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REGULATION OF HEMOPOIETIC STEM CELL TURNOVER AND POPULATION SIZE IN NEONATAL MICE

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FOREWORD
(Nontechnical summary)

It is believed that all blood cells arise from a common stem cell. In the adult mammal this stem cell is found primarily in the bone marrow and resembles a small lymphocyte in its staining characteristics, size and density.

In the physiologically normal adult mouse, stem cells are found in a nonproliferating or slowly proliferating state. However, if the hematological system is stressed and/or the stem cell population is reduced then the remaining stem cells divide and proliferate rapidly. Little is known about the mechanism governing the transition of the stem cell between the nonproliferating and proliferating state. However, an understanding of this regulatory mechanism would be of importance in the development of more effective postirradiation treatment. Thus, in the present study an effort has been made to elucidate the nature of the factors which regulate the proliferative state of the hemopoietic stem cell.

At present there are two theories about the mechanism of hematopoietic stem cell proliferation. One theory postulates that it is controlled by circulating or humoral factors while the other theory postulates that stem cell proliferation is regulated by short-range, tissue-specific chemical factors. In order to evaluate these two theories, a study of stem cell proliferation in neonatal mice was undertaken.

In the neonatal mouse the total stem cell population is shared between two organs, the liver and spleen. Each organ contains approximately an equal number of stem cells. If circulating or humoral factors regulate stem cell proliferation it would be expected that the stem cells present in both liver and spleen would be in a similar

state of turnover. Alternatively, if tissue-specific factors control stem cell proliferation, then it would be likely that stem cells found in the two organs would proliferate at different rates.

In the present work it was found that stem cells in both organs apparently replicate at the same rate, indicating humoral control of neonatal stem cell turnover. Also, it was observed that following birth the stem cell population in the neonatal liver declines rapidly while in the spleen the stem cell population increases exponentially. Therefore, the factors which regulate the size of a stem cell population in a specific organ are to some degree independent of the factors which regulate stem cell turnover.

In conclusion, the results indicate that stem cell turnover in neonatal mice apparently is controlled by a humoral mechanism while the size of the stem cell population appears to be regulated by local tissue-specific factors.

ABSTRACT

Following birth the hematopoietic stem cell population of the liver as measured by the in vivo spleen nodule assay (CFU) declines with a halving time of about 48 hours. The stem cell population of the spleen grows exponentially with a doubling time of about 17 hours. In vitro incubation with high specific activity ^{3}H-TdR and sedimentation velocity studies indicate that CFU in neonatal liver and spleen are in cell cycle and that the distribution of the two CFU populations among the various stages of the cell cycle is similar in these two organs. It was also estimated by serial passage of single spleen colonies derived from neonatal liver and spleen CFU that both stem cell populations have a high self-renewal capacity. Thus, the decline in the neonatal liver stem cell population is not due to a lack of factors necessary to trigger CFU into cell cycle nor is the decline a function of the self-renewal capacity of the stem cell population itself. It is concluded that the factor(s) which triggers neonatal CFU into cell cycle is different from the factor(s) which regulates the size of the neonatal liver CFU population.

I. INTRODUCTION

During ontogeny of the mouse, hematopoietic stem cells or colony forming units (CFU) are found sequentially in the yolk sac, liver, spleen and marrow.[1,12,20] During embryonic and fetal development the habitat of CFU is primarily the liver; while in adult mice, CFU are found almost exclusively in the marrow and spleen. Several observations have raised the possibility that the mechanisms regulating fetal stem cell proliferation and population size differ from those which regulate adult stem cells. In adult marrow, CFU are found in a nonproliferating or G_o state; while in fetal liver, CFU have only been detected in cell cycle.[2] Furthermore, fetal stem cells are believed to generate an erythroid progenitor cell population that differentiates in the absence of erythropoietin; while in the adult, erythroid maturation is dependent on the presence of erythropoietin.[3,9,22] In general, fetal CFU appear somewhat refractory to adult CFU proliferation control mechanisms. When CFU from fetal liver and adult marrow are transplanted and allowed to grow simultaneously in the same lethally irradiated recipient, CFU of fetal origin appear to sustain proliferation for a longer period than marrow CFU and eventually outgrow the latter.[14]

There is some evidence that the rate of CFU turnover and the sizes of the various CFU populations are humorally regulated in adult mice.[4,5,7] However, recent work indicates that stem cell turnover and population size might be controlled locally by a short-range feedback system such as cell-cell interactions or diffusible gradients of short-lived molecules.[6,11,16,19] Whether there is a similar type of local control of CFU proliferation and population size in fetal liver is not known.

Nonetheless, if different mechanisms regulate CFU proliferation and population size in fetal and adult mice then at some time during the development of the hematopoietic system a transition from the former to the latter must occur. During neonatal development the habitat of CFU shifts from the liver to the spleen and marrow.[1,12] Whether this shift is accompanied by or is a result of transitions from fetal to adult types of mechanisms regulating CFU turnover and population size control is not known. Therefore, the purpose of this paper is to report some observations of the behavior of CFU in neonatal spleen as opposed to neonatal liver both in respect to CFU population changes and state of CFU turnover.

II. MATERIALS AND METHODS

Animals. Accurately dated, B6D2F_1/J fetuses and neonatal mice were obtained by ordering timed pregnant females from the Jackson Laboratory (C57BL/6J female x CBA/2J male). Hosts for the in vivo CFU assay were 8- to 12-week-old B6D2F_1/J mice randomized with respect to sex.

Preparation and counting of spleen and liver cells. Pregnant females at various stages of gestation were euthanatized by cervical dislocation and their fetuses dissected free of the uterus. The livers and spleens of these fetuses were removed and cell suspensions were prepared by pressing the livers or spleens against a stainless steel sieve. The cells were collected in an ice-cold balanced salt solution. Neonatal spleen and liver were prepared in a similar fashion. Cell clumps were eliminated from the cell suspensions by moving the suspensions up and down several times through a 26-gauge needle. Nucleated cell concentrations were determined on a Coulter counter Model F following lysis of red blood cells with Zap Isoton (Coulter Diagnostics).

Spleen colony assay (CFU). Cells capable of forming colonies in the spleens of heavily irradiated mice were measured using the technique of Till and McCulloch.[23] Recipient mice were bilaterally irradiated at a distance of 1.82 meters from the AFRRI ^{60}Co source, with a dose rate of 150 rads/min to a total absorbed dose of 900 rads. After exposure and tail vein injection of various cell suspensions, mice were housed three per cage and were allowed acidified water (final pH 2.5) and regular rodent feed ad libitum. Spleens were removed 8 days later and fixed in Bouin's solution. The number of nodules per spleen was then determined by an independent investigator.

In vitro incubation of CFU with high specific activity tritiated thymidine. The in vitro ^{3}H-thymidine suicide technique was carried out using a modification of the method of Becker et al.[2] Cells were suspended in ice-cold Fisher's medium for leukemic cells of mice at a concentration of 1.3×10^{7} to 8.0×10^{7} nucleated cells/ml. One milliliter of the cell suspension was then added to 2.0 ml of isotonic Fisher's medium for leukemic cells of mice containing various concentrations of high specific activity tritiated thymidine (18-20 Ci/M) purchased from Schwarz-Mann. The final solution in 50 ml polycarbonate centrifuge tubes was incubated at 37^{o}C for 30 minutes under 5 percent CO_2. The control cell suspension was identical to the experimental, with the exception of the substitution of cold thymidine for the tritiated thymidine. The reaction was stopped by adding 20 to 40 ml of ice-cold CMRL 1066 cell suspension medium. The cell concentration was then determined and an appropriate aliquot (0.1 to 0.3 ml) was injected I.V. into each of 12-15 irradiated recipient mice.

Velocity sedimentation studies. Cell suspensions were fractionated, by velocity sedimentation, according to the method of Miller and Phillips.[15] The fetal calf serum in phosphate buffered saline gradient was the same as that described for sheer step linear 15-30 percent gradient. The cell load in the starting band (20 ml) was 3.3×10^6 total cells/ml. All sedimentation runs were for 3.5 hours at 6°C. The total gradient was 400 ml and cells were collected in 20-ml fractions. The diameter of the glass sedimentation chamber was 10.37 cm. The number of cells in each fraction was measured with the aid of an electronic Coulter counter Model F.

Serial passage studies. An individual colony design[12,21] was used to study the self-renewal capacity of stem cells from 2-day-old neonatal liver and spleen. Four different cell concentrations from the same cell suspension were injected into four sets of irradiated recipients. After 13 days growth, the spleens were removed and nodules counted. If a linear relationship existed between cells injected and nodules per spleen and the resulting line could be extrapolated through zero then the individual spleen nodules were excised, pooled, and made into cell suspensions. Fractions of the cell suspensions were then injected into two groups of irradiated recipients and the whole procedure repeated until the relationship between number of cells injected and number of colonies formed was no longer linear.

Statistical tests. Two-tailed Student's "t" test was used for all statistical determinations.

III. RESULTS

Changes in cell number and CFU. The number of nucleated cells and CFU in the liver and spleen of fetal and neonatal $B6D2F_1/J$ mice is presented in Tables I and II.

Table I. Liver Cellularity and CFU Number

Days after vaginal plug	Liver cellularity (x 10^{-6})	CFU/10^5 cells	CFU/liver
15	14+	7.3	1,020
16	25	6.8	1,704
17	38	4.1	1,554
18	30	6.1	1,816
19	27.7 ± 3.7‡	8.8 ± 0.5	2,438 ± 202
20*	11.6 ± 2.6	6.7 ± 1.6	708 ± 20
21	15.5 ± 1.5	4.9 ± 0.4	763 ± 146
22	11.9 ± .7	3.4 ± 0.1	413 ± 31
23	25.9 ± 7.0	4.1 ± 0.9	603 ± 511
26	11	2.4	274

* Day 20 is the day of birth
+ One determination on 5 to 12 animals
‡ Mean ± SEM of two to three determinations performed on 5 to 12 animals

Table II. Splenic Cellularity and CFU Number

Days after vaginal plug	Splenic cellularity (x 10^{-6})	CFU/10^5 cells	CFU/spleen
19	.9 ± .2†	9.2 ± .9	86 ± 21
20*	1.8 ± .2	18.9 ± 7.2	268 ± 78
21	2.1 ± .6	29.5 ± 0.5	532 ± 68
22	5.4 ± 2.8	30.5 ± 16.0	952 ± 154
23	12.0 ± 5.1	15.4 ± 1.3	1754 ± 638
26	16.8 ± 1.8	12.9 ± 6.3	2065 ± 835
10 weeks old	135‡	1.9	2565

* Day 20 is the day of birth
† Mean ± SEM of two to three determinations performed on 5 to 12 animals
‡ One determination on five animals

The day of birth, usually the 20th day of gestation, was considered the zero day of neonatal life. From the 19th day of gestation to the 6th day of neonatal life the CFU population of the fetal and neonatal liver declines with a halving time of approximately

48 hours. In the fetal and neonatal spleen, CFU exponential growth is observed with a doubling time of approximately 17 hours. Also, from the 19th day of gestation to the 6th day of neonatal life the nucleated cell population of the liver remains nearly constant while in the spleen, for the same period of time, the nucleated cell population increases nineteenfold.

Proliferative state of CFU found in fetal and neonatal spleen and liver. Cell suspensions from 2-day-old neonatal liver and spleen were incubated in vitro with various concentrations of high specific activity tritiated thymidine (18-20 Ci/M) for 30 minutes at 37^{o}C. This treatment selectively kills cells in the S-phase of the cell cycle. The results graphed in Figure 1 indicate that for ^{3}H-TdR activities above 135 μCi/ml, the fractional survival of CFU from both 2-day neonatal liver and spleen was reduced to a constant value of 0.62. Thus, approximately 38 percent of the CFU found in 2-day-old neonatal liver and spleen are in the S-phase of cell cycle.

The state of proliferation of CFU found in liver and spleen was also determined between the 0 and 3rd day of neonatal life. During this period it was found that the fraction of the CFU population in the S-phase of the cell cycle from these two organs was for the most part between 25 to 50 percent (Tables III and IV). In contrast, only 4 percent of the CFU population in adult marrow and only 20 percent of the CFU population in adult spleen were found to be in the S-phase of cell cycle.

Sedimentation velocities of CFU found in fetal and neonatal liver, and neonatal and adult spleen. The sedimentation velocity profiles of nucleated cells and CFU from 15-day fetal liver, 2-day neonatal liver and spleen, and adult spleen are shown in Figure 2. The sedimentation velocities and half peak width for 15-day fetal liver CFU

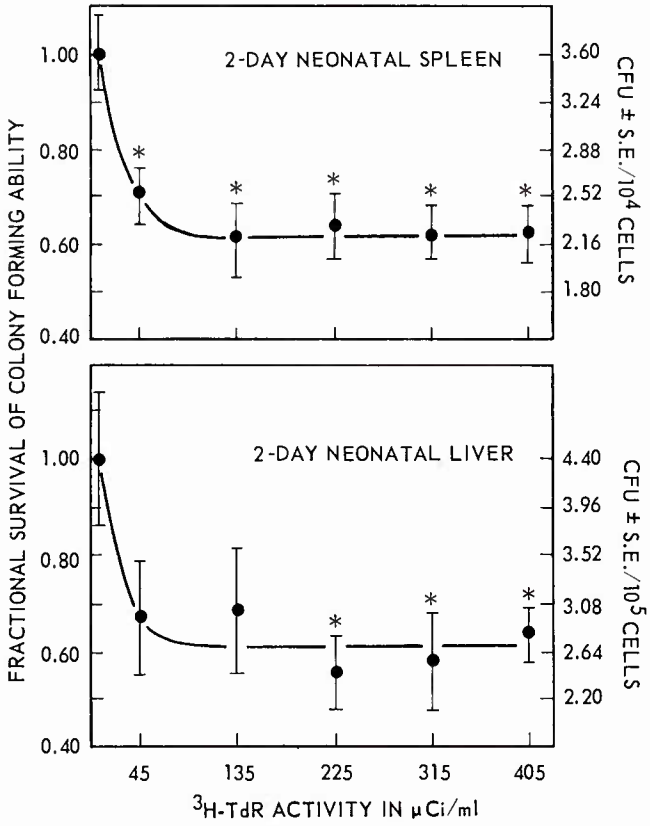

Figure 1. Effect of 30-minute exposure in vitro to various concentrations of ^{3}H-thymidine on the CFU content of 2-day neonatal spleen and liver. Livers and spleens were taken from the same animals. Standard errors of the mean colony counts are indicated. The specific activity of the ^{3}H-thymidine was 18 Ci/mole. Significantly different than control values at the $P<.05$ level (*).

were 5.40 mm/h and Δ2.02 mm/h; for 2-day neonatal liver, 5.75 mm/h and Δ2.70 mm/h; for 2-day neonatal spleen, 5.70 mm/h and Δ3.55 mm/h; and for adult spleen, 4.04 mm/h and Δ1.61 mm/h.

Because cells immediately before mitosis have approximately twice the volume of cells immediately after mitosis the relationship between the sedimentation velocities

Table III. Effect of ^{3}H-TdR on the Colony Forming Ability of Mouse Fetal and Neonatal Liver Cells

Days after vaginal plug	Cells injected ($\times 10^{-4}$)†	^{3}H-TdR (μCi/ml)	Nodules/ spleen‡	Percent survival	P
19	10.6	0	10.00 ± 1.34	65	<.050
	10.6	166	6.50 ± 0.84		
20*	14.2	0	11.90 ± 1.25	64	<.050
	14.2	166	7.66 ± 1.69		
20	7.7	0	5.30 ± 0.53	75	<.200
	7.7	166	4.00 ± 0.65		
20	30.0	0	13.88 ± 2.96	57	<.100
	30.0	166	7.88 ± 1.33		
21	19.4	0	9.18 ± 1.10	94	>.500
	19.4	166	8.68 ± 1.05		
22	10.1	0	4.39 ± 0.70	56	<.025
	10.1	225	2.41 ± 0.37		
23	20.0	0	8.66 ± 1.42	55	<.025
	20.0	166	4.80 ± 0.75		

* Day 20 is the day of birth
† In each experiment 5 to 12 livers were made into cell suspensions and pooled
‡ Mean ± SEM

of cells in the G_1 and G_2 state is given by the equation $V_{G_2} = 1.59\ V_{G_1}$.[15] It has been demonstrated that the adult CFU are in G_o or a long G_1 state.[2] Therefore, by substituting the sedimentation velocity value of adult CFU for V_{G_1} in the above equation, it is possible to calculate the sedimentation velocity for CFU in G_2. These values for CFU in the G_1 and G_2 state are shown by the arrows in Figure 2. Thus, the difference in diameter of CFU from fetal and neonatal mice as compared with adult mice might be indicative of the state of proliferation of neonatal and adult CFU.

Table IV. Effect of ^{3}H-TdR on the Colony Forming Ability of Mouse Neonatal Spleen Cells

Days after vaginal plug	Cells injected ($\times 10^{-4}$)†	^{3}H-TdR (μCi/ml)	Nodules/ spleen‡	Percent survival	P
20*	6.6	0	12.21 ± 1.54	50	<.005
	6.6	166	6.20 ± 0.87		
21	6.8	0	21.27 ± 1.72	53	<.005
	6.8	166	11.33 ± 0.54		
22	1.0	0	3.61 ± 0.30	65	<.005
	1.0	225	2.35 ± 0.23		
22	13.0	0	16.57 ± 3.61	58	<.100
	13.0	166	9.66 ± 7.44		
23	13.5	0	20.75 ± 1.94	72	<.200
	13.5	166	15.00 ± 2.27		
23	9.3	0	13.66 ± 1.97	76	<.200
	9.3	166	10.37 ± 1.04		

* Day 20 is the day of birth
† In each experiment 5 to 12 spleens were made into cell suspensions and pooled
‡ Mean ± SEM

Self-renewal capacity of CFU from neonatal liver and spleen. Using the technique of serial passage of pooled spleen nodules, the self-renewal capacity of CFU from 2-day-old neonatal liver and spleen was estimated. As seen in Table V, nodules derived from neonatal liver CFU contain approximately 58.2-63 CFU per nodule at the 13th day of colony growth while nodules derived from splenic CFU contain between 18.1-65.4 CFU per nodule. Curtailment of CFU self-replication from both liver and spleen began during the 2nd passage. Thus, CFU from neonatal liver and spleen appear to possess a high self-renewal capacity.

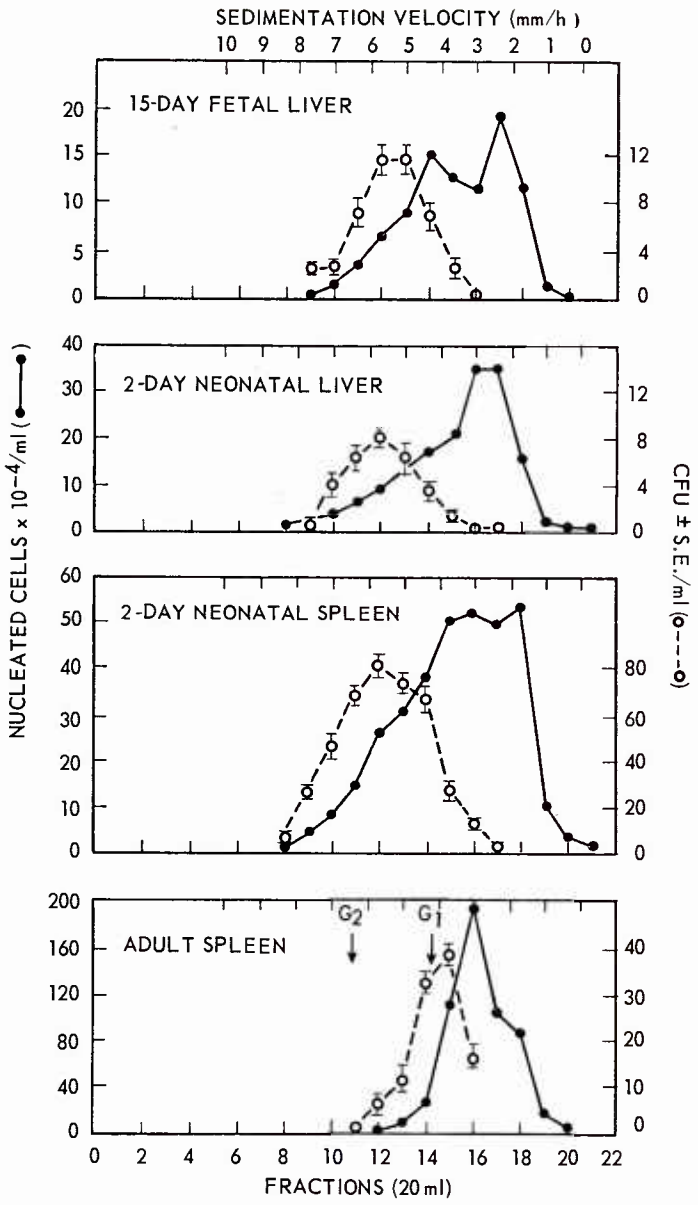

Figure 2. Sedimentation velocity profiles for total nucleated cells and CFU from fetal liver, neonatal liver, neonatal spleen and adult spleen. The CFU are uncorrected for seeding efficiency. Standard errors of colony counts are indicated. For an explanation of G_1 and G_2 see text.

Table V. Serial Passage of Nodules Derived from 2-Day Liver and Spleen CFU

Primary passage				
EXP	Organ*	Organs pooled	CFU/10^5 cells†	Cells/nodule
1	Spleen	5	12.5 ± 4.1	--
	Liver	5	6.2 ± 2.5	--
2	Spleen	8	10.2 ± 1.3	1.72×10^7
	Liver	8	2.5 ± 0.4	1.88×10^7
1^o passage‡				
EXP	Organ	Nodules pooled	CFU/nodule	Cells/nodule
1	Spleen	9	65.4 ± 7.5	--
	Liver	11	63.0 ± 7.0	--
2	Spleen	28	18.1 ± 2.5	1.35×10^7
	Liver	26	58.2 ± 5.4	1.85×10^7
2^o passage‡				
EXP	Organ	Nodules pooled	CFU/nodule	Cells/nodule
2	Spleen	24	11.6 ± 3.3	0.55×10^7
	Liver	19	16.4 ± 3.1	0.80×10^7

* Livers and spleens were taken from the same animals
† Mean ± S. E.
‡ Thirteen-day passage

IV. DISCUSSION

The important conclusion to be drawn from the data reported is that, during the neonatal period, 40 percent of the CFU population in neonatal liver and spleen are in the S-phase of cell cycle. The fractional distribution of the remaining CFU in neonatal spleen and liver between G_1, G_2 and mitosis is not known. However, comparison

of the sedimentation velocity profiles of the CFU population from neonatal spleen and liver suggests that the distribution of CFU among the various stages of the cell cycle is similar in these two organs. Therefore, the majority of CFU in neonatal spleen and liver are in cell cycle; and, if it is assumed that the durations of S and G_2 are characteristic of particular cell types[17] and that major variations in G_1 can be detected by velocity sedimentation, then it can also be assumed that CFU in both neonatal spleen and liver progress from one mitosis to the next at nearly the same rate.

A possible explanation for what appear to be similar states of CFU turnover in neonatal spleen and liver would be that CFU in both organs are triggered into cell cycle indirectly by the same circulating factors[8] which are present at concentrations ensuring maximum CFU turnover in the two organs. An alternative explanation would be that the G_o or prolonged G_1 state in which adult CFU are found[2,10] is a result of a negative short-range feedback system,[16,19] which is inoperative or undeveloped in neonatal animals. In the absence of a negative short-range feedback system, CFU would be found in a nonrepressed state and might enter cell cycle at a predetermined rate regardless of the organ in which they are found.

In view of the data that (1) neonatal liver CFU possess the capacity for extensive self-renewal, (2) the neonatal liver supports CFU in a state of cell cycle and (3) the decline of the liver CFU population takes place during a period of rapid CFU growth in the spleen, it is concluded that the factors governing the rate of CFU turnover in neonatal liver differ from the factors which regulate the size of the liver CFU population. Thus, it appears that the neonatal liver does not support CFU self-renewal at a rate sufficient to maintain the CFU population at a size comparable to that found in

fetal liver. However, neonatal liver supports CFU turnover and, presumably, CFU differentiation.

The concept of different factors regulating CFU turnover and population size finds application in not only explaining the decline of the neonatal liver CFU population but, also, in the development of the CFU population in neonatal spleen. Namely, within 6 to 10 days of neonatal life the splenic CFU population has stabilized at a size comparable to that found in adult mice[12,18] (Table II). Yet, neonatal splenic CFU generate erythrocytic and granulocytic progenitor cell populations that differ from those found in adult spleen. (Metcalf and Stevens[13] reported that neonatal spleen granulocytic progenitor cells cultured in vitro generate different cluster colony ratios than those of adult origin; while Cole et al.[3] reported that neonatal erythropoiesis is insensitive to erythropoietin.) Thus, the neonatal spleen might be described as a "hybrid" in terms of the CFU control mechanisms present. Proliferation and differentiation are apparently regulated in part by mechanisms which differ from those found in adult spleen while the size of the CFU population might be determined by factors common to both neonatal and adult spleen.

In summary, using the techniques of in vitro incubation with high specific activity tritiated thymidine and velocity sedimentation, it was possible to demonstrate that the bulk of CFU found in both neonatal liver and spleen are in cell cycle. Because the liver CFU population is in a state of exponential decline while the splenic CFU population is growing exponentially, it is concluded that in fetal and neonatal liver the size of the CFU population is controlled by the short-range factor(s) which is distinct from the factor(s) necessary for maintaining neonatal liver CFU in a state of cell cycle.

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13. ABSTRACT

Following birth the hematopoietic stem cell population of the liver as measured by the in vivo spleen nodule assay (CFU) declines with a halving time of about 48 hours. The stem cell population of the spleen grows exponentially with a doubling time of about 17 hours. In vitro incubation with high specific activity ^{3}H-TdR and sedimentation velocity studies indicate that CFU in neonatal liver and spleen are in cell cycle and that the distribution of the two CFU populations among the various stages of the cell cycle is similar in these two organs. It was also estimated by serial passage of single spleen colonies derived from neonatal liver and spleen CFU that both stem cell populations have a high self-renewal capacity. Thus, the decline in the neonatal liver stem cell population is not due to a lack of factors necessary to trigger CFU into cell cycle nor is the decline a function of the self-renewal capacity of the stem cell population itself. It is concluded that the factor(s) which triggers neonatal CFU into cell cycle is different from the factor(s) which regulates the size of the neonatal liver CFU population.

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