.


A.
CNC x 10³ / MM³
0
10
20
30
40
B.
% BLASTS
0
1
2
3
0
1
2
3
4
5
6
7
8
9
DAYS POST I.V.

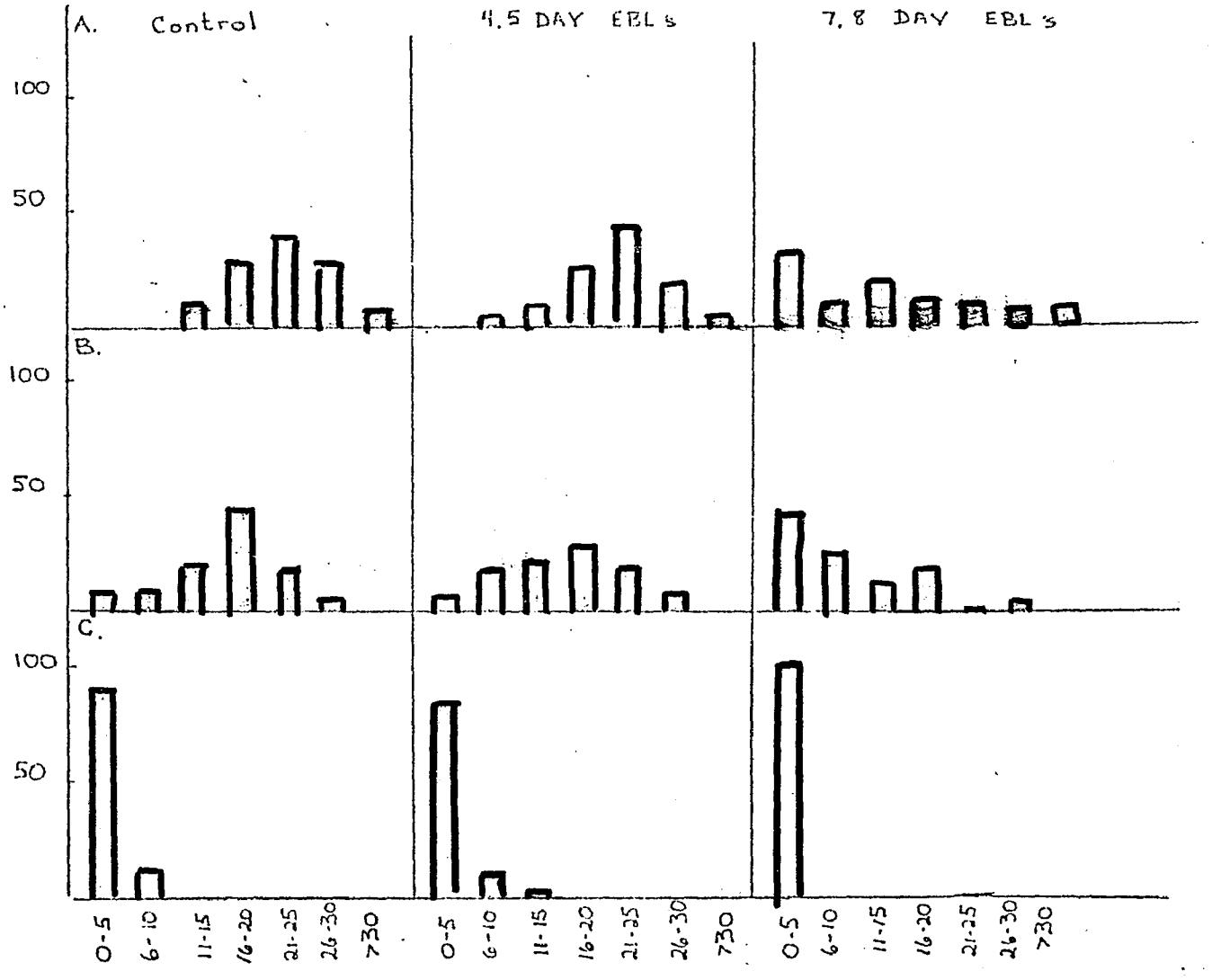
Legend to Figure 4

Frequency distribution of animals falling into the different categories of percent blasts in control, 4,5 day and 7,8 day post-EBL injected animals for bone marrow (row a), spleen (row b) and liver (row c). The frequency is given as the percent of the total animals within the respective block.


CONTROL
4.5, DAY EBL'S
7, 8. DAY EBL'S
A
B
C
100
50
100
50
100
50
% ANIMALS
0-2%
3-5%
0-2%
3-5
6-10
11-20
21-50
0-2
3-5
6-10
11-20
21-50
7.50
% BLASTS

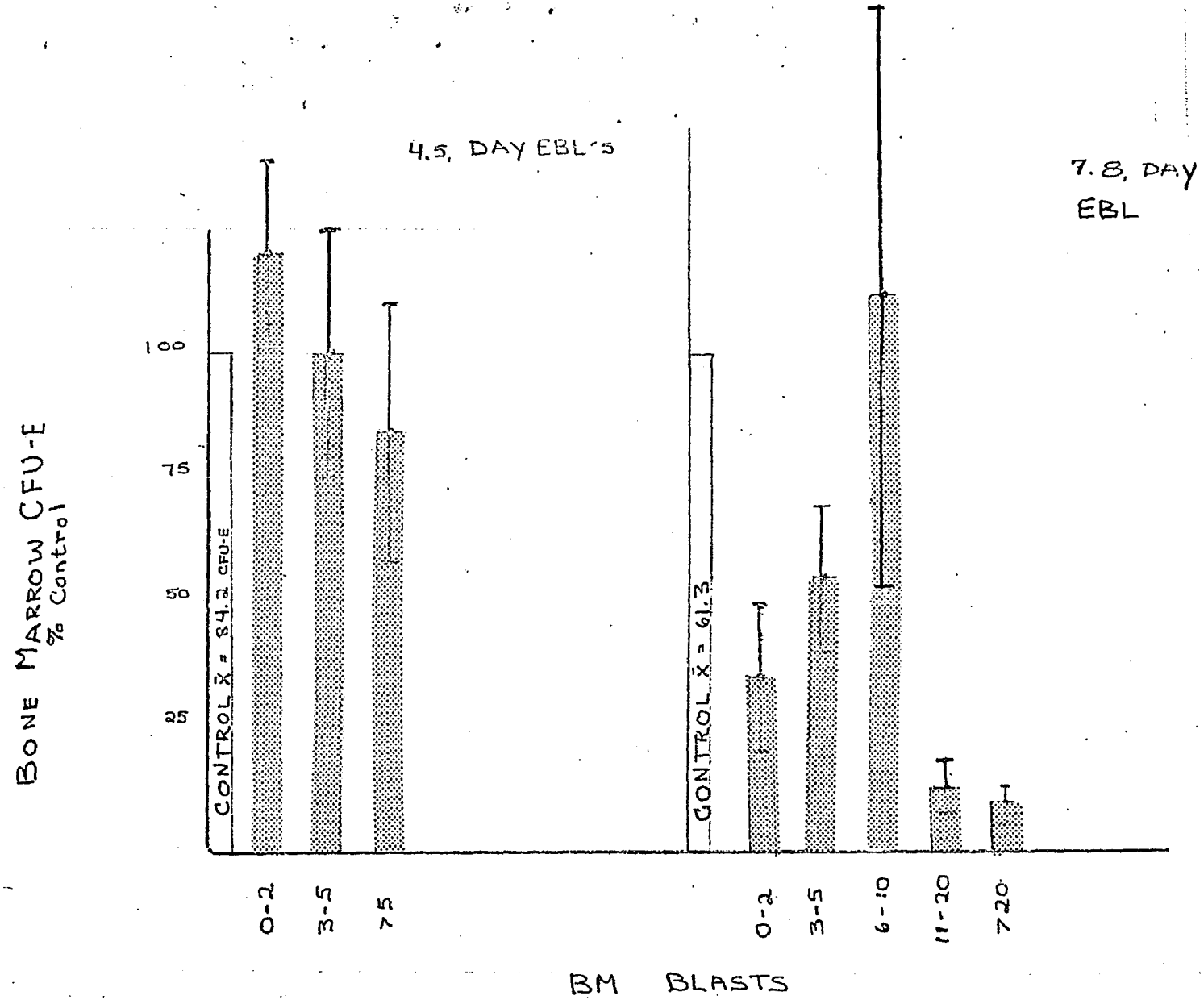
Legend to Figure 5

Frequency distribution of animals falling into the different categories of percent benzidine positive cells in control, 4,5 day and 7,8 day post-EBL injected animals for bone marrow (row a), spleen (row b), and liver (row c). The frequency is given as the percent of the total animals within the respective blocks.


A. Control
4.5 DAY EBL's
7.8 DAY EBL's
100
50
B.
100
50
C.
100
50
0-5
6-10
11-15
16-20
21-25
26-30
>30
% Benzidine Positive Cells

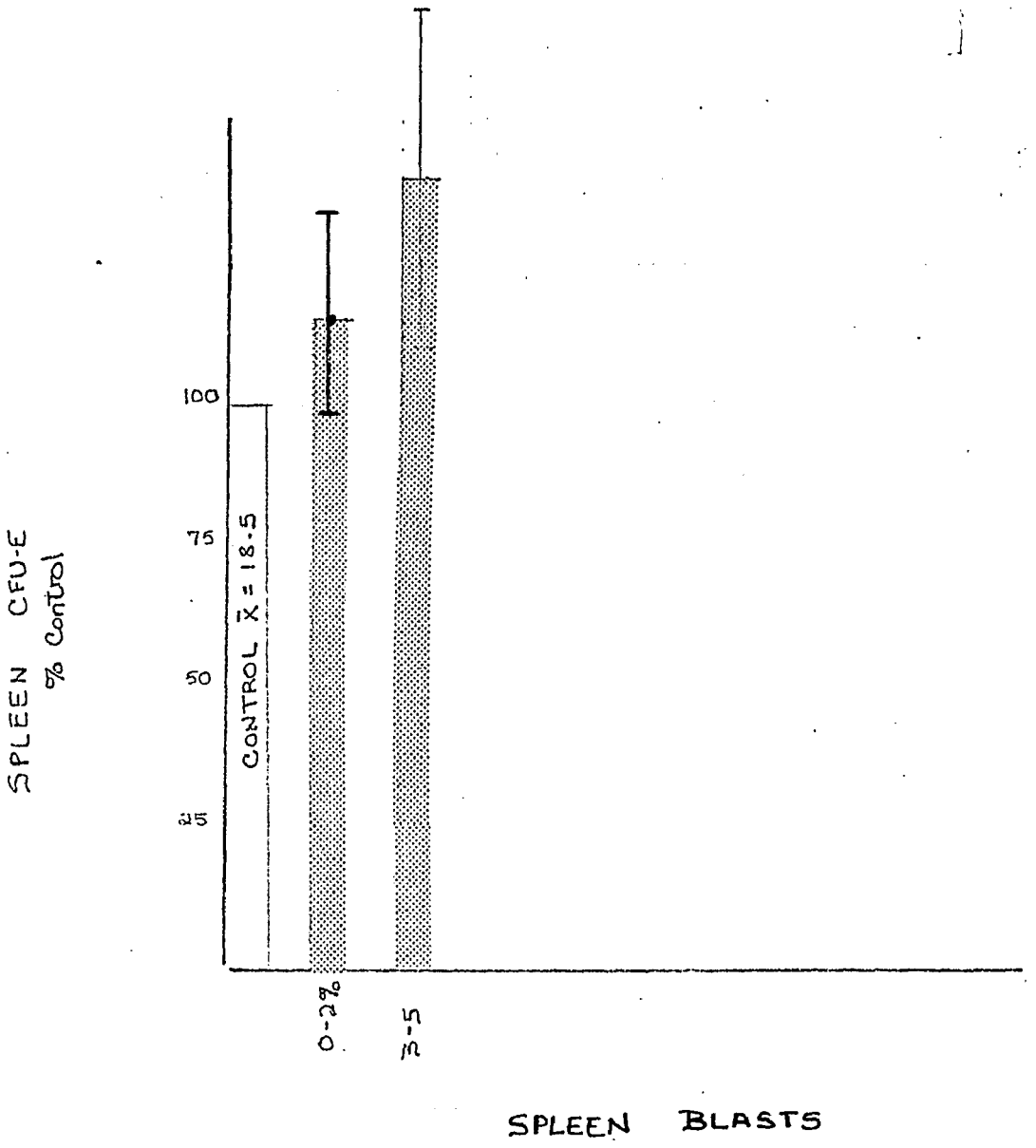
Legend to Figure 6

A frequency distribution of bone marrow CFU-Es (given as percent control) falling into different categories of percent bone marrow for 4,5 day and 7,8 day post EBL injected animals (vertical line: SEM).


4.5. DAY EBL's
7.8. DAY EBL
BONE MARROW CFU-E
% Control
100
75
50
25
CONTROL X̄ = 84.2 CFU-E
CONTROL X̄ = 61.3
0-2
3-5
>5
0-2
3-5
6-10
11-20
>20
BM BLASTS

Legend to Figure 7

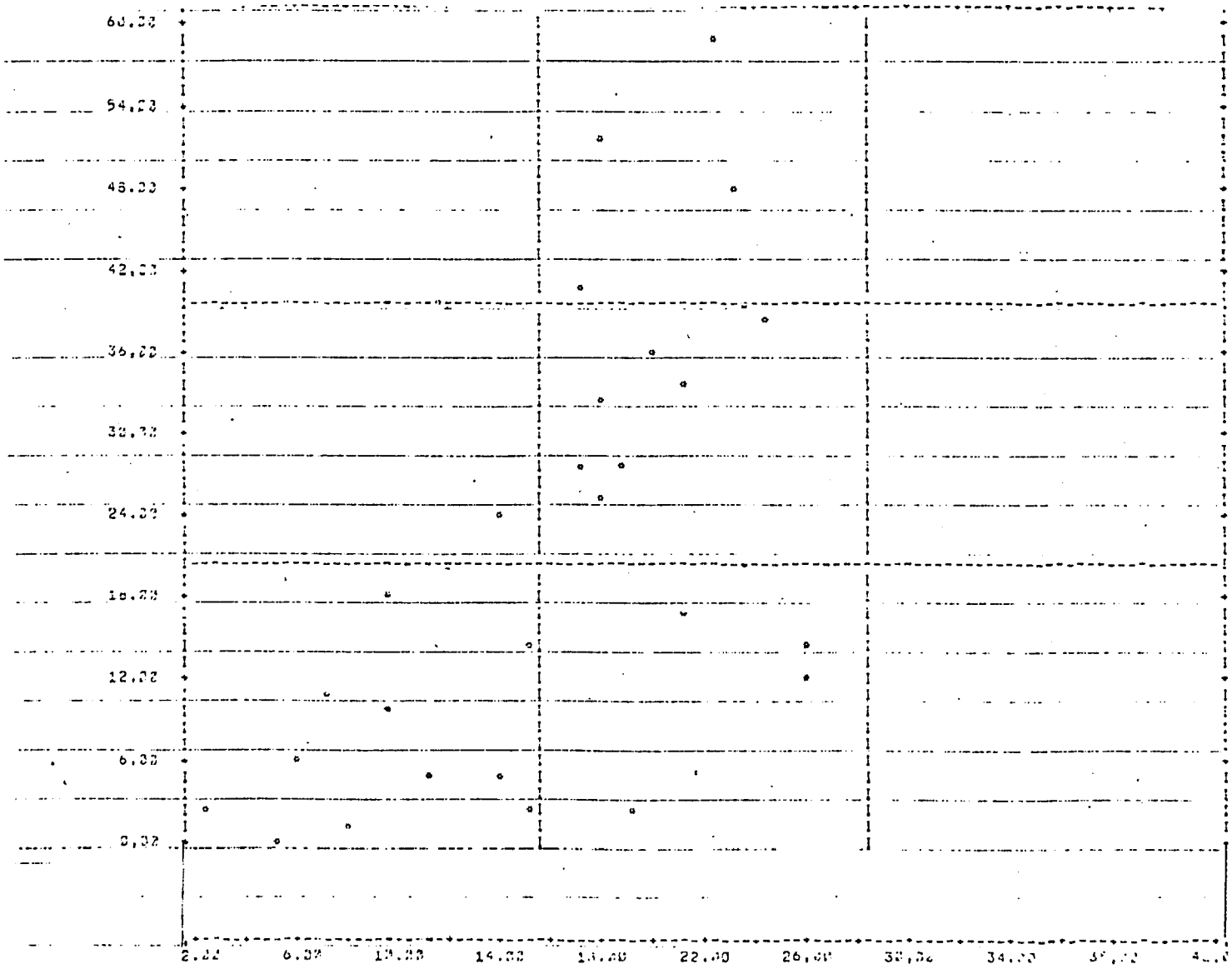
A frequency distribution of spleen CFU-Es (given on ordinate as percent control) falling into the different categories of percent spleen blasts for the early leukemic animal. No spleen CFU-Es were observed in the late leukemic animal (vertical line: SEM).


SPLEEN CFU-E
% Control
100
75
50
25
CONTROL X̄ = 18.5
0-2%
≥5
SPLEEN BLASTS

Legend to Figure 8

Scattergram of number of spleen CFU-Es as a function of the number of spleen benzidine positive cells for the early leukemic animal. Regression analysis is shown at the bottom of the figure.

Scattergram Spleen CFU-E (down) vs. Spleen Benzidine+ cells (across)



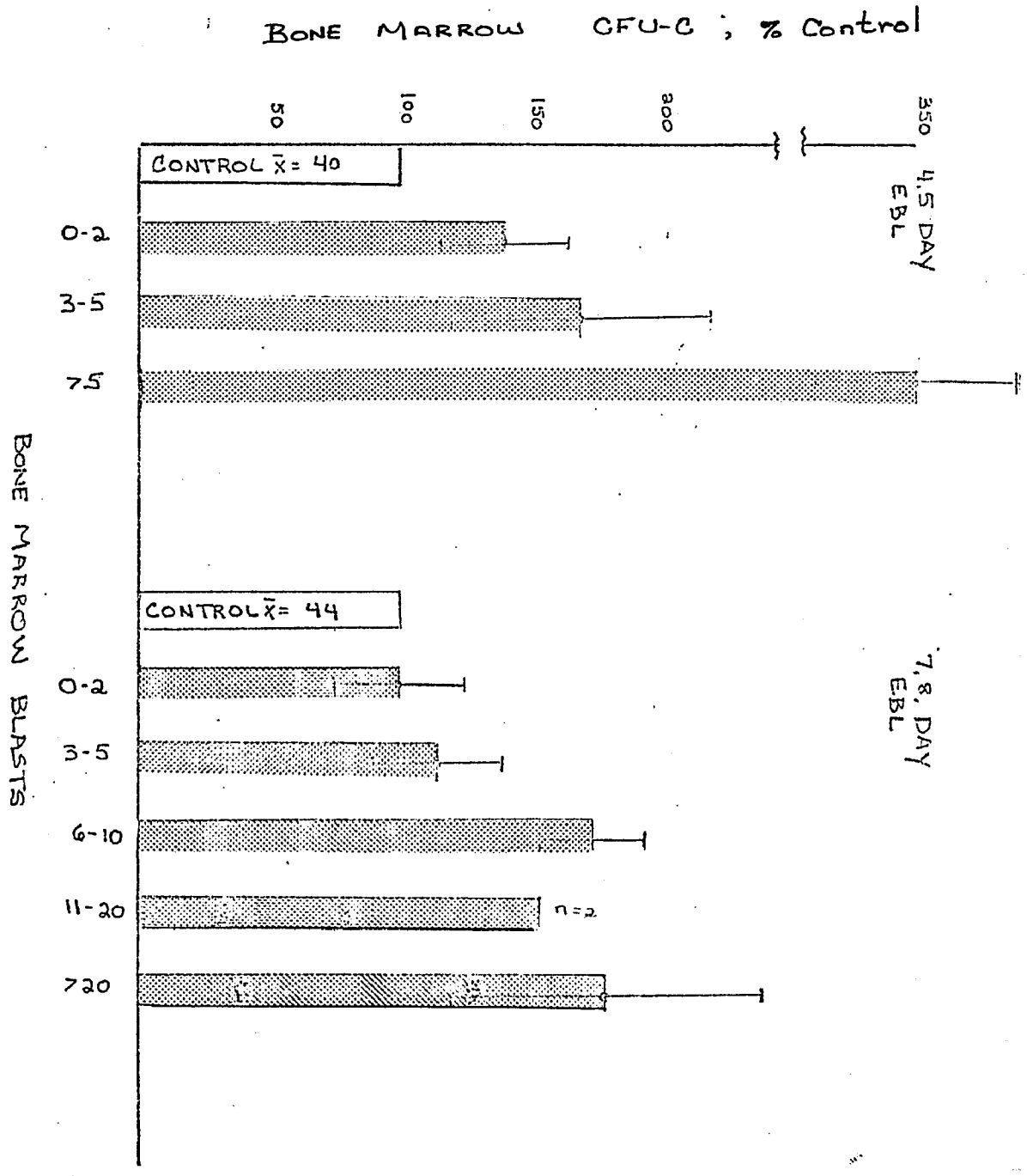
Statistics

Correlation (r) .....o.543 r squared.....o.300

significance.....0.001

Legend to Figure 9

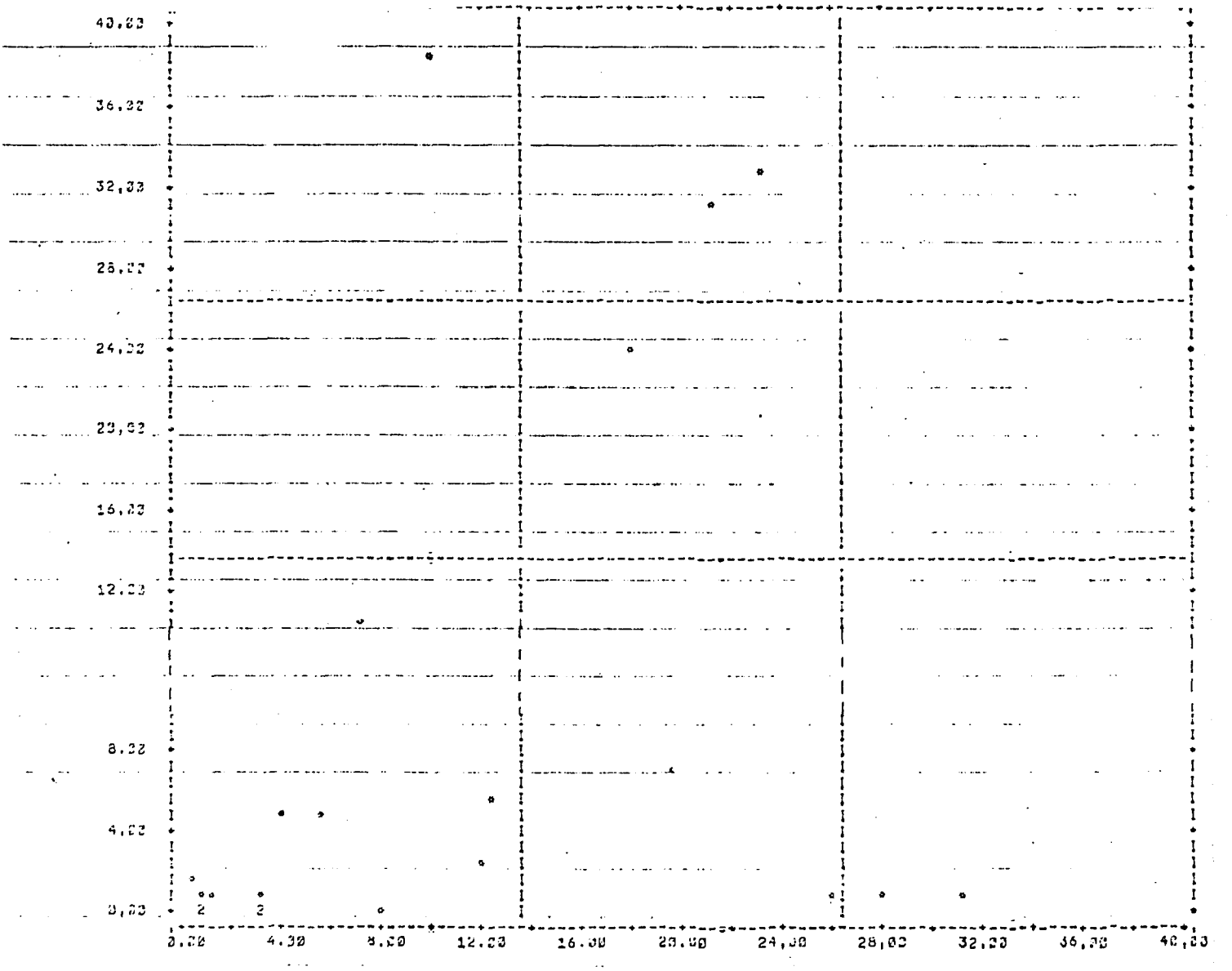
A frequency distribution of bone marrow CFU-Cs (given as percent control) falling into different categories of percent bone marrow blasts for 4,5 day and 7,8 day post-EBL injected animals (vertical line: SEM).


BONE MARROW CFU-C ; % Control
50
100
150
200
350
4,5 DAY EBL
CONTROL $\bar{x}$ = 40
0-2
3-5
7.5
BONE MARROW BLASTS
CONTROL $\bar{x}$ = 44
0-2
3-5
6-10
11-20
n=2
>20
7,8, DAY EBL

Legend to Figure 10

Scattergram of number of spleen CFU-Cs as a function of the number of spleen blasts for the late leukemic animal. Regression analysis is shown at the bottom of figure.

Scattergram of Spleen CFU-C (down) vs. Spleen blasts %



Statistics

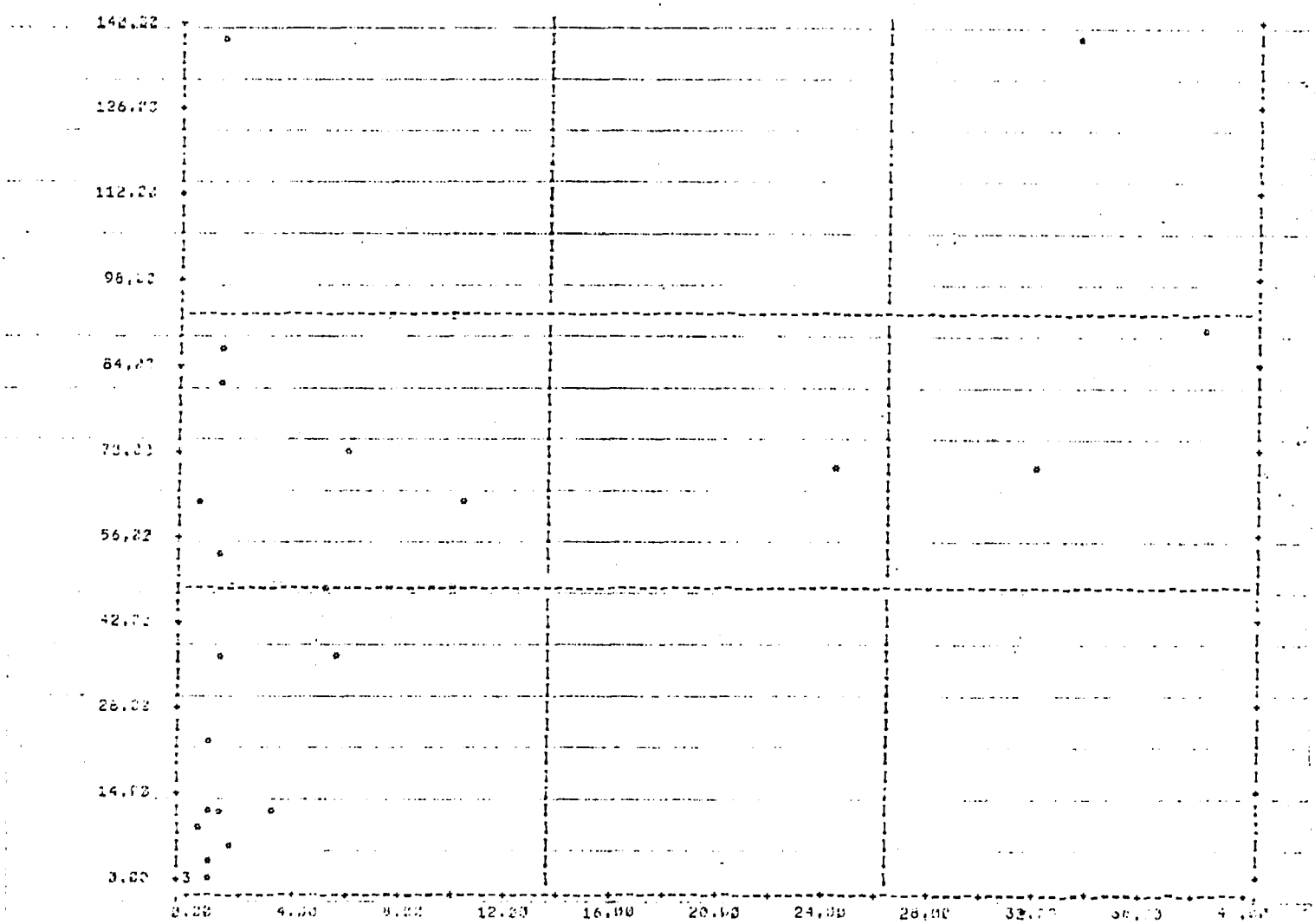
Correlation (r).....0.335 r squared.....0.113

significance 0.068

Legend to Figure 11

Scattergram of number of bone marrow CFU-Cs as a function of the number of spleen CFU-Cs for the late leukemic animal. Regression analysis is shown at the bottom of the figure.

Scattergram of Bone Marrow CFU-C (down) vs. Spleen CFU-C (across)



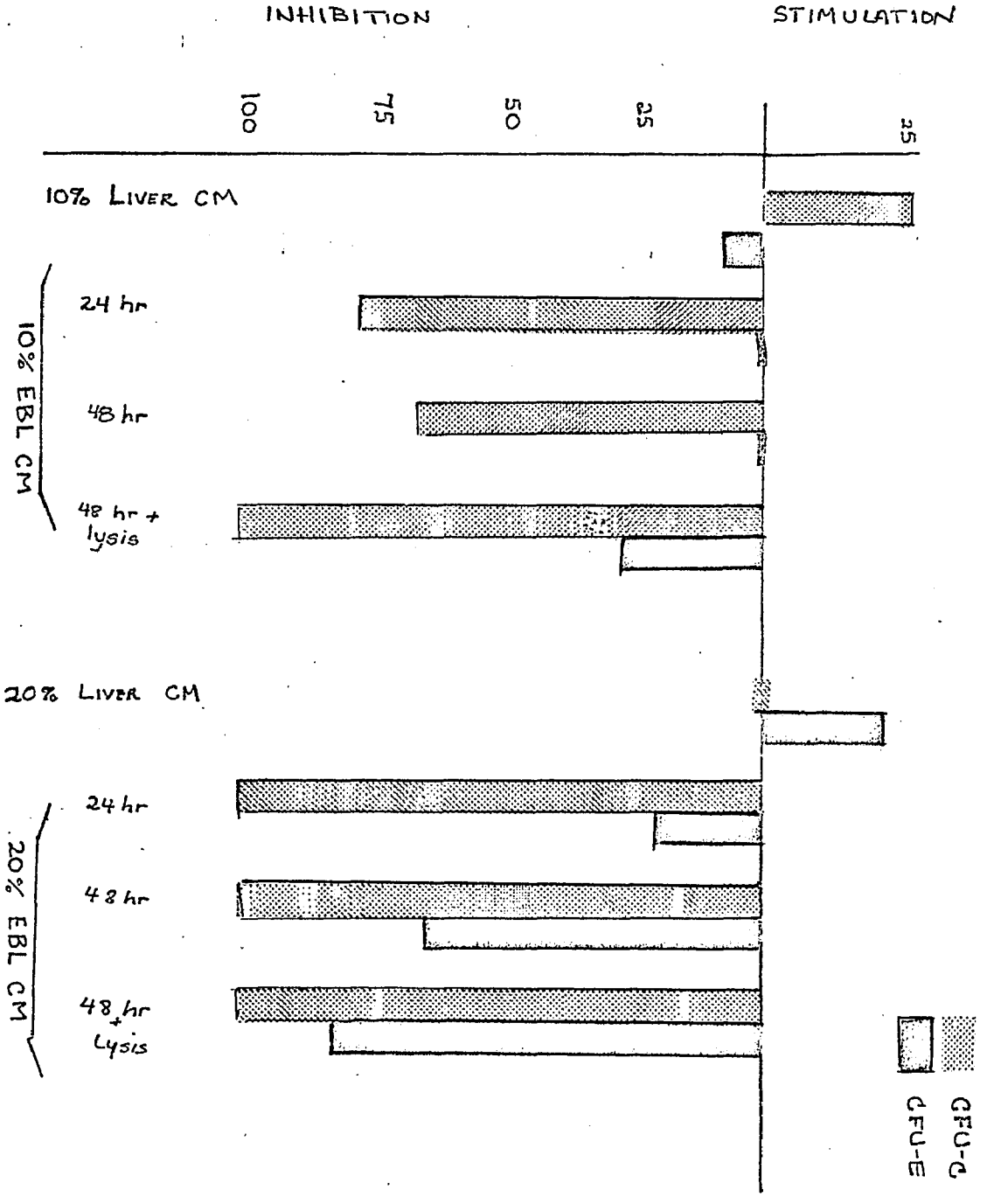
Statistics

Correlation (r).....0.542 r squared.....0.295

Significance 0.0025

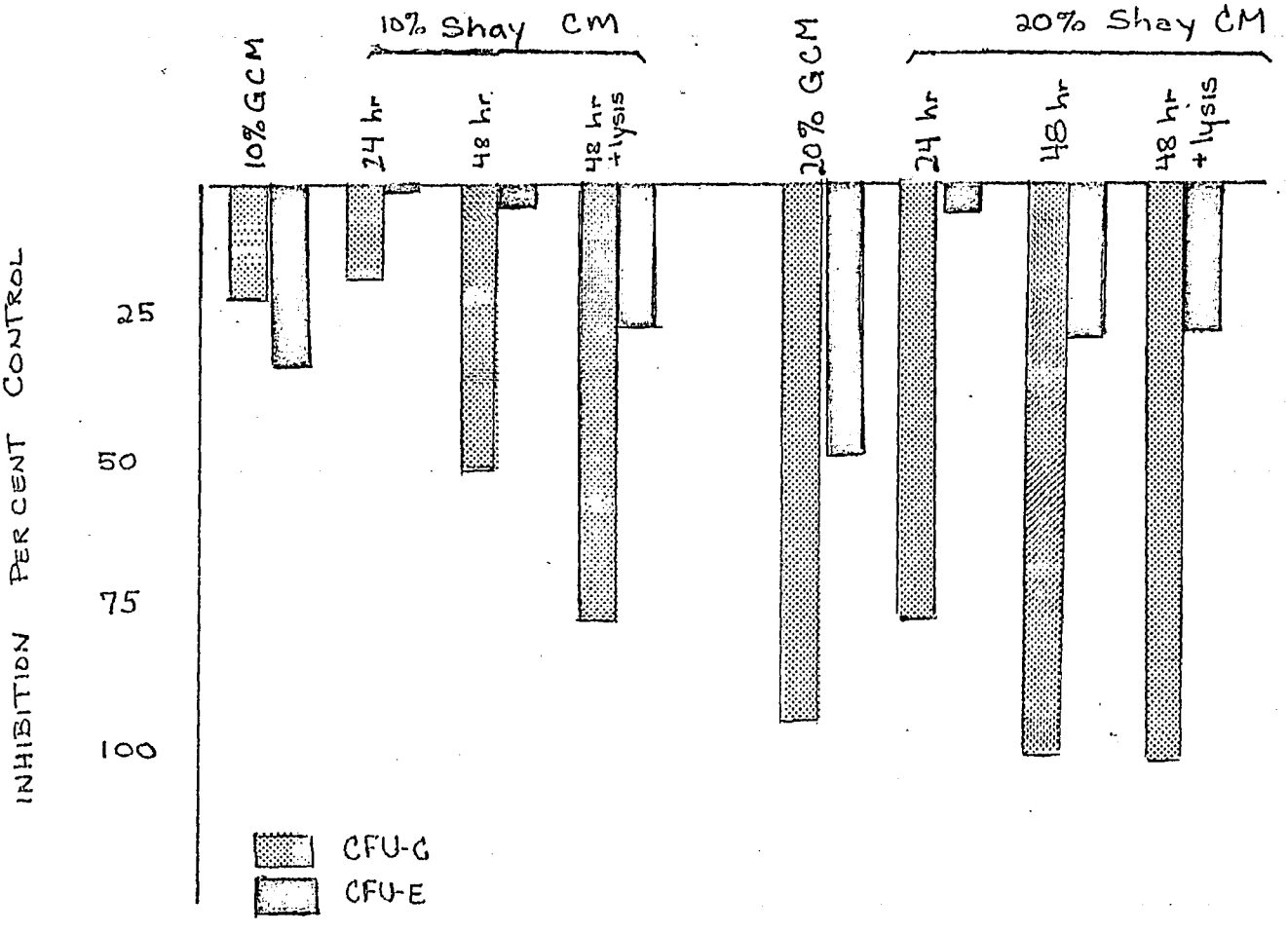
Legend to Figure 12

Degree of inhibition of erythroid and myeloid colony formation induced by 10% and 20% EBL media conditioned for 24 hours, 48 hours and 48 hours plus EBL cell lysis. Inhibition is represented as percent control with each conditioned media group having its own control. Also shown is the inhibition induced by 10% and 20% neonatal liver media conditioned for 24 hours.


INHIBITION
STIMULATION
100
75
50
25
25
10% LIVER CM
10% EBL CM
24 hr
48 hr
48 hr + lysis
20% LIVER CM
20% EBL CM
24 hr
48 hr
48 hr + Lysis
CFU-G
CFU-E

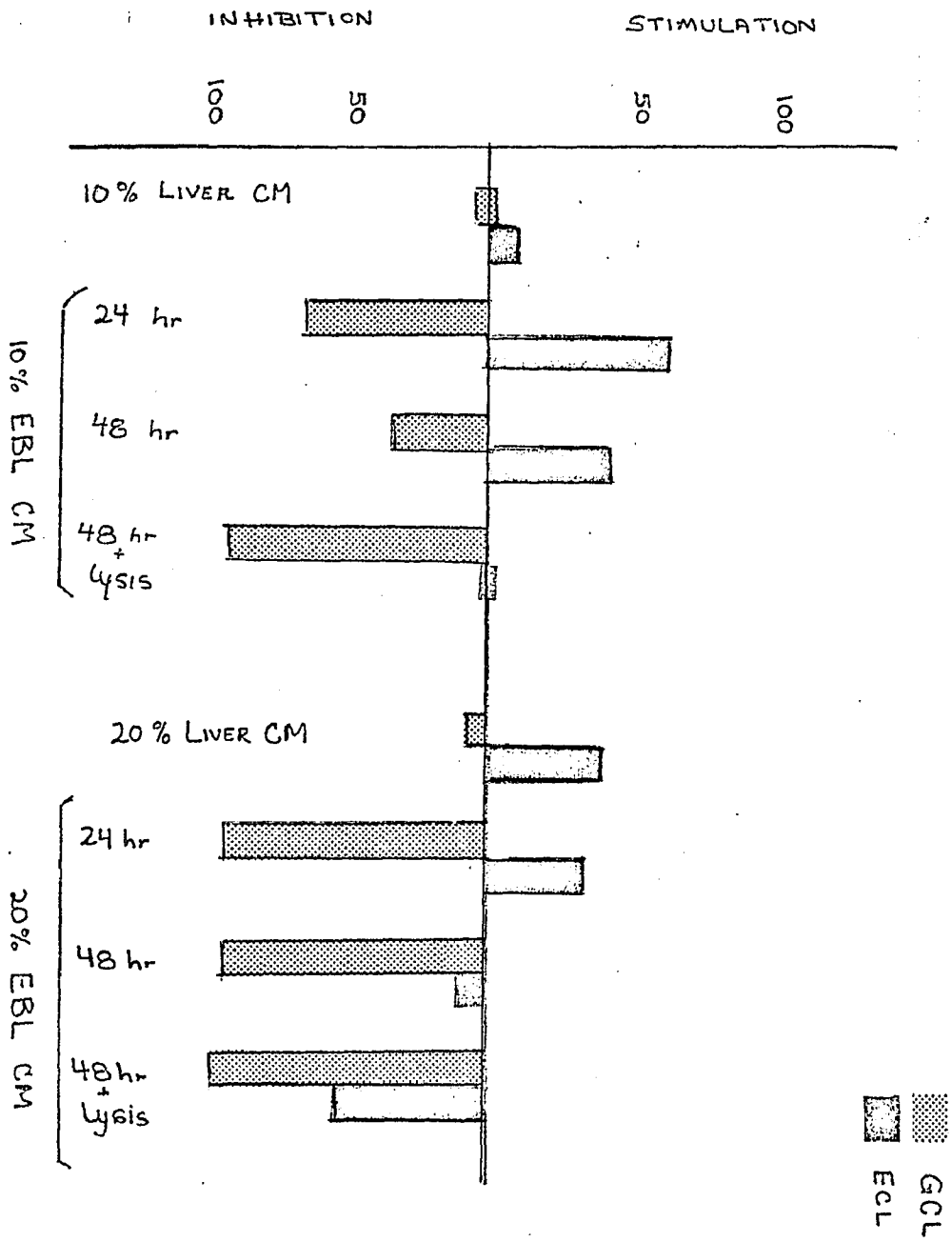
Legend to Figure 13

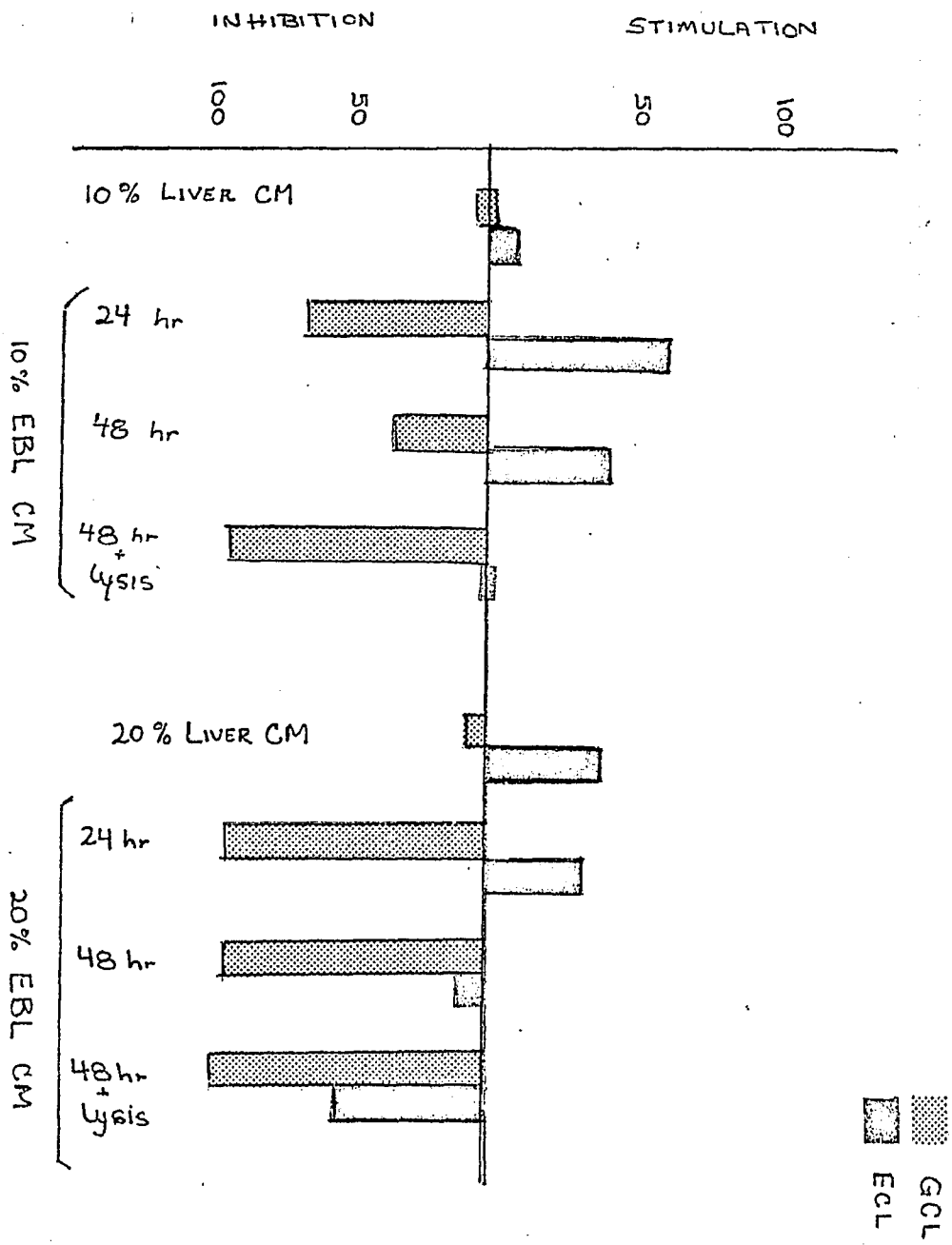
Degree of inhibition of erythroid and myeloid colony formation by 10% and 20% Shay media conditioned for 24 hours, 48 hours and 48 hours plus Shay cell lysis. Inhibition is represented as percent control with each conditioned media group having its own control. Also shown is the inhibition induced by 10% and 20% granulocytic media conditioned for 24 hours.


10% Shay CM
20% Shay CM
10% GCM
24 hr
48 hr
48 hr +lysis
20% GCM
24 hr
48 hr
48 hr +lysis
INHIBITION PERCENT CONTROL
25
50
75
100
CFU-G
CFU-E

Legend to Figure 14

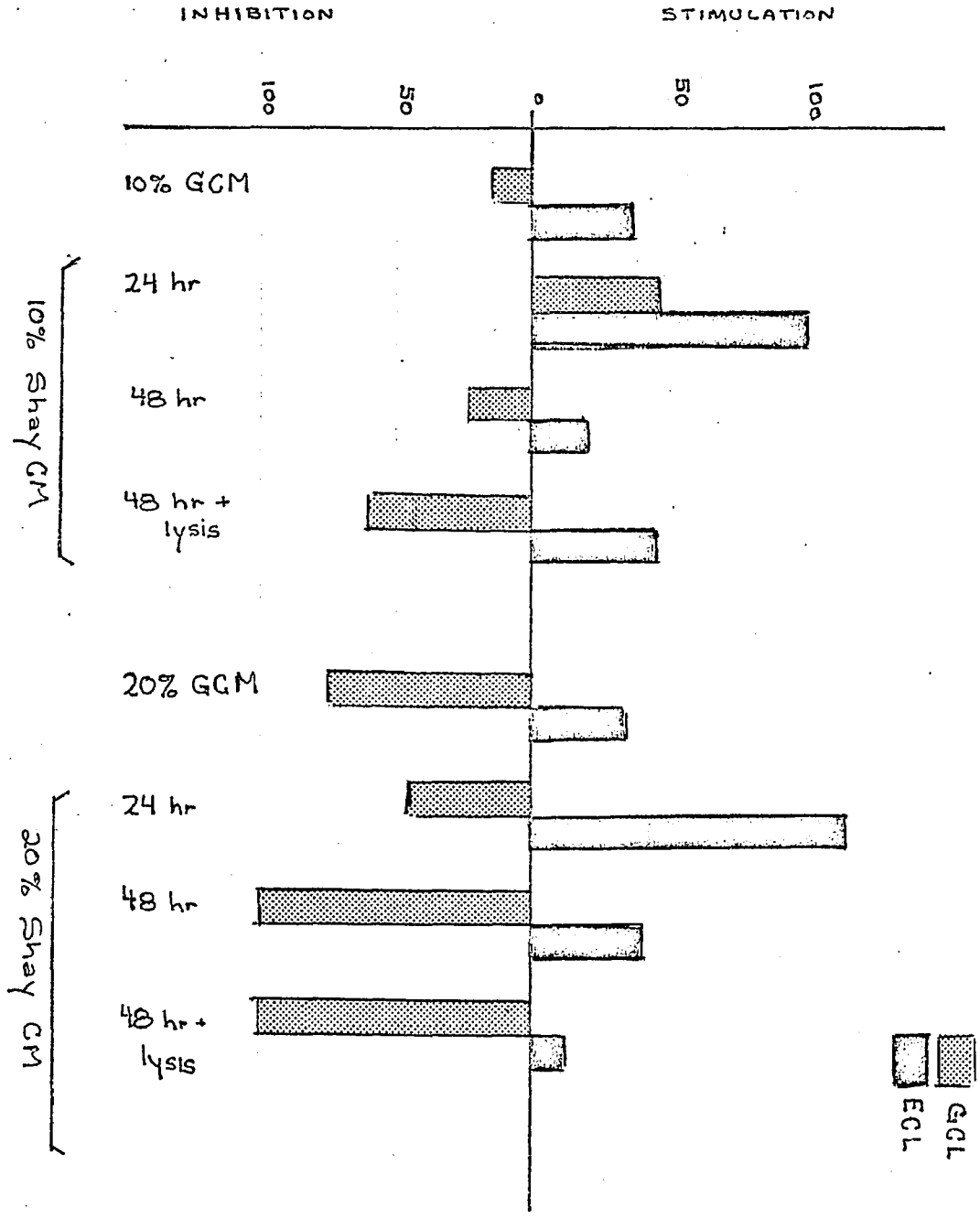
Degree of inhibition and stimulation of erythroid and myeloid cluster formation by 10% and 20% EBL media conditioned for 24 hours, 48 hours and 48 hours plus EBL cell lysis. Inhibition and stimulation is represented as percent control with each conditioned media group having its own control. Also shown is the inhibition/stimulation induced by 10% and 20% neonatal liver media conditioned for 24 hours.


INHIBITION
STIMULATION
100
50
50
100
10% LIVER CM
24 hr
48 hr
48 hr + Lysis
10% EBL CM
20% LIVER CM
24 hr
48 hr
48 hr + Lysis
20% EBL CM
GCL
ECL


INHIBITION
STIMULATION
100
50
50
100
10% LIVER CM
24 hr
48 hr
48 hr + Lysis
10% EBL CM
20% LIVER CM
24 hr
48 hr
48 hr + Lysis
20% EBL CM
GCL
ECL

Legend to Figure 15

Degree of inhibition and stimulation of erythroid and myeloid cluster formation induced by 10 and 20% Shay media conditioned for 24 hours, 48 hours and 48 hours plus Shay cell lysis. Inhibition/stimulation is represented as percent control with each conditioned media group having its own control group. Also shown is the inhibition / stimulation induced by 10 and 20% granulocyte media conditioned for 24 hours.

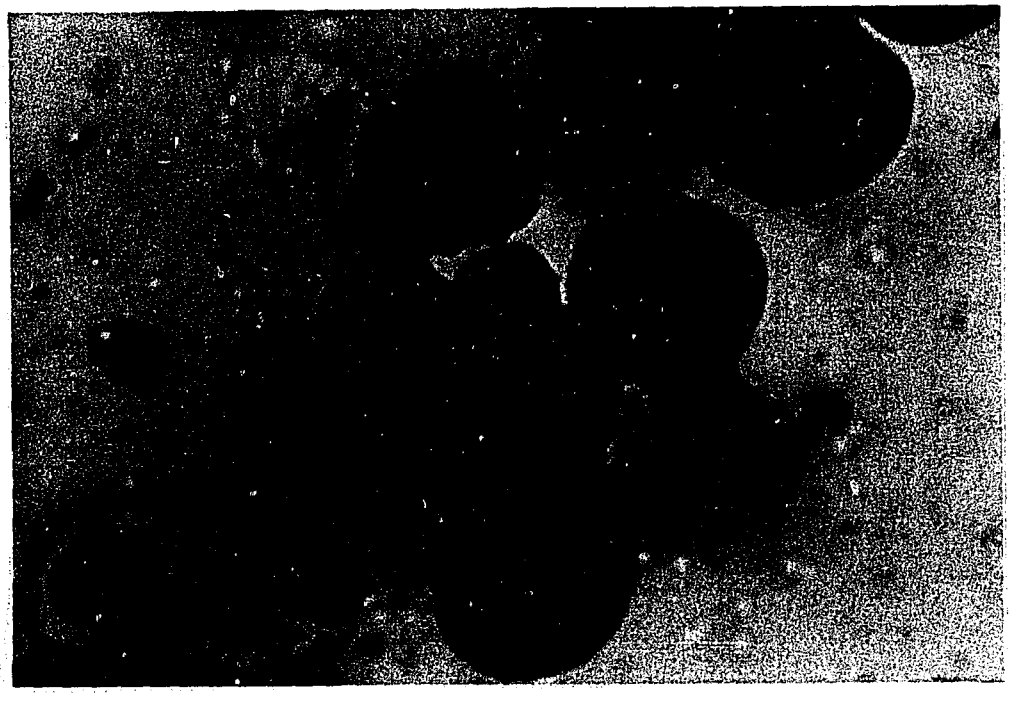

INHIBITION
STIMULATION
100
50
0
50
100
10% GCM
24 hr
48 hr
48 hr + lysis
10% Shay GM
20% GCM
24 hr
48 hr
48 hr + lysis
20% Shay GM
GCL
ECL

PHOTOPLATE 1. Liver section from an EBL animal. Tumor cells on the right are seen disrupting apparently normal liver tissue (H & E stain, magnification 400x).

PHOTOPLATE 2. Spleen section from an EBL animal. Tumor cells are seen in the red pulp, whereas the white pulp (top of plate) appears not to be affected (H & E stain, magnification 400x).

PHOTOPLATE 3. Bone marrow section from an EBL animal. Tumor cells appear to fill the entire hematopoietic sinus. (H & E stain, magnification 400x).

PHOTOPLATE 4. Tumor cells from an EBL spleen smear, stained with Wright-Giemsa (magnification 1000x).



APPENDIX 1

A. Reticulocyte Staining

Materials: New methylene blue, 0.5g of the dye is dissolved in 100ml of distilled water to which 1.6g of potassium oxalate has been added.

Method: Equal volumes of blood and stain were gently mixed on a piece of parafilm by drawing up the liquids into a capillary tube and then expelling them a number of times. The blood and stain was then drawn up into the capillary tube and allowed to stain for 10 minutes. At the end of this time the blood and stain was expelled onto a fresh piece of parafilm, remixed and then a drop was quickly smeared across a pre-cleaned covering the entire surface of the slide. These slides were examined under oil when dry. One thousand red blood cells were counted with the aid of a reticule, and the percent reticulocyte cell was thus determined.

B. Wright's-Giemsa Stain

Materials: Wright's stain, Fisher, Scientific Co., Giordano's buffer, pH 6.4-6.5, Giemsa stain, Hareleco, a 1:50 dilution in distilled water was prepared. This was stable for 8 hours.

Method: The slides were placed flat (smear side up) on a staining rack. Approximately 1½ml of Wright's stain was pipetted onto each slide with a Pasteur pipet. After 1 minute, an equal volume of Giordano's buffer was pipetted onto each slide and gently mixed with the stain by blowing the mixture over the slide. After 3 minutes, the slides were rinsed in running water. A gentle stream of water was used to gently float the stain of the

slide in order to prevent stain precipitation onto the slide. The slides were drained well from water, and then stained with Giemsa. Approximately 3ml of diluted stain was applied to each slide and allowed to remain for 4 minutes. The slides were rinsed as before, allowed to air dry, and then examined under oil at approximately 400x.

APPENDIX 2

A. Methyl Cellulose Preparation

Materials: 125ml distilled water, 4.0g methyl cellulose (Fisher Scientific Co.), 125 ml McCoy's 5A 2x conc. (Gibco)

Method: The distilled water was autoclaved in a 500ml flask, with a large magnetic stirrer enclosed. The flask was removed from the autoclave while the water was still boiling. The methyl cellulose was quickly added to the still boiling water under the sterile hood. The solution was stirred until it became room temperature. Sterile McCoy's 4a 2x was then added to the solution. The preparation then was allowed to stir in the cold for 48 hours. In order to achieve the right consistency the solution must be frozen and then thawed before use. Solution maybe stored at 4°C indefinitely.

B. Medium for CFU-C Growth

Materials: The following quantities are to make up to 300ml of growth media. More or less can be prepared as needed in the same concentrations:

Methyl cellulose with McCoy's 5a (see above)	150 ml
McCoy's 5A 1x (GIBCO)	107.4 ml
Fetal Calf Serum (GIBCO)	30.0 ml
$NaHCO_3$ (7½% solution, GIBCO)	3.0 ml
Sodium Pyruvate (100mm solution, GIBCO)	3.0 ml
Eagle's MEM Vitamins (100x, GIBCO)	1.2 ml
Eagle's MEM Essential Amino Acids (50x, GIBCO)	2.4 ml

Eagle's MEM Non-Essential Amino Acids (100x, GIBCO)	1.2 ml
Eagle's MEM Glutamine (200mM, GIBCO)	1.2 ml
L-Serine (21 mg/ml, GIBCO)	0.12 ml
L-Asparagine (100 mg/ml, Nutritional Biochemical Co., Cleveland, Ohio)	0.48 ml

Method: L-Asparagine is first dissolved in distilled water at a concentration of 10mg/ml. Under sterile conditions, all of the above ingredients with the exception of the methyl cellulose are combined in a large flask. The ingredients are then filtered through a 0.20u Nalge filter. The methyl cellulose mixture is not filtered. Roughly equal volumes of the methyl cellulose mixture and the supplemented McCoy's mixture are combined in a graduate cylinder. To prepare 6 plates, 25 ml of media are needed, 12.5 ml of methyl cellulose and 12.5 ml of supplemented McCoy's are combined in a single flask. The appropriate cell sample is added to each flask, 0.83×10^6 cells/25 ml of media, for a final concentration of 10^5 cells/3 ml (the contents of one small petri plate). The contents of each flask are thoroughly mixed and distributed as described in the materials and methods section.

APPENDIX 3

A. HMEM Media

Materials:

Minimal Essential Media Eagle's with Hank's Balanced Salt Solution (10x concentration, GIBCO)	10 ml
MEM Non-Essential Amino Acids (100x, GIBCO)	1.0 ml
Sodium Pyruvate (100mM, 100x, GIBCO)	1.0 ml
L-Glutamine (200mM, 100x, GIBCO)	1.0 ml
$NaHCO_3$ (5% solution, GIBCO)	1.25 ml
Sterile distilled water	97.75 ml

Method: The above ingredients were mixed together in a 100 ml sterile cylinder, and then poured into a flask. If the media was properly buffered, it appeared reddish-orange. If the media was too basic and appeared too purple, the pH was adjusted by incubating the media in a CO_2 incubator, with the flask cap loosely closed, for about 6 hours. If pre-incubated, the media was filtered through a 0.20u Nalge filter before use. This supplemented HMEM is stable for 2 weeks at 4^oC. For collection of cells, HMEM containing 2% heat-inactivated (60^oC for ½ hour) fetal calf serum was used.

B. Beef Embryo Extract lypholized (GIBCO) was reconstituted to the original volume with supplemented HMEM and distributed in very small aliquots and frozen at -20^oC. Beef embryo extract (BEX) should not be de-

frosted and refrozen more than three times. Prior to use in culture, the beef embryo extract is diluted 1:6 with NCTC-109 (0.1 ml of BEX + 0.5 ml of NCTC-109).

C. Erythropoietin step 3 (Connaught Medical Research Labs. Ltc., Willowdale, Ontario, Canada) was diluted with HMEM to a concentration of 10 units/ml, distributed in small aliquots and frozen until use. Prior to plating the erythropoietin (epo) was diluted 1:4 with NCTC-109 (0.1 ml epo + 0.3 ml NCTC-109).

D. L-Asparagine (Nutritional Biochemical Corp., Cleveland, Ohio) was made up with HMEM to a concentration of 2 mg/ml, filtered through 0.20 μ Nalge filter, distributed and frozen in small aliquots. Prior to plating the asparagine (asp) was diluted 1:10 with NCTC-109 (0.1 ml asp + 0.9 ml NCTC-109).

E. Bovine Serum Albumin

Materials: 50g Bovine Serum Albumin (BSA); 91 ml cold sterile distilled water; 10g resin AG 501x8 (D) 20-50 mesh (Bio-Rad Labs., Richmond, Calif.); 7% $NaHCO_3$; Phenol red; Dulbecco's Ca^{++} and Mg^{++} free phosphate buffered saline.

Method: BSA was slowly sprinkled into the cold sterile water, and gently stirred with a glass rod as the BSA was added to the water. The BSA was stirred in the cold until the major portion was in solution. The solution was refrigerated over night. Five grams of resin was then added to the

BSA solution and stirred. During the first hour, the solution was stirred every 15 minutes, and then kept in the cold for an additional hour. The BSA was then decanted into a flask to which 5 g of fresh resin had been added. The solution was stirred every 15 minutes the first hour, and then allowed to stand at room temperature for 1 hour. If the resin was still in suspension, the solution was centrifuged for 20 minutes at 15,000 rpms and then decanted into a cylinder. In order to obtain a 37% solution of BSA, for each 15 ml of BSA solution, 1.1 ml of 10x PBS was added. The solution was then diluted to 10% by adding 1x PBS (vol)(conc)=(vol)(conc). The 10% BSA was then filtered through #1 Whatman filter paper, and then filtered through a 0.20u Nalge filter to sterilize. The sterile BSA was then frozen overnight, thawed and divided into 100 ml aliquots. Two and one half ml of 7% $NaHCO_3$ and 0.5 ml of phenol red was added to each 100 ml of 10% BSA (Phenol red was made up by adding 0.04g of phenol red to 10 ml of PBS). The BSA was then frozen in small aliquots.

F. NCTC 109 was purchased from Microbiological, Bethesda, Md. Penicillin (5000 u) and streptomycin (5000 ug) (GIBCO) was added to the NCTC to make a 2% solution.

G. CFU-E Plating Procedure

Materials: Each experimental test tube to which 0.1 ml of cells in HMEM with 2% heat-inactivated fetal calf serum, was added contained the following ingredients, which had been diluted appropriately previously, as described above.

Beef embryo extract (BEX)	0.1 ml
Fetal calf serum (heat inactivated at 60°C for 30 minutes)	0.2 ml
Bovine serum albumin (BSA)	0.025 ml
1-asparagine	0.1 ml
Erythropoietin (epo)	0.1 ml
NCTC 109 (with pen/strep, 2% v/v)	0.275 ml

Method: Early in the day the stock solutions were defrosted and diluted appropriately, as previously described. Extra media was prepared to facilitate handling, e.g. if 14 samples were to be prepared, enough media for at least 15 was prepared. The control test tubes were epo-, the volume was substituted with 0.1 ml of NCTC, thus the control test tubes contained 0.375 ml of NCTC. The media was prepared in batches in one large test tube for the experimental and another for the control. From these, 0.8 ml was pipetted into each small test tube, and then kept at 4°C until plated.

H. Dulbecco's Phosphate Buffered Saline (PBS)

Materials:

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

Method: For a 1x solution make up a liter with distilled water, for a 10x solution, make up to a 100 ml with distilled water. Autoclave to sterilize.

I. Gluteraldehyde Fixative: Stock solutions of 0.1 M NaH_2PO_4 (solution A) and 0.1 M $Na_2 HPO_4$ (solution B) were prepared and stored at 4°C. A 0.01 M phosphate buffered solution was prepared by adding 17 ml of solution A to 33 ml of solubion B, and diluting this mixture 1:10 with distilled water. This was made up fresh each time before use. The gluteraldehyde fixative (50% w/w, Fisher Scientific) was diluted 1:5 with the 0.01 M phosphate buffer (10 ml of gluteraldehyde was added to 40 ml of buffer). This fixative was kept in the cold and was stable for at least 3 weeks.

J. Benzidine Stain for Colonies

Materials: $3,3^1$ dimethoxybenzidine (practical, Eastman Kodak, Rochester, N.Y.) was made up in absolute methanol to a 1% solution, and was allowed to age at room temperature for at least 2 days before used.

Hydrogen peroxide 30% (Fisher Scientific, Fairlawn, N.Y.) was diluted to a 2.5% solution by adding 5 ml of 30% H_2O_2 to 55 ml of 70% ethyl alcohol. This H_2O_2 solution was replaced when particles of precipitated stain were found. The reactivity appeared to improve with age. The solution was kept at room temperature until discarded.

Hematoxylin Harris-Lille (Fisher Scientific) was used as a counter-stain.

NH_4OH was used for blueing the slides. For this, a 1% solution of ammonia water was prepared in distilled water.

Method: The clots were stained with benzidine and then counterstained with hematoxylin in the following manner: After the slides with the affixed clots were dry, the slides were immersed in benzidine stain for 2 minutes, the slides were then drained on paper towel and then immersed in the peroxide solution for one minute and then rinsed by immersing the slides in distilled water for one minute. The slides were then drained again and then counterstained with hematoxylin, by immersing the slides in the stain for two minutes. The slides were then rinsed in running water, and then bluded in ammonia water by submerging the slides in the solution for a few seconds. The slides were rerinsed in running tap water, allowed to dry and then mounted with coverslips.

To facilitate scoring, a grid of cellulose acetate, scored in 1 mm boxes was placed over the slide, and secured with scotch tape. The slides were then examined with a microscope at 120x. A group of 8 or more benzidine positive cells, were scored as a colony, 4-7 benzidine positive cells were scored as a cluster.

APPENDIX 4

Conditioning Media Preparation

Materials: To make up 100 ml of media for conditioning, the following ingredients were combined:

McCoy's 5A (GIBCO)	85.8 ml
Fetal calf serum (GIBCO)	10.0 ml
$NaHCO_3$ (7.5%, GIBCO)	1.0 ml
Sodium pyruvate (100mM, GIBCO)	1.0 ml
Eagle's MEM vitamins (100x, GIBCO)	0.4 ml
Eagle's MEM essential amino acids (50x, GIBCO)	0.8 ml
Eagle's MEM non-essential amino acids (100x, GIBCO)	0.4 ml
Eagle's MEM glutamine (200mM, GIBCO)	0.4 ml
1-Serine (21mg/ml, GIBCO)	0.04 ml
1-Asparagine (10 mg/ml, Nutritional Biochemical)	0.16 ml
Penicillin-Streptomycin (5000u, 5000ug, GIBCO)	1.0 ml

Method: The above nutrients were combined under sterile conditions and then filtered through a 0.20 u Nalge filter. Excess media was frozen for future use.

APPENDIX 5

Benzidine Wright's-Giemsa Stain

Materials: A 1% benzidine solution and peroxide solution was prepared as described in Appendix 3J. Wright's stain and the accompanying Giordano's buffer (pH 6.4-6.5) were purchased from Fisher Scientific Co. Giemsa stain (Harleco) was diluted with distilled water 1:50 before use.

Method: The slides were placed in a staining rack, immersed in benzidine solution for 2 minutes, drained on paper towel, immersed in peroxide for 1 minute, and then rinsed in distilled water for 1 minute. The slides were then drained and dried under cold air until thoroughly dry.

The slides were then placed flat up on a staining tray and each slide was covered with approximately 1½ ml of Wright's stain. After 1 minute, 1½ ml of buffer was pipetted onto each slide. The buffer and stain were gently blown around the slide to mix. Care was taken to insure that the entire slide was covered by the mixture. After 3 minutes the stain was rinsed off with a gentle stream of water to float the stain off the slide. The slides were held horizontally for this rinse. The slides were drained and then placed horizontally on the staining rack again for Giemsa staining. Each slide was covered with approximately 3 ml of diluted stain, and allowed to stand for 4 minutes. The slides were rinsed as before, and allowed to air dry.

EXPERIMENTAL DESIGN

I. IN VIVO STUDIES

A. DAILY PERIPHERAL BLOOD DETERMINATIONS AFTER EBL INJECTION THROUGH DAY 9.

1. HEMATOCRIT LEVELS
2. RETICULOCYTE LEVELS
3. CIRCULATING NUCLEATED CELL COUNTS
4. DIFFERENTIALS

B. ORGAN STUDIES FROM ANIMALS SACRIFICED ON DAYS 4,5 OR 7,8.

1. HISTOLOGY
2. CELLULAR COMPOSITION

II. IN VITRO STUDIES FROM ANIMALS SACRIFICED ON DAYS 4,5 OR 7,8.

A. CFU-Es: BONE MARROW, SPLEEN AND LIVER

B. CFU-Cs: BONE MARROW, SPLEEN AND LIVER

III. CONDITIONED MEDIA

NORMAL HEMATOPOIETIC (NEONATAL LIVER AND MATURE GRANULOCYTE) CONDITIONED MEDIA AND TUMOR CELL (SHAY AND EBL) CONDITIONED MEDIA EFFECTS ON NORMAL BONE MARROW CFU-Es AND CFU-Cs.

CHARACTERISTICS OF EBL DISEASE

1. MASSIVE INFILTRATION OF HEMATOPOIETIC ORGANS I.E., BONE MARROW, SPLEEN, AND LIVER BY TUMOR CELLS.

2. REPRESSION OF ERYTHROPOIESIS.

3. INCREASED LEVELS OF GRANULOPOIESIS.

4. COMPENSATORY SPLENIC HEMATOPOIESIS; TRANSITORY ERYTHROPOIESIS SUCEEDED BY GRANULOPOIESIS

INDICANTS OF SPECIFIC REPRESSION OF ERYTHROID DIFFERENTIATION

I. PERIPHERAL BLOOD

A. ANEMIA (HEMATOCRIT DROP) BY DAY 6 POST I.V.

B. RETICULOCYTOPENIA BY DAY 4.

II. HEMATOPOIETIC ORGANS:

DECLINE OF RECOGNIZABLE ERYTHROID PRECURSORS I.E., BENZIDINE POSITIVE CELLS STARTING FROM DAY 4.

III. IN VITRO STUDIES

A. TRANSITORY COMPENSATORY SPLENIC ERYTHROPOIESIS (DAYS 4,5).

B. DECLINE IN BONE MARROW CFU-Es AT DAYS 7.8.

C. INHIBITION BY EBL CONDITIONED MEDIA OF NORMAL BONE MARROW CFU-Es

ADVANTAGES OF THE RAT EBL MODEL

1. RAPID TIME COURSE OF DISEASE DEVELOPMENT (7-8 DAYS).

2. HIGH INFECTION LEVELS WITH LOW DOSAGE OF INJECTED CELLS (>80%).

3. NON-VIRAL ETIOLOGY OF DISEASE WITH NO EVIDENCE OF "HORIZONTAL" INFECTION OF "NORMAL" HEMATOPOIETIC STEM CELLS IN RATS BY LEUKEMIC CELLS.

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