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STUDIES ON THE MOST PRIMITIVE PRECURSOR CELLS OF THE HEMOPOIETIC SYSTEM

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STUDIES ON THE MOST PRIMITIVE PRECURSOR CELLS OF THE HEMOPOIETIC SYSTEM

Abstract

David H. Y. Lin

The colony forming unit (CFU) as assayed by the spleen colony technique is believed to be the most primitive precursor cell of the hemopoietic system. Current evidence suggests that the CFU is the progenitor cell of the granulocytic, megakaryocytic, erythroid and perhaps lymphoid cell lines. It is the purpose of these studies to elucidate the regulatory mechanism which govern the proliferation of the CFU.

The suicidal H^3-thymidine method was used to assess the proliferative state of CFU in the hemopoietic tissues studied. The experiments were designed to examine the possibility of a circulating humoral agent which regulated the CFU population.

Results from the study using partially irradiated and parabiotic mice suggest the presence of a circulating humoral agent which exerts its effect by altering the proliferative state of the CFU population. The changes in the proliferative state of the shielded CFU population are detected as early as two hours after any decrease occurs in the total CFU population and seem to persist as long as any decrease remains. The size of the CFU population is also increased in direct proportion to the need for CFU.

Our studies have also suggested a temporal pattern for the action and release of the circulating humoral agent. Its effect is most pronounced on the initial days following irradiation. The action and release of this humoral agent are detected as long as the CFU population is depressed. It is suggested that any change in the total CFU population may

bring about the release of the humoral agent.

The agar colony forming unit (ACFU) population which gives rise to visible colonies when cultured *in vitro* in a semi-solid agar medium was also examined. The results show a consistent difference in proliferative states of the CFU and ACFU populations before and following irradiation. This observation supports the concept of functionally and physiologically separate CFU and ACFU populations.

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INTRODUCTION

In order to maintain the steady state of the hemopoietic system the movement of mature and functioning cells into the blood stream must be balanced by corresponding influx of cells into the precursor-cell pools. These incoming pluripotential cells have been termed "hemopoietic stem cells".

Though the concept of a hemopoietic stem cell is widely accepted as a working hypothesis, its morphological identity is still unknown. Investigators using morphological criteria to define the stem call have suggested that the cell has the morphological characteristics of a lymphocyte and that the stem cell may be derived from the lymphoid line (Yoffey, 1960, 1962). However experiments showing the failure of lymphocytes or lymph node cells to promote erythropoiesis or confer protection after irradiation (Goodman, 1965; Campbell and Ross, 1951) together with the observation that lymph node cells cannot repopulate the bone marrow after irradiation (Micklem et al, 1966) seem to exclude the stem cell from the lymphoid cell line. It is perhaps due to the fact that these "stem cells" constitute such a small minority of the cells in the hemopoietic tissue that their morphological characterization is understandably difficult.

Direct quantitation of the stem cell did not occur until the advent of the spleen colony technique developed by researchers in Toronto. By studying the nodules developed on the spleen of irradiated mice on day 7-11 after transplanting an inoculum of syngeneic hemopoietic cells (e.g. bone marrow cells) they demonstrated that the cells responsible for the formation of the nodules were closely related or identical to the so-called hemopoietic stem cells postulated by early workers.

The Spleen Colony Technique and The Hemopoietic Stem Cell

The early pioneers of the spleen colony technique refered to the cell(s) which gave rise to the colonies as colony-forming units (CFU). In order to establish the CFU as the primitive precursor cell (the hemopoietic stem cell - HSC) it was necessary , first of all to establish that each spleen colony did arise from a single cell, namely the HSC. Indirect experimental evidence that suggested the clonal nature of the spleen colonies was firstly, that graphs relating the number of spleen colonies to the number of nucleated marrow cell transplanted were linear and the intercept at zero; and secondly, that the radiation survival curves are quite similar to those obtained for single cells in cultures or tumor transplants (Till and McCulloch, 1961; McCulloch and Till, 1962). More direct evidence has been provided by the use of cytological techniques. Becker, McCulloch and Till (1963) have showed that all the metaphase cells within colonies possessing an abnormal karyotype, more than 90% of them contained the same abnormal karyotype. Welshons (1964), Wu et al (1967), and Chen and Schooley (1968) have provided further evidence for the clonal nature of the spleen colonies using stable chromosome markers. Based on these experimental observations, it is the consensus of investigators in this field that the spleen colonies are clonal in nature.

In order for the CFU to be regarded as the HSC it was of utmost importance to establish that all CFU had the functional characteristics of the hemopoietic stem cells: they must possess the capacity of extensive proliferation, they must be capable of self-renewal and be able to differentiate into all the blood elements (McCulloch, Till and Siminovitch, 1965). The capacity of the CFU for extensive proliferation has been de-

monstrated by the fact that after settling in the spleen the cells give rise to colonies of more than 10^6 cells within a period of 10 days. The self-renewal property of the CFU is demonstrated by the heterogenous distribution of CFU among the spleen colonies and the gradual increase in the number of CFU present in the spleen of irradiated and subsequently transplanted animal (Siminovith, McCulloch and Till, 1963). Lewis and Troubaugh (1964) showed that cells from individual colonies can give rise to similar colonies upon retransplantation. The differentiative capacity of the CFU is demonstrated by their ability to give rise to colonies which contain all three types of hemopoietic cells, as well as colonies predominately erythroid, granulocytic or megakaryocytic. Although no colonies of lymphoid cells or macrophages were observed, recently Wu et al (1968), Virolainen and Defendi (1968) and Nowell et al (1970) suggested that these cells are also descendents of the CFU or that CFU and these cells have a common progenitor.

The CFU satisfies all the essential criteria of a stem cell of the hemopoietic system. But rigorous proof of the CFU as the stem cell is still lacking: for instance, in all the chromosome marker experiments, though strongly suggesting a clonal nature of the spleen colonies, the possibility of the settling of two or more cells as a unit to form the colonies is not ruled out. Until the morphological identity of the hemopoietic stem cell and the colony forming cell(s) can be determined and each of their functional characteristics defined we have only the spleen colony technique to quantitate the behavior of some primitive precursor pool of the hemopoietic system. In other words, the CFU is perhaps the most primitive precursor cell pool of the hemopoietic system that we are able to measure. So in the following text, the word CFU will be used to denote a population of cells whose behavior is quantitated by the spleen colony

technique, which areprecursors of all the blood cells, and are perhaps identical to the evasive hemopoietic stem cells.

The Colony Forming Units and Related Precursor Cell Populations

The CFU is pluripotential: i.e., it will under suitable environment or stimuli differentiate into the different blood elements. Investigators have been intrigued by the regulatory mechanism which may govern the behavior and/or determine the differentiative route. Attempts have been made to trace the identifiable blood cells back to the CFU by studying the effects of various factors or humoral agents on the maturation of different blood elements and the CFU.

Red cell production has been shown to be regulated by a humoral agent, erythropoietin (Jacobson et al, 1957; DeGowen et al, 1962; Garcia and Schooley, 1963; Schooley et al, 1968). An extensive review of erythropoietin and red cell production has been given by Goldwasser (1966). However, experiments have suggested that erythropoietin does not act directly on the CFU population to differentiate but on an intermediate population, existing between the CFU and the identifiable erythroid precursors, which is now termed the "Erythropoietin-sensitive Cell (ESC)" population. The existence of this population of cells was postulated initially after the observation of Bruce and McCulloch (1964) that the wave of erythropoietic activity resulting from prolonged hypoxic conditions was not preceded but followed by a decrease in CFU numbers in the spleen. The idea of a separate ESC population was further strenghtened by Schooley (1966) who showed that the growth of CFU in spleens of irradiated plethoric animals was unaffected by the presence or absence of erythropoietin. However, in contrast to the CFU, the ESC is unipotential

and is dependent on the CFU population to replenish its numbers. Evidence for this has been reviewed recently by Schooley (1969).

Another CFU-dependent population that has been proposed is that which gives rise to the granulocytic and monocytic-macrophage cells. Quantitation of these cells is achieved through an *in vitro* agar technique and they are suitably called the agar colony forming units (ACFU). Initial observation of these cells and their ability to form agar colonies was reported independently by Pluznik and Sachs (1965) and Bradley and Metcalf (1966). Both laboratories described the *in vitro* method for cloning hemopoietic cells in soft agar over a feeder layer of neonatal kidney cells or embryonic cells. The colonies which develop are granulocytic or monocytic in nature and at the time of scoring usually contain 50 - 200 cells. Wu *et at* (1968), using chromosome markers, showed that many individual spleen colonies were capable of forming colonies both *in vivo* (CFU) and *in vitro* and thus demonstrated a close relationship between the two precursor cell populations. However, Bennett *et al* (1968) have shown that the CFU and ACFU populations are separate, at least in the genetically anemic mice of the W series. Chen and Schooley (1970), based on recovery patterns after 200R irradiation, have provided further evidence for the concept that these are functionally distinct populations. It has been suggested (Bennett *et al*, 1968; McCulloch, 1968) that ACFU are an intermediate population between CFU and myeloblasts. Based on these studies and the fact that the ACFU population seems to be committed to the granulocytic or monocytic lines, the ACFU population can be classified as a dependent stem cell population.

Regulation of the CFU population

In the steady state, the generation time of the CFU has been assumed to

be relatively long (40 hours); when the population is regenerating (e.g. after irradiation) the estimation of its doubling time has been on the order of 20 hours (McCulloch and Till, 1964).

These observations have illustrated another property of CFU which fits the hypothesized hemopoietic stem cell - and that is the CFU population's ability to respond to varying demands of hemopoiesis. Becker et at (1965) have suggested that one of the ways the CFU responds is by varying its cell cycle: e.g. a shortened cell cycle in response to a greater demand for CFU. Lajtha and co-workers (1962, 1964) have used another model to explain the CFU reponse to varying hemopoietic demands. The model postulates that the CFU exist in two physiological states: some of the cells may be in cell cycle whereas the other cells exist in a state of prolonged interphase (the prolonged G_1 or G_o phase) during which they neither synthesize DNA or undergo mitotic division. The fraction of cells that undergo division in any given period depends on the current physiological demand, and may be in direct proportion to that demand.

Whether the CFU population responds to a hemopoietic stimuli by shortening the cell cycle or by increasing the fraction of the dividing population will be discussed in a later section, but it is evident that the population is governed by some regulatory mechanism. However, the nature of the regulatory mechanism is not known.

Ford et al (1966) observed that in a partially irradiated animal, transplanted bone marrow cells identified by marked chromosomes preferentially colonized irradiated portions of the skeleton and lymph nodes. The marked cells persisted almost exclusively in the irradiated limbs throughout the period of observation whereas the cells which settled in the lymph

nodes were observed to migrate into the unirradiated nodes and thymus. The persistent graft within the marrow and the futile attempt in our laboratory (Schooley and Chen, unpublished) to locate transplanted marked cells within the marrow of normal unirradiated animals suggest to us that the settling and proliferation of bone marrow cells may be regulated by the animal.

The nature of the regulatory mechanisms which govern the CFU population is not known. Wolf and Trentin (1968) have proposed that the proliferation and subsequent differentiation of the CFU is dependent largely upon the local environment. But preliminary studies by Knospe et al (1969) have suggested the involvement of a humoral factor which stimulates the proliferation of CFU after irradiation. The object of these studies is to examine the nature of the regulatory substance which acts upon the CFU population and to determine the temporal patterns of this action.

STUDY I: REGULATORY MECHANISMS OF THE COLONY FORMING UNIT POPULATION

In light of the humoral nature of the regulatory agent, erythropoietin, for erythroid precursors and recent reports on probable similar "poietins" for other hemopoietic series (Rytömaa and Kiviniemi, 1968, 1969; Evatt and Levin, 1969) it would be logical to examine whether the CFU population may be similarly regulated by a circulating humoral agent.

It was our working hypothesis that the action of the humoral agent is either to shorten the cell cycle or to induce mitosis in a greater fraction of CFU. In either event, the end result would be a greater number of cells entering the S-phase of the cell cycle at any particular time and becoming susceptible to the killing effect of high specific activity tritiated thymidine. Therefore throughout this study the suicidal thymidine technique was employed to determine any changes in the proliferative state of the CFU population in hemopoietic tissues.

MATERIALS AND METHODS

Animals 8 - 10 week old LAF_1 (C57BL x A/J) female mice were obtained from Bar Harbour, Maine. All animals were acclimatized and given water containing terramycin for a week in our animal facilities before use. The animals used for the experiments were 12 - 16 weeks old. The animals were housed usually at 5 mice per cage and given standard mouse feed (from Feedstuff, San Francisco, California) and chlorinated water ad libitum.

Irradiation For shielding experiments a 250kv x-ray source was used. The unit was operated at 250kv and 15ma with a filtration of 0.5mm Cu and 1.0mm Al. Dose rates were measured each time before irradiation with Victoreen ionization chambers. Animals were anesthetized each time with a 10% sodium pentobarbital solution (Diabutal, Diamond Laboratory Inc., Des Moines, Iowa) and placed on a rotating lucite disc directly under the beam. Shielding was accomplished by using either a jacket over the tibia or coffin over the whole animal with one limb protruding from a prefabricated hole in the coffin. Both the jackets and coffins were made from ½" lead. Efficiency of the shielding was checked by periodic histological examination of both irradiated and shielded limbs. For irradiation of large numbers of animals, a 1500 Curie Co^{60} source was used. Animals were placed in groups of 60 or less in a lucite container at 1.22 meters away from the source. Irradiation was accomplished at a dose rate of 26.6 R/min.

Suicidal thymidine technique High specific activity tritiated thymidine (> 18 Ci/mM) was obtained from New England Nuclear Corp. The solution was adjusted to contain 0.9% sodium chloride by adding appropriate amounts of sodium chloride just prior to use. Suitable aliquots of the isotonic

tritiated thymidine (H^3-thy) were added to the cell suspensions prepared in CMRL 1066 medium without thymidine or co-enzymes (GIBCO, California) to give a final specific activity of 500 μCi/ml. The cell suspensions thus treated were gently agitated at 37°C for 30 minutes and prior to use, were either diluted at least 10 times with Eagle's minimum essential medium (MEM, GIBCO, California) or washed and centrifuged and resuspended in MEM. As a control, an aliqout of each suspension was treated with normal saline without thymidine. Cell counts were made on all suspensions prior to injection.

Preparation of parabiotic mice 8 - 12 week old LAF_1 female mice were paired by weight and caged together 3 - 5 days before the operation. On the day of operation the animals were weighed again and anesthetized with a 10% sodium pentobarbital solution. The parabiosis technique was the method of Bunster and Meyer (1933) modified by the use of wound clips instead of skin sutures. Briefly, after intravenous anesthesia the animals were shaved and matching skin incisions were made along the right flank of one animal, the left flank of the other from the base of the tail to the rear of the ear. The adjacent front and hind limbs were united by sutures of chromic catgut. The adjoining skin edges were united sutures and wound clips. Animals were kept on a wet diet for a period of 10 days and used 4 weeks after the operation.

Anesthesia 10% sodium pentobarbital solution: prepared by mixing 10ml Diabutal (60 mg/ml sodium pentobarbital), 10ml 100% ethyl alcohol, 20ml ethylene glycol and 60ml sterile water. Dose given either intraperitoneally or intravenously is 60 mg/kg body weight.

Spleen colony technique The method used was similar to that of Till and McCulloch. Single cell suspensions were prepared from either the

spleen or bone marrow as described. Suitable dilutions were made to give a final colony count of 10 - 25 colonies per spleen. The cells were injected intravenously in a 0.5ml volume via the lateral tail veins of animals which had been given a whole body irradiation of 1100R not more than 3 hours previously. 15 - 20 animals were used for each dilution or group. On the 9 - 11 day after the injection the animals were sacrificed by cervical dislocation and their spleens excised and placed immediately into Bouin's fixative. After a 24 hour fixation period the spleens were washed successively in 60% and 70% ethyl alcohol and the colonies were scored under a 10X dissecting scope.

The endogenous colony level was checked occasionally by irradiating groups of animals without giving a bone marrow or spleen cell transplant. At the dose (1100R) which was given to our recipients the number of endogenous colonies was found to be less than 0.1 colony per spleen.

Preparation of cell suspensions Bone marrow suspensions were prepared from either femurs or tibias. The marrow plug was removed from the shaft by first making a hole in the distal end of the bone and flushing MEM through the cavity by means of a 23-gauge needle inserted in the proximal end of the bone. (If the cell suspensions were to undergo the suicidal H^3-thy treatment, they were prepared in medium CMRL 1066 without thymidine or co-enzymes instead of MEM.) The plug or cell clusters were broken up by passing the suspension about 10 times through a 26-gauge needle and finally the cell suspension was filtered through a nylon gauze sack into a siliconized tube kept in ice.

Spleen suspensions were prepared by piercing a hole at one end of the spleen and gently pushing the cells out of the capsule by means of two pairs of forceps. The cell clumps and clusters were broken by flushing

as described for the bone marrow suspensions.

Cell counts were determined in a hemocytometer chamber after suitable dilution with Turk's diluting fluid.

RESULTS

The suicidal H^3-thymidine technique and proliferative states of CFU from different sources. Drew and Painter (1959) have shown that cells undergoing DNA synthesis lose their colony-forming ability when they are exposed to high specific activity tritiated thymidine. This technique has been referred to as the "suicidal thymidine technique". Becker et al (1965) and Guzman and Lajtha (1970) have used this technique to determine the proliferative state of the CFU population. To assess our ability to employ this technique, CFU from normal bone marrow, regenerating marrow and fetal liver were subjected to the suicidal thymidine technique. The 7th day regenerating marrow was chosen because the time is approximately the midpoint of the exponential growth curve of CFU following irradiation (Valeriote and Bruce, 1967; Schooley, 1966). The results are summarized in Table I. The results support the concept that CFU in the normal steady state have long cell cycles and relatively few cells are undergoing DNA synthesis at any time and therefore the suicidal thymidine treatment has little effect on the steady state CFU population. However, when the bone marrow and the CFU are regenerating after irradiation or undergoing rapid proliferation as in the case of the fetal liver, more cells undergo division and thus resulting in a greater percentage killed by the suicidal thymidine technique. It is also noted that the CFU in the regenerating marrow are proliferating more rapidly in irradiated animals which have received no transplant.

Effect of suicidal thymidine treatment on unirradiated normal marrow In order to dtect any changes in the proliferative state of the bone marrow CFU it was necessary to establish the baseline value using unirradiated marrow as control. The results are given in Table II. It may

TABLE I

SURVIVAL OF COLONY FORMING UNITS DERIVED FROM NORMAL MARROW, REGENERATING MARROW AND FETAL LIVER AFTER A 30-MINUTE INCUBATION WITH HIGH SPECIFIC ACTIVITY (>18Ci/mM) H^3-THYMIDINE

CFUs From	H^3-thy at 500 uc/ml	No. of cells injected	CFU/spleen	Fractional survival	Percent Kill
Normal Marrow	present	$5X10^4$	14.7±1.0*	1.04	0
	absent	$5X10^4$	14.1±0.5		
7th Day Regenerating Marrow with Transplant	present	$1.5X10^5$	10.8±1.0	0.56	44%
	absent	$1.5X10^5$	19.3±1.3		
7th Day Regenerating Marrow without Transplant	present	10^5	3.43±0.7	0.34	66%
	absent	10^5	9.92±1.1		
18th Day old Fetal Liver	present	$1.5X10^5$	8.22±1.3	0.52	48%
	absent	$1.5X10^5$	15.7±1.6		

* - Mean ± 1 Standard error

TABLE II

SURVIVAL OF COLONY FORMING UNITS DERIVED FROM NORMAL MARROW AFTER A 30-MINUTE INCUBATION WITH HIGH SPECIFIC ACTIVITY (>18Ci/mM) H^3-THYMIDINE

Normal Marrow Suspension No.	H^3-thy at 500 mc/ml	No. of cell injected	CFU/spleen	Fractional Survival	Percent Kill
1	present	$5X10^4$	14.7±1.0*	1.04	
	absent	$5X10^4$	14.1±0.5		
2	present	$5X10^4$	13.5±1.1	0.98	2%
	absent	$5X10^4$	13.7±0.9		
3	present	$5X10^4$	14.9±0.9	0.99	1%
	absent	$5X10^4$	15.0±0.9		
4	present	$5X10^4$	15.4±0.8	0.95	5%
	absent	$5X10^4$	16.3±0.8		

* - Mean ± 1 Standard error

be seen that the baseline value of kill of CFU by the suicidal thymidine technique is less than 5%. This is in good agreement with values obtained by other investigators (Becker et al, 1965; Guzman and Lajtha, 1970) and with the basic concept that the CFU population in the normal steady state is either undergoing very slow division cycles or the fraction of CFU undergoing division is very small.

Therefore, in the following results, if the suicidal thymidine killing effect exceeds 5%, it would suggest that the proliferative state of the CFU population treated has been altered.

Effect of whole body irradiation or one tibia irradiation on the proliferative state of CFU in shielded limbs Two groups of animals were used. They were treated as follows:

Group I: The animals were given 1100R whole body x-ray with one tibia shielded. $2x10^6$ bone marrow cells were given to each animal following irradiation. Animals so treated will be referred to as WBR (Whole Body Irradiated) animals.

Group II: 1100R x-ray was delivered to only the left tibia of these animals while the rest of the body was shielded. $2x10^6$ bone marrow cells were given to each animal following irradiation. Animals so treated will be referred to as LTR (Left Tibia Irradiated) animals.

At appropriate times following irradiation, groups of these animals were sacrificed. Both irradiated and shielded tibias were removed and the suicidal thymidine treatment was performed on the bone marrow suspensions prepared from the respective tibias and then the CFU content of the suspensions were assayed by the spleen colony technique. A schematic representation of the experimental procedures is shown in Figure I. The killing effect on the CFU from the irradiated bone marrow served as

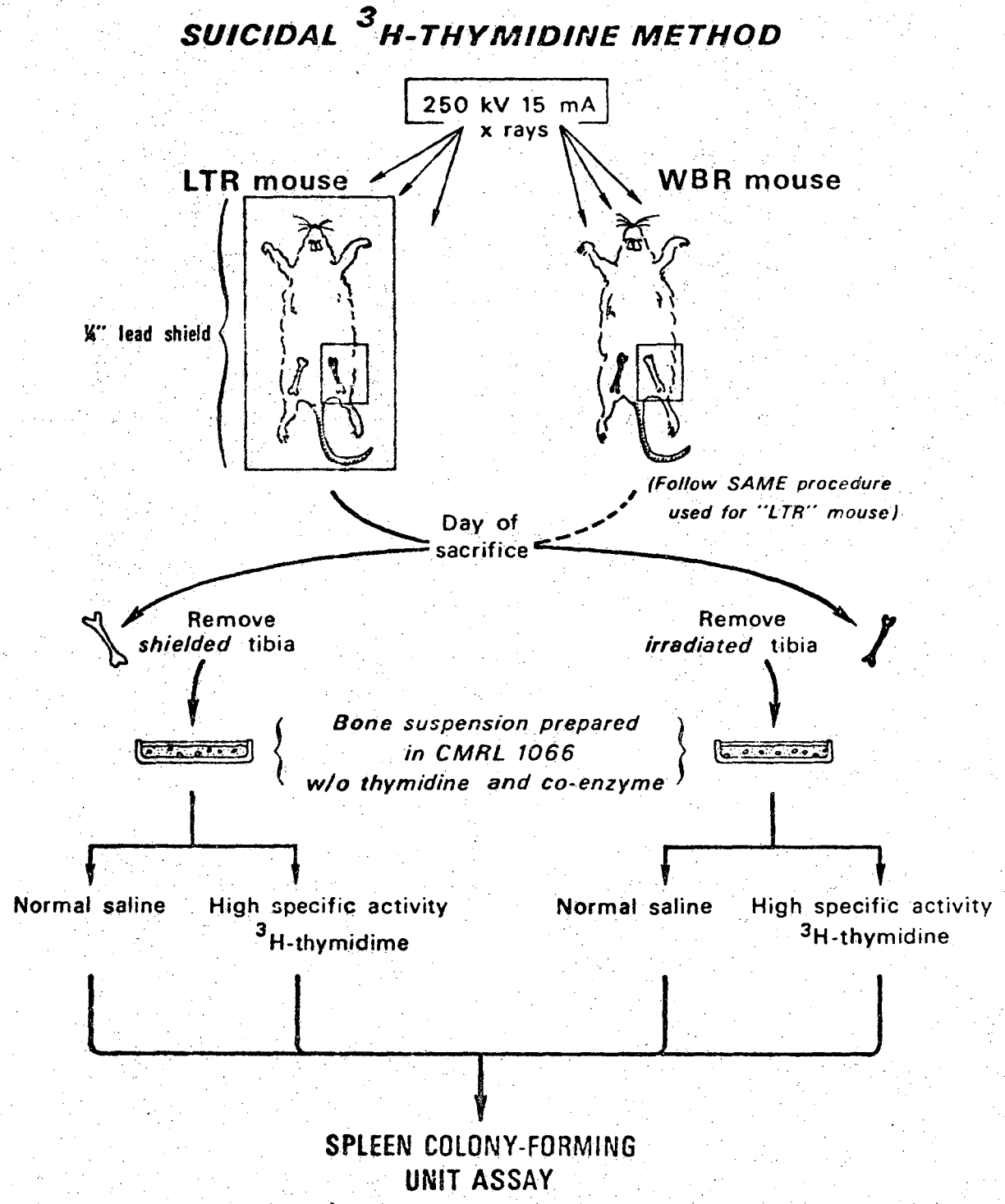

FIGURE I. SCHEMATIC REPRESENTATION OF EXPERIMENTAL PROCEDURES IN THE DETERMINATION OF PROLIFERATIVE STATES OF CFUs IN TIBIAS OF WBR AND LTR MICE BY THE SUICIDAL H^3-THYMIDINE METHOD

TABLE III

EFFECT OF THE SUICIDAL H^3-THYMIDINE TREATMENT ON THE SURVIVAL OF COLONY FORMING UNITS TAKEN FROM THE SHIELDED TIBIAS OF WBR[1] ANIMALS AT VARIOUS TIMES AFTER IRRADIATION

Time After Irradiation	CFU Observed/Spleen		Percent Kill as a Result of H^3-thy Treatment
	Normal Saline Treated Control	H^3-thy Treated	
2 Hours	13.9±0.6*	11.3±0.9	17%
	16.6±0.7	14.2±0.8	
3 Days	19.8±0.8	14.0±0.7	25%
	17.3±1.0	14.8±0.8	
	19.3±0.8	13.3±0.8	
7 Days	24.7±1.4	21.6±1.1	18%
	22.9±0.8	18.3±1.0	
	19.4±0.8	17.5±1.9	
	22.9±1.1	19.8±0.9	
	23.8±0.7	15.4±1.0	

* - Mean ± 1 Standard Error

1. - WBR - Whole body irradiated with one tibia shielded and given $2X10^6$ bone marrow cells

TABLE IV

EFFECT OF THE SUICIDAL H^3-THYMIDINE TREATMENT ON THE SURVIVAL OF COLONY FORMING UNITS TAKEN FROM THE SHIELDED TIBIA OF LTR[1] MICE AT VARIOUS TIMES AFTER IRRADIATION

Time After Irradiation	CFU Observed/Spleen Normal Saline Treated Control	H^3-thy Treated	Percent Kill as a Result of H^3-thy Treatment
2 hours	15.5±1.1*	11.7±0.6*	24%
3 days	14.8±0.6	9.38±0.5	23%
	14.7±1.1	13.4±0.8	
7 days	15.0±1.1	12.0±1.0	17%
	17.9±0.8	13.4±0.6	
	16.9±1.0	14.0±0.7	
	16.1±0.8	15.1±0.6	

* - Mean ± 1 Standard Error

1. LTR - Whole body shielded with one tibia irradiated and given $2X10^6$ bone marrow cells

a check on the suicidal thymidine treatment and in each experiment it was greater than 45%. The killing effect on the CFU in the shielded tibias are summarized in Table III and IV.

The percentage kill shown in the tables is the average of the number of experiments shown. Originally the assay was performed solely on day 7 animals with the reasoning that the effect should be most pronounced when the CFU are doubling most rapidly; later it was found that similar killing effect could detected as early as two hours post-irradiation. It should also be noted that the suicidal thymidine killing effect on shielded CFU population in the case of both the LTR and WBR animals are quite similar and are significantly different ($p < 0.001$) from the killing effect of high specific activity H^3-thymidine on normal marrow (ref. Table II).

Effect of whole body irradiation or one tibia irradiation on the cellularity and colony forming unit content of the shielded tibia The two groups of animals (LTR and WBR) were similarly treated as in the previous section. The cellularity and colony forming unit content was determined by pooling 4 or 5 shielded tibias from animals of each group at 2 hours, 3 days and 7 days after irradiation. The results are presented in Table V. Most of the values obtained are averages of three to six different experiments; the exceptions are the 2 hour and 3 day LTR animals where only one experiment was performed.

The results show very little change in the cellularity for both whereas there is a significant ($p < 0.001$) increase in CFU content of shielded tibias from whole body irradiated animals (WBR). We also noted that this increase of CFU content is less dramatic in the WBR group if the animals were given 20×10^6 bone marrow cells instead of 2×10^6: the day 7 tibial

TABLE V

CHANGES IN CELLULARITY AND COLONY FORMING UNIT (CFU) CONTENT OF THE SHIELDED TIBIA AT VARIOUS TIMES AFTER IRRADIATION (1100R) AND BONE MARROW TRANSPLANTATION

Treatment	Time After Irradiation	10^6 Cells/Tibia	CFU/10^6 Cells	CFU/Tibia
Normal	-	9.70±0.2*	296±17*	2870±180*
WBR[1]	2 Hours	9.29±1.0	305±27	2830±400
	Day 3	10.9±1.4	430±34	4680±700
	Day 7	11.4±0.6	444±33	5060±460
LTR[2]	2 Hours	10.2	310±23	3150±230
	Day 3	10.8	320±23	3460±250
	Day 7	9.49±0.7	298±21	2830±290

1. - Whole body irradiated with one tibia shielded and given 2 x 10^6 bone marrow cells
2. - Whole body shielded with one tibia irradiated and given 2 x 10^6 bone marrow cells
* - Mean ± 1 Standard error

CFU content is this case was 3969±452 instead of 5057±458.

The results in Table V seem to indicate that there is an increase in CFU content when more CFU are destroyed as in the case of the whole body irradiated (WBR) animals. To further investigate this observation, a series of animals was given varying doses of irradiation with one tibia shielded; but these animals did not receive any bone marrow transplant. The results are shown in Table VI. It is seen again that while there is little change in cellularity the increase in CFU content is directly related to the irradiation dose and thus perhaps directly related to the surviving CFU population of the animal.

Effect of suicidal thymidine treatment on spleen and bone marrow colony forming units in parabiotic mice One of the parabiotic mice was shielded with a ½" lead coffin while the other partner received 1100R x-ray. After irradiation, each aprtner was given $4x10^6$ bone marrow cells. The marrow transplant was given to minimize the migration of stem cells from the shielded parabiont to the irradiated one (Carroll and Kimeldolf, 1969). At the third and seventh day after irradiation 2 - 4 pairs parabiotic animals were sacrificed and their spleens and tibias and femurs removed. The suicidal thymidine treatment was performed on both the spleen and bone marrow suspensions (Figure II). The results are summarized in Table VII and VIII.

The results indicate that irradiation of one member of a parabiotic pair affects the proliferative state of the colony forming unit population in both the spleen and bone marrow of both members. Whereas the killing effect of suicidal thymidine is predictable in the case of the irradiated parabiont, it was surprising to observe such pronounced killing effects in the unirradiated one. It should be noted that the killing effect is more pronounced on the third day.

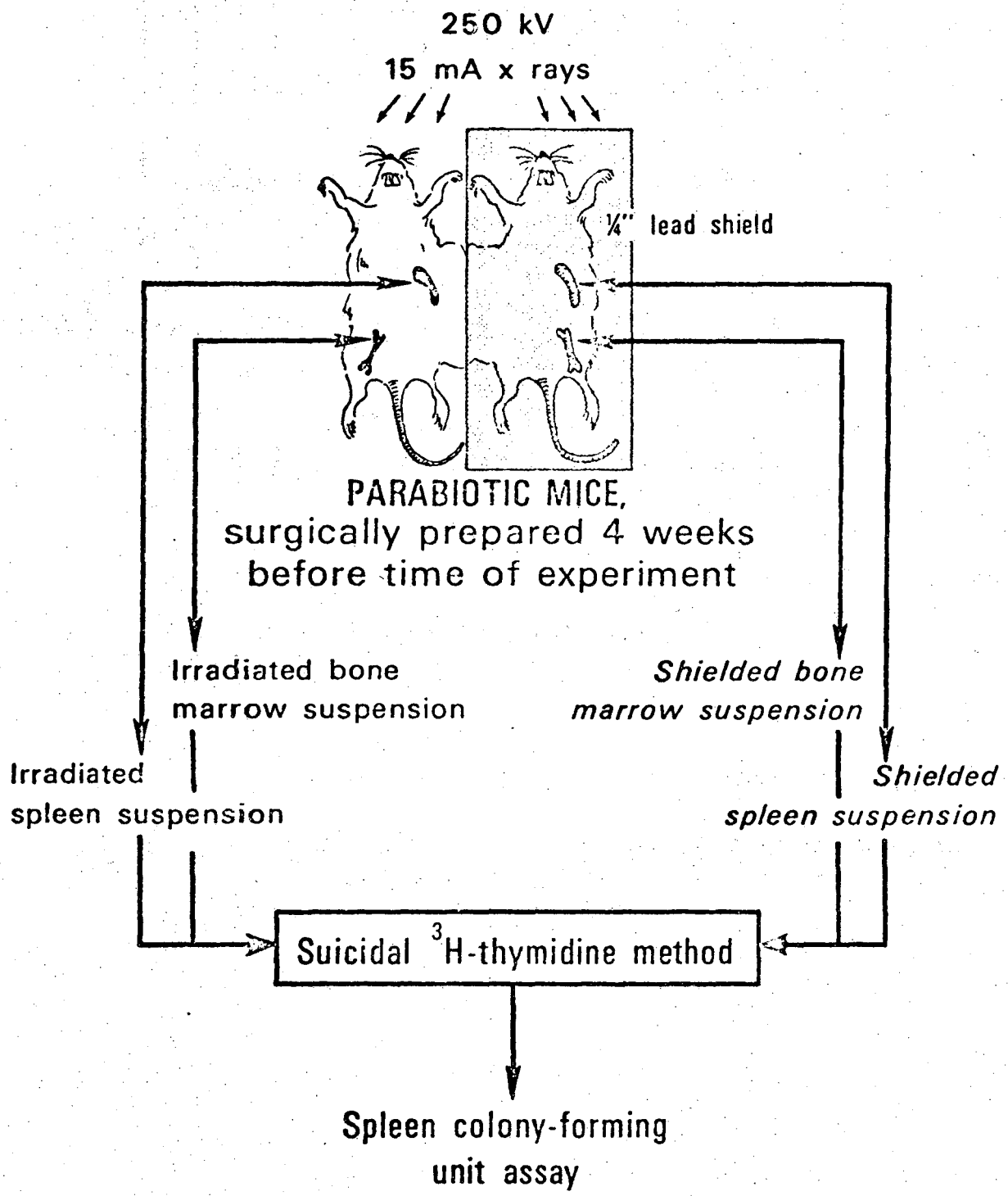

FIGURE II. SCHEMATIC REPRESENTATION OF EXPERIMENTAL PROCEDURES IN THE DETERMINATION OF PROLIFERATIVE STATES OF CFUs IN SPLEENS AND TIBIAS OF PARABIOTIC MICE

TABLE VI

CELLULARITY AND CFU CONTENT OF THE SHIELDED TIBIA OF WBR[1] MICE 7 DAYS AFTER DIFFERENT IRRADIATION DOSES

Dose	10^6 Cells/Tibia	CFU/10^6 Cells	CFU/Tibia
200R	10.1	355±26*	3590±260*
500R	10.5	442±22	4630±240
1100R	9.18	572±27	5250±250

1 - Whole body irradiated with one tibia shielded and given no bone marrow
* - Mean ± 1 standard error

TABLE VII

EFFECT OF SUICIDAL H^3-THYMIDINE TREATMENT ON SPLEEN AND BONE MARROW COLONY FORMING UNITS IN THE IRRADIATED PARTNER OF PARABIOTIC MICE

Time After Irradiation	Irradiated Parabiont					
	Bone Marrow			Spleen		
	Normal* Saline Control	H^3-thy*	Percent Kill	Normal* Saline Control	H^3-thy*	Percent Kill
Day 3	7.42±0.9	2.57±0.6	65%	18.9±1.1	11.1±1.1	41%
Day 7	9.93±0.9	4.69±0.7	57%	40.6±1.2	27.3±1.2	32%
	17.3±0.8	9.96±1.0	42%			

* - Mean ± 1 Standard error

TABLE VIII

EFFECT OF SUICIDAL H^3-THYMIDINE TREATMENT ON SPLEEN AND BONE MARROW COLONY FORMING UNITS IN THE SHIELDED PARTNER OF PARABIOTIC MICE

Time After Irradiation	Shielded Parabiont					
	Bone Marrow			Spleen		
	Normal* Saline Control	H^3-thy*	Percent Kill	Normal* Saline Control	H^3-Thy*	Percent Kill
Day 3	15.1±0.9	9.06±0.6	40%	12.2±1.3	8.78±0.9	28%
Day 7	16.7±1.6	14.3±0.9	14%	31.5±1.1	23.7±1.7	25%
	18.4±1.2	15.3±1.2	17%	21.5±1.0	18.0±0.9	17%

* - Mean ± 1 Standard error

DISCUSSION

The suicidal H^3-thymidine technique has been employed to assess the proliferative state of the CFU population in the shielded tibias of animals which have received whole body irradiation or whose contralateral limbs have been irradiated. In both cases (Table III, IV) the proliferative state of the CFU is significantly different ($p < 0.001$) from that of normal unirradiated marrow. The change in proliferative state may be detected as early as 2 hours after the irradiation and persists through the 7th day. More active proliferation is also observed in the CFU population of parabionts whose partners have been irradiated.

The results suggest that the rate of division of CFU may be governed by a humoral agent that is released as a result of irradiation of the animal and is circulated and exerts its effect on the CFU population not only locally but perhaps throughout the whole system.

McCulloch & Till (1962) have reported the D_o of CFU population to be 95-110 rads. Van Bekkum (1969) estimated the size of the bone marrow population in the mouse to be approximately 6×10^8 cells. Noting that the tibia in our animals contain 10^7 bone marrow cells, our results indicate that the irradiating less than 2% ($10^7/6 \times 10^8$) of the total bone marrow population, as in the case of single tibia irradiated animals (LTR animals),would bring about measurable changes in the CFU population in the contralateral limbs.

The study on the changes in cellularity and CFU content resulting from irradiation of either one limb (LTR animals) or the whole body (WBR animals) reveals that (Table V) whereas there is little change in total cellularity of the shielded limb, there is a striking increase in CFU

content and concentration when the whole body has been irradiated. The CFU changes in the WBR and LTR animals may be more readily understood if one realizes that the WBR animal has a greater demand for CFU, whereas the LTR animal has more than 98% of its CFU virtually intact. Further evidence that suggests that the increase in CFU content of the tibia could be related to fraction of surviving CFU is shown in Table VI which shows a direct increase in CFU content after increasing whole body doses.

Our results here do not agree with the observation reported by other investigators: Gidáli et al (1969) reported a decrease in CFU in the shielded tibias of BALB/c mice given 500R whole body irradiation; and Carsten and Bond (1968) observed no increase in CFU content in the hind legs of animals given a 100 rads exposure to the upper body. The discrepancy may be due to the fact that their animals are receiving a lower dose (100 rads) and that the observation by Gidali et al may be unique to the BALB/c mice which have been reported to react differently than other strains at least in its non-responsiveness to hypoxia (Nohr, 1967). It should be noted at this time that the initial pilot experiments for this study were performed on $BC3F_1$ mice and the results were similar to those obtained using our present strain (LAF_1). This would rule out the possibility of these observations being unique for the animals used.

However, in order for the shielded limb to respond to different demands for CFU, there must be a mechanism by which the CFU may detect changes in the total CFU population. One way by which the CFU population could respond to changes is by means of a negative feedback chemical substance similar to the chalone (a mitotic inhibitor) isolated for the epidermis

(Bullough and Laurence, 1964). But our data showing similar killing effects resulting from the suicidal thymidine treatment in both the WBR and LTR animals seem to rule out a chalone or mitotic inhibitor as the sole governing mechanism for the CFU population.

We are performing experiments (unpublished) to determine the effect of irradiated hemopoietic tissue extract on the proliferative state of the normal CFU population. Preliminary results show that the irradiated tissue extract does not alter the fraction of CFU killed by the suicidal thymidine treatment. This has led us to suspect that the humoral agent or chalone is either very labile or not located within the major hemopoietic tissues.

Our results in this study do however suggest a circulating humoral agent which regulates the proliferative states of the CFU population, and reveal the ability of the CFU compartment within a shielded limb to change its size in response to differing CFU demands. Whether the increase in CFU population is due to the action of the humoral agent is not known. But the fact that the CFU population can vary its cell cycle and increase its compartment size simultaneously suggest that the opinions of Becker et al (1965) and Lajtha et al (1962, 1964) on the response of CFU to hemopoietic demands may be both correct. Our observations here also refute the suggestion that the control of the proliferation of the CFU population is entirely local.

STUDY II: THE GROWTH AND PROLIFERATIVE STATES OF COLONY FORMING UNITS AND AGAR COLONY FORMING UNITS FOLLOWING IONIZING RADIATION

Results from the previous study reveal the existence of a circulating agent which affects the proliferative state of colony-forming units. Furthermore studies with parabiotic animals suggest that there may be a temporal pattern of the release of this substance.

The growth curves of CFU in the marrow (Valeriote & Bruce, 1967) and in the spleen (Schooley, 1966; McCulloch & Till, 1964) have shown an initial "lag phase" from Day 1 - 3 before the beginning of exponential growth at Day 3. During this lag phase, the CFU are believe to be undergoing rather rapid proliferation and should this proliferation be governed by the "circulating agent" there would be a greater effect exerted during this time (Day 2 - 3) on normal unirradiated marrow. Our results in Tables III, IV and VII, VIII, seem to suggest that there may be indeed a greater effect of this circulating agent on Day 3. This study is therefore conducted to determine whether there is a temporal change in proliferative state resulting from this circulating agent. Also examined in this study was the change in proliferative states of the agar colony-forming units.

MATERIALS AND METHODS

Animals 12 - 16 week old LAF_1 female mice were used both as recipients and donors in the following experiment. Acclimatizing and animal care were as previously described.

Irradiation Irradiation was accomplished by a 1400 Curie cobalt-60 source. Animals were place in a lucite container at a distance of 1.22 meters from the source and given 1100R. The dose rate was 26.6R/min.

Preparation of cell suspensions Same as previously described.

Suicidal thymidine technique Same as previously described.

Spleen colony technique Same as previously described.

Agar colony forming unit (ACFU) assay

Materials:

a. E2020 Medium: Total 400ml consisting of the following: 10X Minimum Essential Medium (MEM) with L-glutamine and without sodium bicarboante (Earles Salts) 100ml; 7.5% sodium bicarbonate, 30ml; 100X sodium pyruvate, 10ml; 100X non-essential animo acids, 10ml; penicillin/streptomycin (5000 units of each/ml) 2ml; fetal calf serum, 100ml (All of the above were procured from Grand Island Biological Co., GIBCO, Oakland, Calif.); triple glass distilled water 148ml.

b. Trypticase soy broth (TSB): 3mg trypticase soy broth powder (Baltimore Biological Lab., Baltimore, Md.) in 100ml of triple glass distilled water auto-claved 15 minutes at 121°C and 15lb. pressure and stored at 4°C for no more than 4 weeks.

c. Bacto-agar (Difco Labs., Detroit, Michigan) 0.6% solution: 0.6gm of bacto-agar was placed in 100ml of triple distilled water and boiled for 10 minutes in water bath. The melted agar was held in

45°C water bath until use.

d. E2020 & TSB solutions: 4 parts E2020 and one part TSB.

e. Conditioned Media: Kidneys from 6 - 14 days old Swiss Webster mice were removed aseptically and placed in Eagles MEM (GIBCO) with 60 units/ml of penicillin-streptomycin. The kidneys were then minced with a chopper and digested in 0.25% trypsin solution (GIBCO) with 50 units/ml penicillin streptomycin and 0.4% D'Nase solution (in phosphate buffered saline) for 1½ hour at room temperature. The solution was digested with constant gentle stirring by magnetic stirrer and occasional pipetting to break up larger tissue clumps. After the digestion and settling of larger clumps the cell suspension was washed in E1010 (E2020 & TSB + equal volume triple distilled water), centrifuged and counted after suitable dilution with Turk's diluting fluid. The cell suspension was then adjusted to contain $4x10^6$ cell/ml of E1010 plated in 15x100mm petri dishes at 15ml/dish, and incubated at 37° in a humidified incubator with a constant flow of 5% CO_2 in air. The conditioned media cell suspension was harvested after 7 - 10 days by centrifuging to remove all cells and passing the solution through a 0.45u millipore filter. The solution was stored at 4°C.

Assay for agar CFU: an aliquot usually 50-200µl of the marrow or spleen cell suspension was taken and placed in a plastic tube. The suspension was then diluted with 4ml of E2020 and TSB, 1ml conditioned media, 5ml of 0.6% agar, and then mixed. 2ml of the mixture was plated in 35x60mm tissue culture dishes (Falcon plastic) and incubated for 9 - 10 days at 37° in a humidified incubator with a constant flow of 5% CO_2 in air. The colonies on each plate were scored under 10X magnification.

Experimental Procedure After whole body irradiation of 1100R sixty LAF_1 female mice were given 10^7 bone marrow cells from LAF_1 donors. On designated days after irradiation and transplantation, 5 - 10 animals were sacrificed and cell suspensions from pooled tibias and spleens were prepared in CMRL 1066 without thymidine or co-enzymes. The bone marrow suspension and spleen cells suspensions were subjected to the suicidal thymidine treatment and assayed for spleen colony forming unit and agar colony forming unit content.

RESULTS

The results of the experiment are summarized in Table IX and X. Graphic representation of the results are given in Figure III and IV. To avoid confusion, cells assayed by the spleen colony technique are designated as spleen colony forming units (SplCFU) and the cells assayed by the *in vitro* agar technique are designated as agar colony forming units (ACFU). Whereas the recovery pattern of SplCFU and ACFU are similar in both the spleen and bone marrow, the suicidal thymidine killing effect is markedly different for the two populations. It should be noted also that the SplCFU content of the tibia does not recover to normal values. The thymidine killing effect was observed to be more pronounced in the initial days following irradiation.

TABLE IX

RECOVERY OF SPLEEN COLONY FORMING UNITS (Spl CFU) AND AGAR COLONY FORMING UNITS (ACFU) IN TIBIAS OF IRRADIATED (1100R) MICE

Day Post Irradiation	Spleen CFU		Agar CFU	
	CFU/Tibia	Percent Killed by H^3-thy Treatment	ACFU/Tibia	Percent Killed by H^3-thy Treatment
Control	2870±180*	5%	12000±1400	40%
1	10.0±1.0	53%	88.5	59%
3	15.1±1.9	38%	N/A	N/A
7	415±24	49%	4850	61%
10	725±25	31%	10400	67%
14	732±39	18%	9500	65%
17	1120±97	14%	14400	58%
21	600±100	12%	14600	48%
35	733±76	N/A	N/A	N/A

* - Mean ± 1 Standard error
N/A - not available

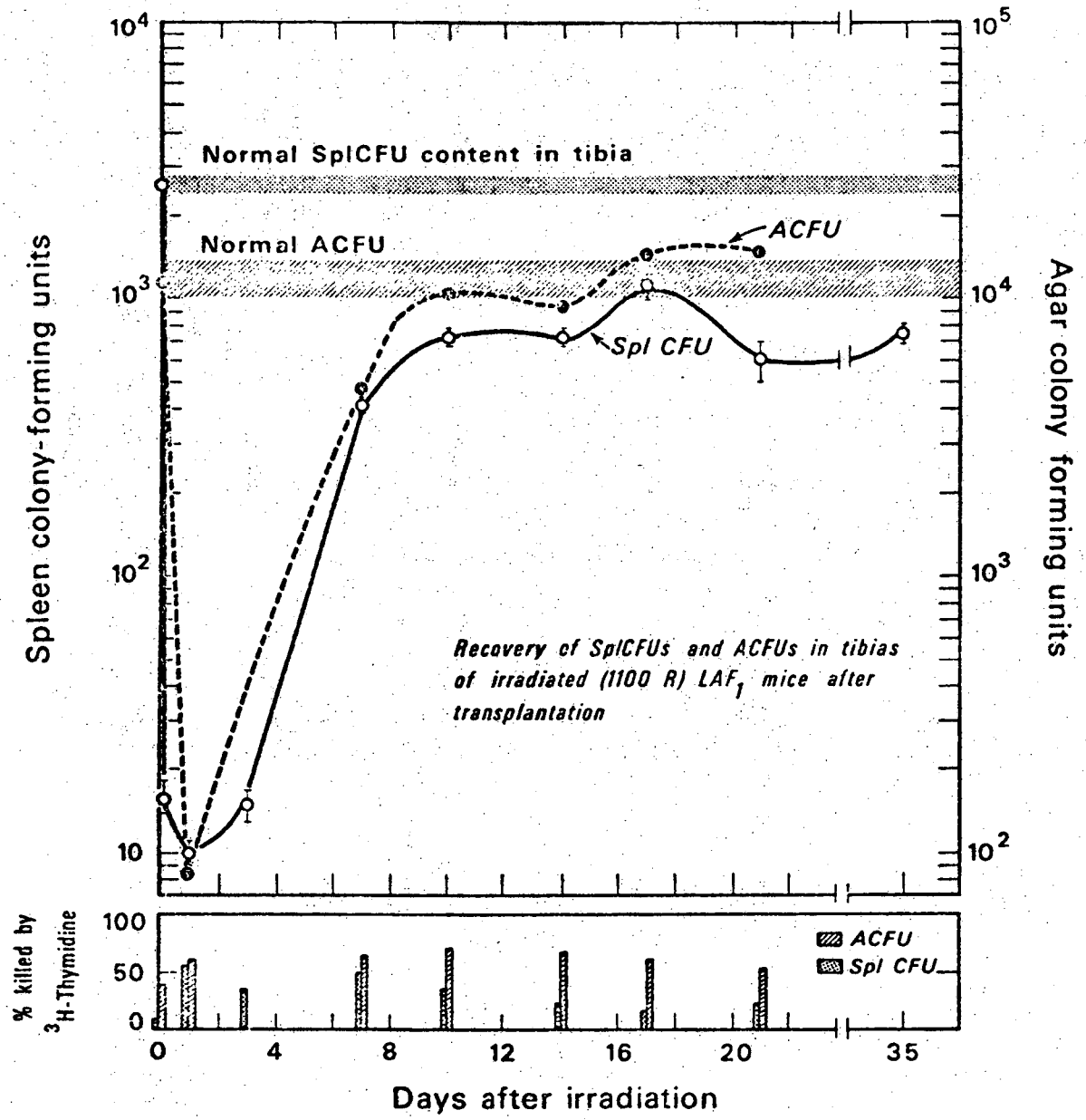

FIGURE III. RECOVERY AND PROLIFERATIVE STATES OF SplCFU AND ACFU IN TIBIAS OF IRRADIATED (1100R) LAF_1 MICE

TABLE X

RECOVERY OF SPLEEN COLONY FORMING UNITS (Spl CFU) AND AGAR COLONY FORMING UNITS (ACFU) IN SPLEENS OF IRRADIATED (1100R) MICE

Day Post Irradiation	Spleen CFU		Agar CFU	
	CFU/Spleen	Percent killed by H^3-thy Treatment	ACFU/Spleen	Percent killed by H^3-thy Treatment
Control	2500±200*	8%	30000±4000	40%
1	40±2	43%	648	82%
3	715±50	37%	2680	95%
7	7240±210	13%	46400	52%
10	8380±200	11%	34100	52%
14	7000±260	11%	113000	58%
17	4210±260	50%	20000	64%
21	3070±270	19%	54000	32%
35	2446±165	N/A	N/A	N/A

* - Mean ± 1 Standard error
N/A - not available

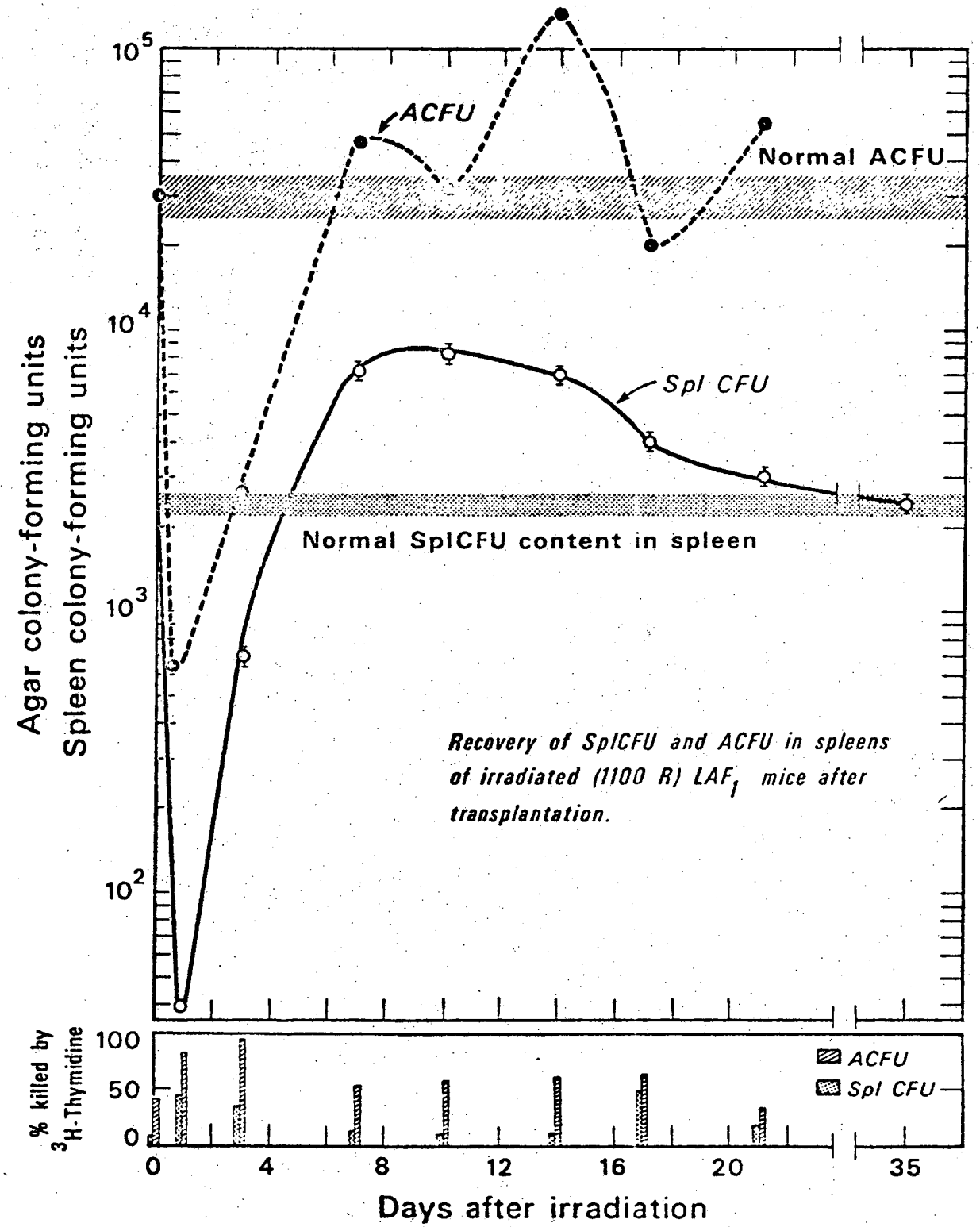

FIGURE IV. RECOVERY AND PROLIFERATIVE STATES OF SplCFU AND ACFU IN SPLEENS OF IRRADIATED (1100R) LAF_1 MICE

DISCUSSION

Several observations are made in this experiment:

a. During the initial days of recovery the SplCFU in both tibia and spleen are in an active proliferative state;

b. The recovery pattern of the SplCFU are different in the spleen and the tibia;

c. There is a distinct difference in the proliferative states of SplCFU and ACFU.

Table IX and X show that during days 1 - 3 the suicidal thymidine killing effect is more pronounced. These results are consistent with the changes in proliferative patterns observed for parabiotic animals in the previous study . (ref. Table VII and VIII) Higher proliferative states are also suggest by the data on the WBR and LTR animals. (ref. Table III and IV) These observations lead us to believe that the regulating humoral agent is released in greater quantities during the initial days after irradiation; but as the SplCFU population recovers to near normal values, the concentration of the agent falls and its effect diminishes.

It should be noted that the proliferative state of SplCFU in the spleen and in the tibia remains slightly altered through day 21. During this time the SplCFU numbers of the spleen have fully recovered but the CFU content of the tibia remains depressed. A similar depressed SplCFU content in the femur has been reported by Valeriote and Bruce (1967). The sustained change of proliferative state and the depressed SplCFU values in the marrow lead us to suspect that the release of the circulating humoral agent is brought about by a depressed SplCFU population. If this were indeed the case then it would be likely that a drop in the total

SplCFU population may serve as the triggering device for the release of the humoral agaent. Detection of a change in the proliferative state of shielded tibias of LTR animals also supports this hypothesis.

Guzman and Lajtha (1970) reported a difference in suicidal thymidine killing effect on normal SplCFU derived from bone marrow and those derived from the spleen. Our results also suggest a difference. Schofield (1970) suggested that the difference may be due to the existence of two types of SplCFU, each possessing a different doubling time and each being differentially attracted to the spleen or bone marrow. We attribute the difference to the possibility that the SplCFU populations from the spleen and tibia may have different demands placed upon them. The splenic SplCFU population may be actively proliferating to sustain sustain intermediate hemopoietic precursor cell populations whereas the SplCFU population in the bone marrow may serve mainly to replenish the SplCFU populations located in other hemopoietic tissues. During the steady state conditions the bone marrow SplCFU population is not active but during the recovery and replenishing period it is actively proliferating to seed other SplCFU populations. It is not known however whether the depression of SplCFU population in the marrow following irradiation is the result of seeding of SplCFU from the bone marrow. In other words the splenic SplCFU population serves as a reservoir for the SplCFU whereas the bone marrow SplCFU population serves as the major source. Observations of Bruce and McCulloch (1964) that a gradual decrease in the splenic SplCFU during the first fifteen days of exposure to hypoxia was not accomplished by any significant change in the SplCFU content of the femur seem to support this hypothesis. Schooley's finding that the spleen is a major reservoir of erythropoietin-sensitive cells, an intermediate hemopoietic precursor

cell population, also provides support for this hypothesis. (Schooley,1970) The results on the recovery of ACFU does not offer any information on the relationship between the regulatory mechanisms governing the ACFU and SplCFU population. A doubling time of 20 - 24 hours was observed for ACFU during the exponential recovery phase.

Bennett et al (1968) suggested that the ACFU population, though closely related to the SplCFU population, may be an intermediate stage of differentiation between the SplCFU and the myeloblast and is distinctly different. Chen and Schooley (1970) have provided further evidence in support of this concept. Our observation in both the spleen and the tibia that the proliferative states of the SplCFU and ACFU are markedly different throughout the recovery period and in the control animals offers support to the concept that the two populations are not only different functionally but also physiologically.

SUMMARY AND DISCUSSION

The purpose of these studies was to elucidate the nature of the regulatory mechanism(s) which govern the proliferation and growth of the colony forming unit (CFU) population.

Results from the study using partially shielded and parabiotic mice suggest the presence of a circulating humoral agent which acts by altering the proliferative state of the CFU population. The effects of the agent are detected as early as two hours after any change occurs in the total CFU population of the mouse and seem to persist as long as the change remains.

In addition to its proliferative state being altered to meet the demands for CFU, the size of the CFU population is also adjusted. The extent of adjustment is directly related to need for CFU.

In our studies, radiation was used to perturb the CFU from its steady state. Since ionizing radiation is known to affect the other hemopoietic populations the changes observed may be an indirect effect of the differentiation of CFU to replenish the intermediate hemopoietic cells populations depleted by radiation. But our results showing a persisting alteration in the proliferative states in shielded tibia of animals which have had only one tibia irradiated seem to support the concept of a circulating agent.

The relation of ionizing radiation and the release of various hemopoietins has not been well studied; so it is difficult at this time to discuss the possible release of such hemopoietins after radiation and to assess their effect upon the proliferative state of the CFU population. Schooley (1966) did observe a steady increase of erythropoietin in the plasma of mice following whole body irradiation; but he also resported

that erythropoietin had little or no effect on the growth of CFU. McCulloch et al (1965) reported a genetically controlled "host-factor" found in the mice of genotype Sl/Sl^d for the regulation of CFU; however this factor was observed not to be cross-circulated when a Sl/Sl^d was joined to a normal animal by parabiosis. It would be highly speculative at this point to discuss the "host-factor" in relationship to our observations but it is not inconceivable that the gene locus sl which determines the production of the "host-factor" is closely linked to the gene locus which controls the circulating "stem cellpoietin" that our results suggest.

Our studies have also suggested a temporal pattern for the action and release of this circulating agent. The effect on the CFU population is most pronounced on the initial days following irradiation. The action and release of this agent is continuous as long as the CFU population is depressed. This observation leads to the speculation whether a change in the total CFU population at any time brings about the release of this humoral agent. Results from the LTR mice (Table IV) gives support to this possibility.

The results in the study of the change in the proliferative states of ACFU and CFU offer further support for the concept of a functionally and physiologically separate ACFU and CFU population. Whether the regulatory mechanisms for the two compartments are similar is not evident in our study.

A possible mechanism by which the primitive precursor cell population is regulated has been presented in our studies. However, much work remains to be done to characterize the nature of the substance and to understand its action.

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