BNL-18319

CULTURE OF NORMAL AND LEUKEMIC CELLS IN DIFFUSION CHAMBERS*

Eugene P. Cronkite, M.D., Arland L. Carsten, Ph.D.,
Gundabhaktha Chikkappa, M.D., J. A. Laissue, M.D. and Siegfried Ohl, M.D.

Medical Research Center
Brookhaven National Laboratory
Upton, New York
11973
USA

Running Title: Cell Culture in Diffusion Chambers

To be presented at a workshop entitled, "Prognostic Factors in Human Acute Leukemia", to be held at the International Institute for Scientific Cooperation, Schloss Reisensburg near Ulm, Germany, on October 1-3, 1973.

*This work is supported by the U. S. Atomic Energy Commission, the National Cancer Institute, Grant #1R01 CA14742-01 and the American Cancer Society.

THE PROBLEM:

The purpose of this paper is to demonstrate the types of information that have been obtained through the culture of normal and malignant hemopoietic cells in diffusion chambers (DC). The culture of cells in DC is another technique to investigate the kinetics and regulation of proliferation of the stem cell pool, the differentiated hemopoietic pool, and malignant hemopoietic cells. Through these studies, in parallel with other techniques, it is hoped to verify experimentally some model for the stem cell pool and to ascertain the factors that regulate its self-maintenance and differentiation into the diverse committed stem cells (CSC).

Stem cell survival and function is crucial in chemotherapy. The objective is to eliminate the malignant cell line and preserve the capability of the pluripotent stem cell pool (PSCP) to regenerate and differentiate into all normal hemopoietic cell lines.

The problem of the stem cell was first clearly considered by Osgood in 1957 (19). He thought that stem cells divided asymmetrically to produce one stem cell and a differentiated cell. He perceived the necessity for repletion of the PSCP after damage in order to restore normal numbers. His concept invoked a temporary suppression of differentiation with geometric growth of the stem cell pool to restore its size. Since the initial formulation of a model for stem cell behavior, examples of various models have been proposed by Lajtha, et al. (13); Kretchmar, et al. (12); Newton (17); Till, et al. (23); and Cronkite (7).

Evidence derived in a study of murine stem cells and hematopoiesis leads one to believe that the stem cell pool is divided into a PSCP and CSC for each hemopoietic cell line. The experimental evidence for committed stem cells is reviewed by Stohlman (21). The PSCP, under normal

steady state conditions, has a small fraction in cycle and a large fraction either in a resting G_0 state or prolonged G_1. The committed stem cell pool (CSCP) has a much larger fraction in cycle as indicated by ^{3}H-thymidine (^{3}H-TdR) suicide or hydroxyurea and is presumably the pool upon which humoral factors act. The PSCP is believed to produce increased numbers of stem cells by diverting cells from the quiescent phase into cycle. The nature of the stimulus that regulates the flow of cells from the quiescent compartment to the active cycling compartment is not known but it is believed to be related to cell-to-cell interactions. McCulloch and Till[15, 16] and Goodman and Grubbs (11).

MATERIALS AND METHODS:

Murine bone marrow is obtained from the femora of mice using a grinding technique (22). The diffusion chambers are those described by Benestad (1) and the methods by which they are used and cells prepared are essentially unchanged. Mice received 750 R one day before chamber implantation.

Caprine bone marrow culture in DC is a modification of the murine method. Details will be published by Laissue et al. (14). Bone marrow aspirations are made in the standard manner. The aspirate is diluted with an equal volume of phosphate-buffered saline without calcium and magnesium containing 0.5% EDTA and flushed 2-3 times through a 22 gauge needle. Four ml of this suspension are layered over 3 ml of a mixture of Isopaque and Ficoll D = 1.077, as described by Boyum (4) in a glass tube 14 ml in diameter and centrifuged for 15 min at 500 g. Following removal of part of the plasma layer, the nucleated bone marrow cells at the plasma-Isopaque-Ficoll interface are removed by aspiration. The cells are washed twice. The washed cells are suspended in ice-cold complete

medium 199 with an EDTA concentration reduced to 0.004% supplemented with autologous goat serum to a final concentration of 10%. 100 µl of the suspension containing approximately 250,000 nucleated bone marrow cells are pipetted into each chamber, sealed, and stored at 4°C in vials containing complete medium.

The goat in which the chambers are to be implanted is irradiated after the bone marrow has been aspirated. An absorbed midline dose of 270 R gamma radiation at a dose rate of 72.4 R/min was delivered from a nuclear reactor gamma converter plate.

Under general anesthesia and aseptic surgical technique, three small incisions are made into the peritoneal cavity. A Teflon holder containing 5 DC's is inserted through each incision into the peritoneal cavity and anchored near the incision by a silk suture. The abdominal wall is sutured in layers. The procedure is repeated on the other side of the goat. At intervals, under anesthesia, a Teflon chamber holder is removed, cells are harvested, counted, smeared, and identified as described by Benestad (1).

Human bone marrow is aspirated and treated in a similar manner to caprine bone marrow and implanted into 750 R irradiated mice.

For radioactive labeling of the DC contents, the mice are injected intraperitoneally with either 0.1 µCi/g body weight of ^{3}H-TdR or 0.4 µCi/g body weight of ^{125}IUdR at various times after injection. Autoradiographs are made in standard manner and stained with Giemsa.

Autologous Caprine Hemopoietic Cell Growth in DC:

These studies will be published in detail by Laissue et al. (14). Bone marrow is aspirated from the animal and prepared and placed in the DC's. In the interim the goat is given 270 R whole-body irradiation.

Control animals receive no irradiation. DC are implanted in the normal and irradiated goats at similar time intervals following aspiration. Exponential growth of cells commenced in the DC 3 days after implantation in the irradiated goats. The exponential growth involves granulocytopoiesis and mononuclear cell production. When thrombopenia develops in the host, approximately 9 days after irradiation, increased numbers of megakaryocytes appear in the DC.

Proliferation in the DC implanted in the non-irradiated goats proceeds since mitoses are seen and the number of macrophages and granulocytic cells in the DC increases. Granulopoiesis involves myeloblasts, promyelocytes, myelocytes, metamyelocytes, bands and segmented neutrophils in the control and irradiated hosts. The growth rate of granulocytic and erythropoietic cells is significantly greater in the DC in the irradiated hosts, e.g., doubling time of granulocytic cells is about 36 hours compared to a small increase in DC in non-irradiated goats. After 10 days in culture the total number of granulocytes begins to decrease in the DC in the irradiated mice.

Erythropoiesis faded out quite rapidly in the DC implanted in non-irradiated goats. By the third day immature erythroblasts were not seen, indicating that neither PSC nor CSC in the DC were being directed along the erythropoietic pathway. The large normoblasts proliferated and matured into small normoblasts. The former disappeared by the third day and the latter by the eighth day in culture. However, brisk erythropoiesis was observed in DC implanted in irradiated goats. Both mature and immature normoblastic proliferation commenced by the third day after implantation. From the fifth to the tenth day post implantation, the immature erythroblasts increased with a doubling time of about 18 hours.

In order to investigate whether whole-body irradiation is required to produce the stimulus for erythropoiesis and granulopoiesis, marrow was taken from animals which were then given extracorporeal irradiation of the blood (ECIB) or sham-ECIB to see whether depletion of lymphocytes and peripheral stem cells would influence the growth in the DC In preliminary studies, it does, in fact, appear that ECIB is sufficient to produce a stimulus for the growth of cells in the DC.

Growth of Human Peripheral Blood Cells in DC:

These initial studies will be summarized very briefly and reported in detail later by Boecker et al. (3). There are similarities in growth among the studies on peripheral blood, the autologous caprine studies described above, and the growth of human bone marrow in DC by Boyum et al. (5). Whether all peripheral blood cultures will grow in a similar manner is not known.

The segmented neutrophilic granulocytes implanted in the DC decrease as a two-component exponential curve. The half times of the two-component exponential curve for segmented neutrophils are roughly 8 and 16 hrs, respectively. Neutrophilic precursors appear for the first time on the ninth day in culture. From 9-11 days the doubling time is roughly 24 hrs. After the 11th day in DC culture, the doubling time is longer. The doubling time of the non-proliferating pool of neutrophilic granulocytes between days 9-15 is about 52 hrs. From the appearance on day 9, the metamyelocytes (M_5), band (M_6), and segmented neutrophils (M_7) progressively increase in number indicating that these cells are proliferating and maturing in the DC. The presence of the non-proliferating (M_5-M_7) cells at 100-150 hours indicates that at least some of these cells are surviving as long as the normal marrow (Cronkite et al. 8). The time from

reappearance of M_5 in the DC to termination of the culture was 168 hrs. During this time there was a steady increase in M_5-M_7 granulocytes.

Lymphocytoid blasts increase explosively from essentially zero on day 1 to about 10^5 on day 11. A total of 1.1×10^5 small lymphocytes were implanted. The number dropped to 4×10^4 during the first day in culture. The cell number then remained relatively level for 8 days followed by an exponential rise with doubling time of 36 hrs. There were no plasma cells in the original inoculum. On day 9 they appeared and increased exponentially for 3 days with a doubling time of about 6 hrs. The large loss of small lymphocytes in the first 24 hrs (50%) cannot be accounted for by transformation, since only 200 lymphocytoid blasts appeared in this interval. The bulk of the small lymphocytes disappearing may have adhered to the membrane remaining then uncounted or undergone dissolution. During the first 6 days only 700 lymphocytoid blasts appeared, perhaps all by transformation. However, between 6-11 days there was an exponential growth from 700 to 80,000 blasts, accountable for primarily by proliferation with a doubling time of about 18 hrs. After 10 days in culture the small lymphocytes increased in number to about the initial implant. This increase could be due to release of the cells stuck on the membrane or to reversion of the proliferating lymphocytoid blasts to small lymphocytes producing immunoglobulins presumably against murine proteins.

Growth of Normal Human Bone Marrow in DC:

These studies will be reported in detail by Ohl et al. (18).

One to 4 hrs after implanting DC containing human bone marrow, 25 to 53% can be harvested dependent upon the size of the inoculum. This is presumably due to adherence to the filters and sticking in corners between filters and the Lucite ring. The number harvestable, the % recovery,

is nearly a linear function of the number placed in the DC. The non-recovered cell type has not been determined as yet.

Figure 1

In Figure 1 total numbers of different cell types are plotted. The lymphocytes peak successively at 40, 140 and 210 hrs. Myeloblasts (M_1) plus promyelocytes (M_2) peak at 40 hrs and then fall to a low at 90 hrs and later attain a new maximum at 200 hrs following which they decline for the next 60 hrs. The culture was terminated at 260 hrs. Myelocytes (M_{3-4}) increase rapidly peaking at 40 hrs and then decline to a minimum at 130 hrs followed by a slow increase throughout the rest of the culture period. M_5 peak at 40 hrs and thereafter slowly decline. M_7 increase rapidly during the first 40 hrs and then more slowly through 130 hrs and then decline almost linearly for the rest of the time in culture.

^{3}H-TdR was given to the mice immediately after the DC were implanted, labeling those cells in DNA synthesis and thus allowing observation of the flow of labeled cells from proliferative pools to and through the non-proliferative pool as shown in Figure 2.

Figure 2

From the first observation point at approximately 2.4 hrs, labeled lymphocytes, proliferating neutrophils (myeloblasts, promyelocytes and myelocytes) increase in number for 20 and 40 hrs, respectively. A small number of labeled M_5 were seen at 2.4 hrs (minimum <u>in-vivo</u> myelo-meta-myelocyte transit time is 3 hrs). The wave of labeled cells moved progressively from M_5 through M_6 and to M_7. Labeled M_7 peaked at 120 hrs, remained at a plateau for an additional 40 hrs and then declined slowly. The maximum number of labeled M_7 was 21×10^3 compared to 205×10^3 total M_7.

The silver grains overlying the cells were counted. The diminution in the grain count is influenced by time between successive mitoses

and the degree of reutilization of ^{3}H-TdR from dying cells. The average grain count over the M_{3-4} of the 3 subjects diminished as a single exponent with a half-time of 80 hrs. In one individual ^{3}H-TdR and ^{125}IUdR were used to label the cells in the DC's. Least square analysis indicates that the ^{3}H-TdR curve had a half-time of 67 hrs and the ^{125}IUdR had a half-time of 47 hrs.

Curves for labeled monocytes and macrophages are not shown. However, the fraction of monocytes labeled increased for 40-50 hrs and then decreased strikingly for the rest of the culture period. Initially there were no labeled macrophages. Labeled macrophages appeared at 70 hrs, increased in number attaining a maximum at 190 hrs and thereafter decreased in number.

In Table 1 the minimum transit time from M_{3-4} to M_5 to M_6 to M_7 is compared for growth of human cells in DC and in-vivo (Cronkite, et al., 8). The minimum transit time from the M_{3-4} to each stage of the M_{5-7} granulocytic pool is shorter in the DC than in-vivo. However, the rate of replacement of M_5 is not faster - 2.5% compared to 2.5-3.3% in-vivo.

Leukocyte alkaline phosphatase (LAP) activity of M_7 increases for the first 140 hrs to a peak, then decreases with a second peak at 210 hrs. This coincides roughly with the number of M_7.

Eosinophilic Growth in DC:

In pilot studies (2) it has been shown that eosinophilic proliferation proceeds. An estimate of generation time in cultures for eosinophilic myelocytes is 76 hrs compared to 61 hrs in-vivo in a patient with eosinophilia. Total eosinophil counts peak at 60 to 70 hrs and again at about 180 hrs. There is an orderly flow of labeled eosinophils from the eosinophilic myelocyte to band and segmented cells. Labeled eosinophilic myelocytes peaked at 42 hrs, metamyelocytes at 88 hrs and segmented at about 160 hrs.

Blood Dyscrasias:

Chronic granulocytic leukemia (CGL) cells have been grown and the results published by Chikkappa, et al. (6). The CGL cells proliferated as shown by ^{3}H-TdR labeling. The presence of Ph 1 chromosome was observed for several days together with and the orderly flow of labeled cells from myelocyte to the segmented neutrophil. LAP activity of newly produced cells increased strikingly suggesting that the environment provided by the fatally irradiated mouse switches on the genomes responsible for LAP synthesis. Since only mitotic figures with Ph 1 chromosome were seen, it is assumed that the LAP + cells are progeny of the leukemic cell line and not normal cells.

Acute myeloblastic leukemia and multiple myeloma cells also grow but data are not sufficiently complete to provide insight into their DC growth. One patient with severe myelofibrosis has been studied in which in-vivo proliferation after ^{3}H-TdR injection was compared to proliferation in DC cultures. After *in-vivo* ^{3}H-TdR, labeled metamyelocytes appeared between 10 and 22 hrs and then increased steadily to 22% labeled metamyelocytes after 65 hrs at a rate of 0.33% per hr. When the cells were cultured in DC labeled metamyelocytes were seen within one hr, increasing slowly for the first 20 hrs and then more rapidly until 40 hrs in culture at which time about 27% were labeled. On the steep part of the upward slope the labeled metamyelocytes appeared at a rate of about 1.35% per hr.

DISCUSSION:

The DC system is a non-steady state system. Initially some cell types decline in number. The proportion of immature to mature types becomes smaller. This in itself may by diminishing chalone effect from mature cells in the environment be stimulatory to growth and

differentiation at the PSC and CSC level. Cell numbers then increase with production of mature cells. As the latter increase in number, the number of less mature cells begin to decrease. Whether this is due to chalone suppression or to loss of PSC and CSC through some mechanism is not clear. The system does not permit exit of cells hence it is a non-steady state system with changing proportions of cells resulting in changing probabilities for cell to cell interaction and for chalone effects. Despite these inherent differences from in-vivo steady state situations the DC system has some advantages over in-vitro culture. All cell lines to some extent can be cultured. Humoral factors probably both inhibitory and stimulatory can be studied in general. Specific observations will be considered next.

The studies on growth of caprine bone marrow cells in DC demonstrates, as shown earlier by Boyum et al. (5) that the growth rate is faster when DC are implanted in the irradiated host as compared to the normal host. In the autologous caprine system there is brisk erythropoiesis showing that the DC allows humoral regulating factors to enter. Since the erythropoietic growth begins before the host becomes anemic and there is no erythropoietic growth in the DC in normal hosts one assumes that radiation hypoplasia of the host erythropoiesis suppresses utilization of erythropoietin allowing plasma concentration to increase and enter DC in effective amounts. In addition the internal environment of the DC provides the necessary cell to cell interaction or hemopoietic inductive environment for differentiation in the absence of splenic or bone marrow stroma. Megakaryocytopoiesis starts after the host becomes thrombopenic, hence the necessary humoral and local environments are sufficient to initiate the process which might later lead to platelet production. Whether

proliferation originates from the PSC or CSC cannot be answered in these studies. ECIB destruction of circulating PSC, CSC and lymphocytes may also provide a growth stimulus for hemopoietic cells in DC.

The successful culture of all hemopoietic cells from peripheral human blood cells shows that all CSC and/or PSC are present in the blood. Some information is derived on the senescent survival of M_7 in DC. Random loss from DC as known in-vivo is not possible, hence all disappearance is from senescence. The age distribution of M_7 in the sample introduced into the DC is distributed exponentially from zero to maximum in-vivo survival. If there is an in-vivo finite life span of 30 hrs as suggested by Fliedner et al. (10) the life span is longer in DC because M_7 are seen as long as 4-6 days after being placed in DC culture. The reappearance of new M_5 and their progression through the non-proliferating pool is fairly consistant with in-vivo observations.

In the first study M_7 of the blood disappeared from the DC by the 6th day in culture. In a second study there was longer survival. Non-proliferating granulocytes (M_{5-7}) reappeared on the 8th day of culture and steadily increased for the next 7 days when the cultures were discontinued. If the newly produced M_7 had half-times in the DC of 8 and 16 hrs respectively as those which were placed in the DC, one would not have expected an exponential increase with doubling time of 52 hrs - the approximate generation time of the myelocyte in-vivo (9). These observations suggest that many newly produced M_{5-7} survive at least 7 days (168 hrs) in culture. The normal in-vivo transit time from M_5 through M_7 in bone marrow is 100-150 hrs with an average time in the blood of nearly 10 hrs for a maximum total life from M_4 to loss of blood M_7 of 110-160 hrs.

The initial doubling time of the proliferating granulocytes M_{1-4} was

24 hrs or less than half of the generation time for in-vivo steady state (9). We therefore suggest that this supports the hypothesis of Cronkite and Vincent (9) that generation time of myelocytes (M_{3-4}) decreases under stress by shortening G_1 phase of the cell cycle and of course a concomitant decrease in ^{3}HTdR labeling index. The latter was not determined in these studies.

In culturing peripheral blood cells the appearance of lymphocytoid blasts, the increase in small lymphocytes, their later increase, and the appearance of plasma cells leads one to believe that the DC may be useful for many immunological studies. The doubling time for plasma cells is about the same as generation time for lymphocytic cells. The longer doubling time of lymphocytoid cells suggests either cell death or diversion of cells from this stage into cell lines. On arithmetic balance the former is more likely.

Human bone marrow culture has introduced some problems of interpretation when growth is compared to the peripheral blood culture just discussed. The first problem that arises is the comparative growth of the lymphocytic series when peripheral blood cells or bone marrow are introduced into the DC. The total number of cells in culture is equal to the number introduced plus the difference between the number produced and the number dying in culture. When bone marrow was grown in DC lymphocyte peaks were seen at 40, 140 and 210 hrs and labeled cells were seen throughout the culture period. However, the explosive growth of lymphocytoid blasts observed when peripheral blood cells were cultured was not seen. The difference might be due to implantation of 1) a smaller number of cells, or 2) predominately bone marrow derived lymphocytes when marrow is grown. It suggests that a mixture of B and T cells with phagocytes may be

necessary for launching immunological transformation and proliferation of lymphocytic cells and plasma cells as one would expect in a suspension of blood lymphocytes.

The difference in behavior of labeled cells in the DC and the total number of cells gives valuable information. The flow of labeled cells gives direct information on minimum transit time primarily of the cohort of labeled M_{3-4} through successive mitoses and then through the M_{5-7} pool. From the mean grain count overlying M_{3-4} one can estimate M_{3-4} generation time and derive some insight into the number of myelocytic mitoses since there is no large labeled precursor compartment feeding into the M_{3-4} compartment. The half-time for diminution of the grain count of 80 hrs is much greater than the 52 hrs of Cronkite and Vincent (9). This suggests that there is either reutilization of ^{3}H-TdR due to death of cells or that growth is slower. The more rapid diminution when labeled with ^{125}IUdR suggests reutilization. The half-time of the latter 47 hrs is close to 52 hrs derived from other logic (9). If one assumes about a 50 hr half-time the ^{125}IUdR (no reutilization) goes to 2 grains in nearly 100 hrs implying two serial mitoses of the myelocytes. However, as will be seen later there is great difficulty in rationalizing these estimated generation times with cell production rates.

The first M_1 plus M_2 peak at 40 hrs represents a two-fold increase in absolute numbers which could be due to 1) input from neutrophilic CSC or 2) additional mitosis at this stage with suppression of maturation. In the same interval M_{3-4} numbers increase two-fold which could be due to additional mitosis with suppressed maturation rate. However, M_5 is also increasing showing that there is some progression from M_{3-4} to M_5. The successive increases in M_6 and M_7 shows that there is an orderly

progression through maturation. M_7 increases from 58×10^3 to 190×10^3 in the first 40 hrs proving a very rapid transit from M_4 to M_7.

The model for steady state granulopoiesis of Cronkite and Vincent (9) visualizes one mitosis for M_1, one at M_2 and two successive mitoses in M_{3-4}. With this structure the ratio of $M_1:M_2:M_3:M_4$ if there is equal time in each compartment would be 1:2:4:8 for a total of 15 cells. Each terminal myelocyte will produce 2 M_5 or twice the number of M_4 placed in the DC. If there is no input from the stem cell pool then the total number of M_5 that can be produced by the M_{1-4} placed in the DC is $\Sigma M_{1-4} \times \frac{64}{15}$.

Since 41×10^3 M_{1-4} were recovered at 2.4 hrs a total of 175×10^3 M_5 can be produced during an unspecified time if there is no cell death. These will proceed to M_7 at a rate determined by the intensity of the maturation stimulus. Labeled M_7 were seen as early as 42 hrs setting a minimum transit time from M_4 through M_6. During the first 20 hrs M_7 increased from 58×10^3 to 150×10^3. The sum of M_5 plus M_6 in the chamber was about 52. If all matured to M_7 in 20 hrs the total would be about 110×10^3. To this can be added a maximum of 175×10^3 commencing after 42 hrs the minimum transit of labeled cells from M_4 to M_7. If there were no death of M_{5-7} the minimum number in the DC would grow to the initial $(M_7+M_6+M_5)$ + number produced by M_{1-4} mitoses of the Cronkite and Vincent model for a total of 58+26+26+ 175 = 285. The maximum observed was about 205×10^3 at 120 hrs, hence one believes that there was death of some M_7 during the 120 hrs of culture.

The preceeding discussion did not consider time. From the Cronkite and Vincent steady state model the average myelocyte generation time is 52 hrs. The number of M_4 is $8/15 \times 41 = 22 \times 10^3$. This will be followed by waves from M_3, M_2 and M_1 producing a total of 44×10^3 M_5 for each wave. If there were no CSC or PSC input, production of M_5 would cease at 208 or fewer hrs.

If the system is not stimulated and there is no death in transit the maximum M_7 could not be seen before 208 hrs. M_7 would rise to 110 by maturation of the M_5 and M_6 introduced in the DC and be reached at about 20 hrs. From 20 to 40 hrs the number increased another 80 or nearly 2 generations. The generative cycle must have decreased to nearly the DNA synthesis time of 12 hrs. At the same time M_{3-4} numbers increased as did M_{1-2} suggesting shorter cycle times and increased numbers of mitoses in order to account for increase in M_7 and concomitant increase in the M_{1-2} compartments.

The preceding considerations are totally incompatible with the grain count diminution observed.

The relative plateau in the M_7 from 40-140 hrs is similar to the observations on survival of newly produced granulocytes when one cultures peripheral blood leukocytes.

The sequence of events in flow of ^{3}H-TdR labeled cells is not really representative of the total population. The initial labeling index of M_{3-4} is about 0.20. There are about 7×10^3 labeled M_{3-4} initially. This grows to about 23×10^3 in 40 hrs. If the unlabeled grow at the same rate they will expand to 88×10^3. Labeled M_7 appear after 40 hrs in culture. During this interval the absolute numbers of M_7 increased from 58×10^3 to 190×10^3 proving that there is substantial production from the unlabeled precursors in the DC. From 40-120 hrs there is an increase in labeled M_7 forms from $0\text{-}21 \times 10^3$ per DC but very little increase in the total number suggesting that somewhat less than the number of labeled segmented forms have died and indergone dissolution. In conclusion one cannot explain the total yield of M_7 on the basis of the labeled population. The yield from unlabeled myelocyte population would be 4 x that from the labeled population

or an additional 80 x 10^3 M_7. The sum of this and the labeled M_7 is close to the net increase from 50 x 10^3 to nearly 190 x 10^3.

The labeling by ^{3}H-TdR gives one an insight into the behavior of the flash labeled population (primarily M_{3-4}) whereas the total cell numbers provide insight into behavior of the labeled and unlabeled cells of the proliferating and non-proliferating pools plus a glimmer on the input from the PSCP. The second wave of unlabeled M_1 and M_2 presumably represents input from PSCP into granulopoiesis. However, it is reflected only by a small increase in M_{3-4} and very little input into the M_{5-7} pool since these commence rapid decline from 140 hrs onward when the second wave of M_1 and M_2 commences, suggesting earlier chalone inhibition.

The more rapid decrease of the grain count when ^{125}IUdR is used as a label than when ^{3}H-TdR is used indicates that ^{3}H-TdR is being reutilized from dying labeled cells probably at M_1 - M_4 stage. One must consider the possibility of radiation induced death as an ineffective granulopoiesis as suggested by Patt (20). One cannot really distinguish between the two options. However behavior of the labeled population in the DC seems not representative of the total population and can't explain the yield of cells counted.

The increase in absolute numbers of labeled monocytes can be explained only by their proliferation in an enclosed space. As labeled macrophages appear the number of labeled monocytes decreases suggesting monocytes change into macrophages by proliferation followed by differentiation. The number of labeled monocytes increases by a factor of 4 before labeled macrophages appear suggesting 2 mitoses of the labeled cells.

The increase in LAP in neutrophils with time, with peaks at 140 and 210 hrs follows at nearly the predicted time for waves of new non-

proliferating cells from the M_1 plus the M_2 peaks.

The appearance of labeled neutrophils after 40 hrs is as rapid as was observed in-vivo in a patient with infection (Fliedner et al., 10). This plus the fact that caprine and murine cells grow more rapidly in the irradiated host than in the non-irradiated host clearly demonstrates that growth rates in the irradiated host are accelerated and clearly different from in-vivo steady state growth. This limits the usefulness of the DC system to investigate steady state growth and its regulation just as growth of CFU's to produce splenic colonies is an exponential, non steady state expanding system. However, the system is still useful for study of differentiation and assay of stem cells and their growth rates.

To date leukemic, multiple myeloma cells and cells from myelofibrosis with myeloid metaplasia have been grown with success. The data are not sufficiently extensive as yet for any meaningful interpretation.

SUMMARY:

Normal murine, caprine, and human bone marrow and peripheral blood cells can be grown in DC's. The irradiated host provides a stimulus to erythrocytic and granulocytic growth over what is seen in the DC's implanted in the non-irradiated host. The stimulus is most likely humoral and affects erythropoiesis, granulopoiesis and megakaryocytopoiesis. It is suggested but not proved that the stimulus for erythropoiesis is an increased level of plasma erythropoietin. It is tempting to suggest that CSF and thrombopoietin are responsible for the stimulation of the other cell lines. Growth of normal human cells in irradiated mice shows a marked reduction of the transit time from the myelocyte to the segmented neutrophils. Growth does not follow steady conditions in terms of rates and transit times. Since all cells produced are retained in the DC's after the time they

would normally be extruded into the blood, a non-steady state system exists. Senescent death of non-proliferating cells occurs.

Human leukemic and multiple myeloma cells grow in DC's as do blood cells from patients with myelofibrosis and myeloid metaplasia. Data are insufficient to characterize aberrations in growth.

Pilot studies show that human eosinophils will grow, proliferate and mature.

REFERENCES

1. Benestad, H. B.: Formation of granulocytes and macrophages in diffusion chamber cultures of mouse blood leukocytes. Scand. J. Haemat. 7, 279-288 (1970).
2. Beyer, J., G. Chikkappa, E. P. Cronkite. To be published.
3. Boecker, W., E. P. Cronkite, G. Chikkappa and A. L. Carsten. In preparation.
4. Böyum, A.: Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. Suppl. 97, p. 109 (1968).
5. Böyum, A., W. Boecker, A. L. Carsten and E. P. Cronkite: Proliferation of human bone marrow cells in diffusion chambers implanted into normal or irradiated mice. Blood 40, 163-173 (1972).
6. Chikkappa, G., W. R. Boecker, G. Borner, A. L. Carsten, K. Conkling, L. Cook, E. P. Cronkite and S. Dunwoody: Return of alkaline phosphatase in chronic myelocytic leukemia cells in diffusion chambers. Proc. Soc. Exp. Biol. Med. In press (1973).
7. Cronkite, E. P.: Enigmas in hemopoietic cell proliferation. Federation Proc., 23, 649-661 (1964).
8. Cronkite, E. P., V. P. Bond, T. M. Fliedner and S.-A. Killmann: The use of tritiated thymidine in the study of hemopoietic cell proliferation. CIBA Foundation Symposium on Hemopoiesis, pp. 70-92 (Ed. by G.E.W. Walstenholme and M. O'Connor, Pub. J & A. Churchill, Ltd., London 1960.
9. Cronkite, E. P. and P. Vincent: Granulocytopoiesis. Series Hemat. 2, 3-43 (1969).
10. Fliedner, T. M., E. P. Cronkite and J. S. Robertson: Granulocytopoiesis I Senescence and random loss of neutrophilic granulocytes in human beings. Blood 24, 402-414 (1964).

11. Goodman, J. W. and C. G. Grubbs: The relationship of the thymus to erythropoiesis. In: Stohlman, F., Jr. (Ed.) Symposium on Hemopoietic Cellular Proliferation. Grune & Stratton, New York 1970, pp. 26-35.

12. Kretchmar, A. L., T. P. MacDonald and R. D. Lange: Mode of action of erythropoietin in polycythemic mice III. The dose-response curve. In: Stohlman, F., Jr. (Ed.) Symposium on Hemopoietic Cell Proliferation. Grune & Stratton, pp. 150-159, New York 1970.

13. Lajtha, L. G., R. Oliver and C. W. Gurney: Kinetic model of a bone marrow stem cell population. Brit. J. Haemat. 8, 442-460 (1962).

14. Laissue, J., E. P. Cronkite and D. D. Joel: In preparation.

15. McCulloch, E. A. and J. E. Till: Effects of short term culture on populations of hemopoietic progenitor cells from mouse marrow. Cell Tissue Kinet. 4, 11-20 (1971).

16. McCulloch, E. A. and J. E. Till: Regulatory mechanisms acting on hemopoietic stem cells. Am. J. Pathol. 65, 601-619 (1971).

17. Newton, D. M.: Medeling the stem cell system - current status. Ann. Acad. Sci. 128, 781-789 (1966).

18. Ohl, S., G. Chikkappa, E. P. Cronkite and A. L. Carsten: In preparation.

19. Osgood, E. A.: A unifying concept of the etiology of the leukemias, lymphomas and cancer. J. Nat. Cancer Inst. 18, 155-166 (1957).

20. Patt, H. M. and M. A. Maloney: A model of granulocyte kinetics. Ann. N. Y. Acad. Sci., 113, 515-522 (1964).

21. Stohlman, F., Jr.: Regulation of red cell production. In: Formation and Destruction of Blood Cells. Ed. T. J. Greenwalt and G. A. Jamieson, pp. 65-85, J. P. Lippincott Co., Phil. & Toronto, 1970.

22. Stoner, R.D. and V. P. Bond: Antibody formation by transplanted bone marrow, spleen, lymph node and thymus cells in irradiated recipients. J. Immunol., 91, 185-190 (1963).

23. Till, J. E., E. A. McCulloch and L. Siminovitch: A stochastic model of stem cell proliferation, based on the growth of spleen colony forming cells. Proc. Nat. Acad. Sci. USA (Wash.) 51, 29-36 (1964).

Table 1 Flow of ^{3}HTdR labeled cells from the myelocyte through the non-proliferating pool of cells.

	Hours			
	In Vivo*		In Diffusion Chamber	
	Zero Label	First Label	Zero	First Label
Metamyelocyte	2	3	2	4
Band	36	48	21	27
Segmented	48	72	40	42

The minimum transit times lie between zero label and first label.

*Cronkite et al (8)

LEGENDS

Figure 1:

Changes observed in the absolute cell counts of lymphocytes and different stages of granulopoiesis.

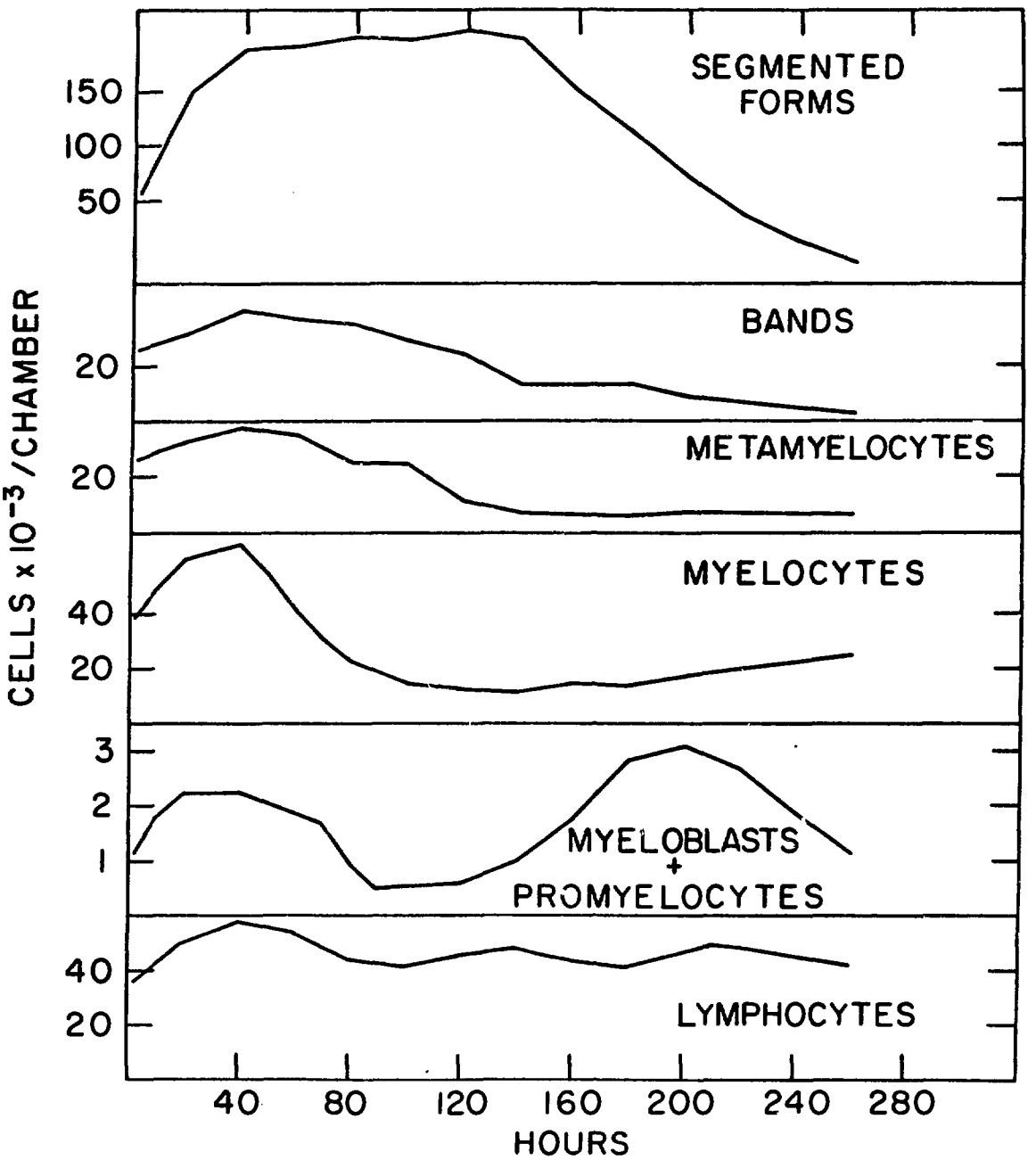
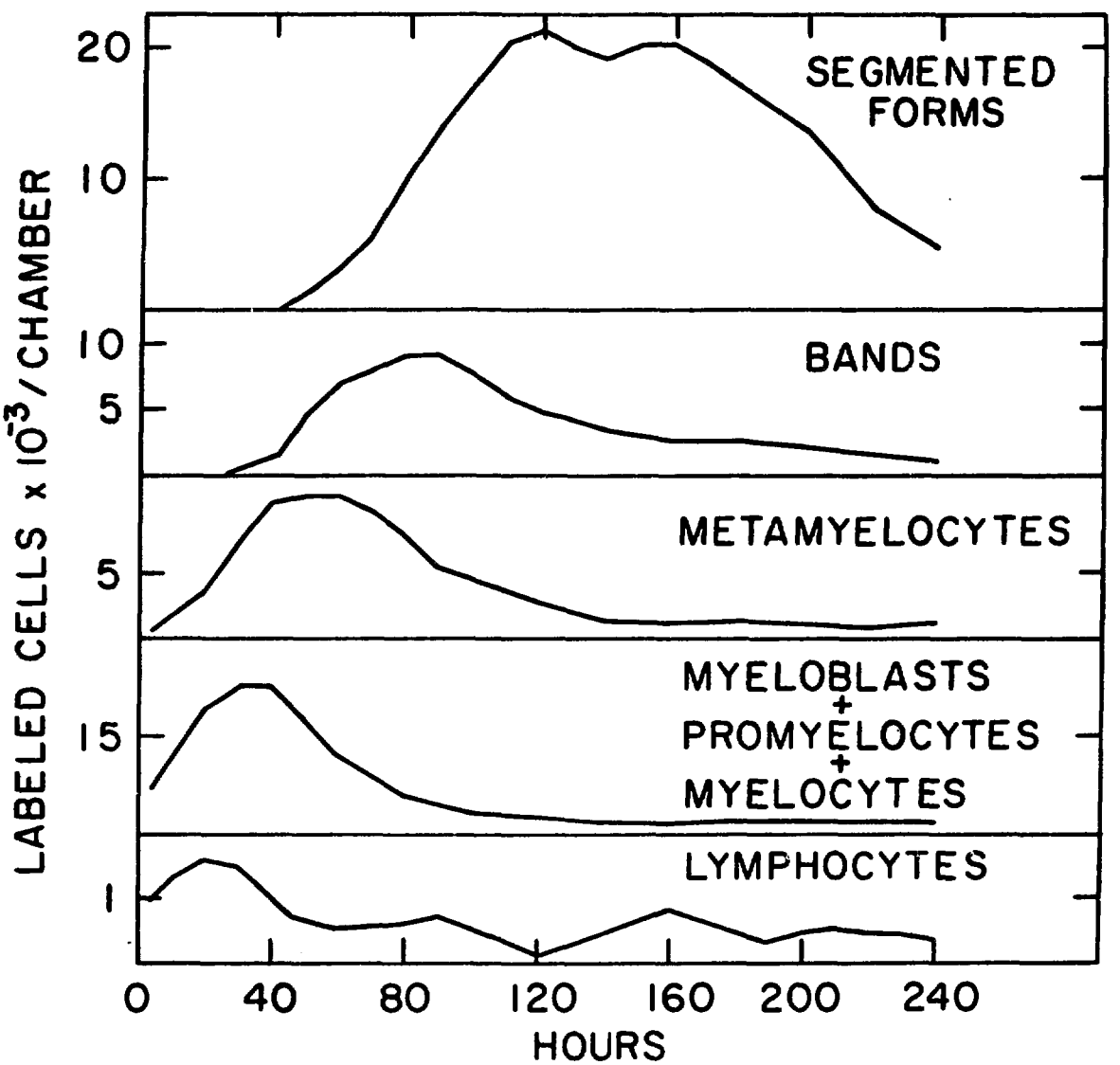

SEGMENTED FORMS
150
100
50
BANDS
20
METAMYELOCYTES
20
MYELOCYTES
40
20
3
2
1
MYELOBLASTS + PROMYELOCYTES
40
20
LYMPHOCYTES
CELLS x 10⁻³/CHAMBER
40
80
120
160
200
240
280
HOURS

Figure 2:

Incidence of ^{3}H-TdR labelling. Zero time is immediately after implantation, changes represent the result of proliferation and flow of cells to successive stages of granulopoiesis. Values normalized to unit value (1.0) for initial appearance of labelled cells.


LABELED CELLS x 10^{-3}/CHAMBER
20
10
SEGMENTED FORMS
10
5
BANDS
5
METAMYELOCYTES
15
MYELOBLASTS + PROMYELOCYTES + MYELOCYTES
1
LYMPHOCYTES
0
40
80
120
160
200
240
HOURS