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Induction of Splenic Granulopoiesis *in Vitro*[1] (39436)

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The adult murine spleen is composed predominantly of lymphoid cells with some macrophages and granulocytic elements also present. Although the spleen accounts for only a small fraction of the blood cell production of the adult animal, it is an important hematopoietic organ containing both pluripotent stem cells (CFU-S) and committed granulopoietic progenitors capable of colony formation in soft-gel culture (CFU-C) (1-3). The concentration of CFU-C in the normal spleen is low relative to that in the bone marrow, and unlike the bone marrow CFU-C many splenic granulopoietic progenitors appear to be resting and not in active cycle (2, 4). Under conditions of increased demand for blood cells, the spleen of the adult, like that of the fetus, can become an actively hematopoietic organ. Although erythropoietin is clearly important in initiating splenic erythropoiesis, the signals for activation of splenic leukopoiesis are not clearly defined. In order to examine the characteristics of splenic granulopoiesis, we studied the generation of CFU-C and of mature granulocytes and mononuclear phagocytes in suspension cultures of spleen cells induced by colony-stimulating activity (CSA). In this system, CSA stimulation resulted in a marked proliferation of CFU-C and differentiation of granulopoietic progenitor cells.

Materials and methods. Young adult female DBA-2 mice (21-25 g) were used for all experiments. The spleen was removed aseptically and a single-cell suspension prepared by first mincing the tissue in culture medium, and then passing it through progressively smaller-bore needles until the cells passed easily through a No. 25 needle. Cell viability was greater than 99% as determined by trypan blue exclusion (0.4% dye over 4 min). McCoy's 5A medium containing 15% fetal calf serum and antibiotics was used throughout (5, 6).

Pregnant mouse uterus extract (PMU) was used as the source of colony-stimulating activity (CSA). The extract was prepared as described by Bradley *et al.* (7) and added to cultures at a concentration of 100 μl/ml. This concentration was previously shown to yield maximal colony formation in agar cultures of mouse bone marrow and spleen cells.

Spleen cells were cultured in liquid suspension with the Marbrook *in vitro* diffusion system previously described (6, 8). Cultures were initiated with 3×10^6 spleen cells in 1 ml of medium with or without the addition of 0.1 ml of PMU.

The suspension cultures were terminated at intervals up to 14 days for total viable cell counts (hemacytometer), and 200-cell differential counts were performed on Giemsa-stained cytocentrifuge preparations. Cultures were pulsed with 1 μCi of tritiated thymidine ([^{3}H]TdR) (sp act, 2 Ci/mmole) 60 min before harvest and autoradiographs prepared as previously described (6). A cell was considered labeled if its nucleus contained five or more grains above background. Labeling indices were expressed as the percentage of cells taking up the tracer and total labeled cells in culture calculated from the viable cell counts. At each period of harvest, a portion of the cultured cells was transferred to agar culture in order to assay the number of CFU-C present. Occasionally, there was spontaneous blastic transformation of lymphocytes *in vitro*. These cultures were discarded.

Agar cultures were prepared by the method of Pike and Robinson (5, 9). One-tenth milliliter of PMU was incorporated into an underlayer of 0.5% agar, and the cultured spleen cells were dispersed in a 0.3% agar overlayer. The number of cells

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plated was adjusted so that the number of colonies per plate ranged between 30 and 100. Duplicate agar cultures were incubated in a humidified environment of 7.5% CO_2 in air and colonies containing 50 cells or more enumerated after 7–10 days of culture. The total number of CFU-C per liquid culture was calculated by multiplying the plating efficiency in agar by the total viable cell count.

Results. The pattern of viable cell counts in liquid culture is shown in Fig. 1. There was a rapid fall in cell numbers until Day 4, both in control cultures and in those containing CSA. Thereafter, cell numbers increased prominently in cultures to which CSA had been added, reaching a peak (four times control) at 9 to 10 days and remaining substantially elevated above controls after 2 weeks *in vitro*. The increase in cell numbers

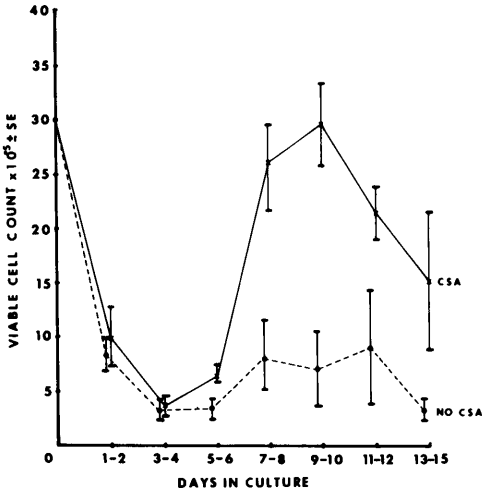

FIG. 1. Viable cell counts in liquid cultures of spleen cells. Data represent means and standard errors of seven experiments.

observed in CSA-stimulated cultures was due to cellular proliferation as reflected by [^{3}H]TdR labeling studies (Table I). Prior to culture, there was only modest cellular DNA synthetic activity as indicated by the mean labeling index of 2%. In CSA-stimulated cultures the labeling index rose to a mean high of 47% at 5–6 days and total labeled cells increased by approximately 10-fold after 1 week of culture. Control unstimulated cultures showed considerably less increase in total labeled cells.

Differential cell counts revealed an increase in immature cells in CSA-stimulated cultures at 5–7 days, followed by the appearance of mature granulocytes and macrophages (Table II). At Day 5, about two-thirds of the cells were undifferentiated blast cells (Fig. 2). These cells subsequently differentiated appropriately to morphologically normal mature granulocytes and macrophages (Fig. 3). The mature cells were phagocytic for heat-killed *Candida albicans* and demonstrated cytochemical reactions typical of granulocytes and macrophages (10).

Data on the generation of CFU-C are given in Table III. Unstimulated cultures showed a modest increase in CFU-C with time. In CSA-stimulated cultures there was a mean 70-fold increase in granulopoietic progenitors capable of colony formation in agar. The rise in numbers of CFU-C preceded the appearance of morphologically identifiable blast cells which in turn preceded the increase in mature leukocytes (Fig. 4). The peak of the CFU-C curve roughly paralleled that of blast cell numbers. The increase in labeling index coincided with the increase in blast cells and CFU-C (Table I, Fig. 4).

TABLE I. [^{3}H]THYMIDINE LABELING IN LIQUID CULTURES OF SPLEEN CELLS (SEVEN EXPERIMENTS)

	With CSA		Without CSA	
Days in culture	Mean labeling index (%)	Total cells labeled ($\times 10^{-3} \pm$ SE)	Mean labeling index (%)	Total cells labeled ($\times 10^{-3} \pm$ SE)
0	2	60 ± 1	2	60 ± 1
1-2	5	50 ± 4	3	20 ± 6
3-4	30	120 ± 22	9	27 ± 9
5-6	47	300 ± 34	18	58 ± 14
7-8	25	650 ± 78	19	152 ± 24
9-10	10	290 ± 106	4	28 ± 7
11-12	5	111 ± 21	4	36 ± 20
13-16	3	42 ± 11	3	9 ± 3

TABLE II. TOTAL DIFFERENTIAL CELL COUNTS × 10^{-3} ± SE (SEVEN EXPERIMENTS)

Days in culture	CSA	Immature cells	Mature granulocytes	Macrophages	Lymphoid cells
0		120	120	60	2700
5	+	448 ± 63	64 ± 9	38 ± 5	90 ± 13
	−	156 ± 41	14 ± 4	27 ± 7	143 ± 38
7–8	+	884 ± 136	854 ± 116	702 ± 108	160 ± 45
	−	304 ± 133	264 ± 99	152 ± 57	80 ± 35
9–10	+	510 ± 63	930 ± 114	1410 ± 174	150 ± 19
	−	91 ± 39	238 ± 102	322 ± 138	49 ± 21
11–12	+	88 ± 8	770 ± 84	1298 ± 142	44 ± 5
	−	9 ± 9	216 ± 120	648 ± 360	27 ± 15
13–15	+	120 ± 48	435 ± 174	945 ± 180	0
	−	0	26 ± 8	303 ± 92	0

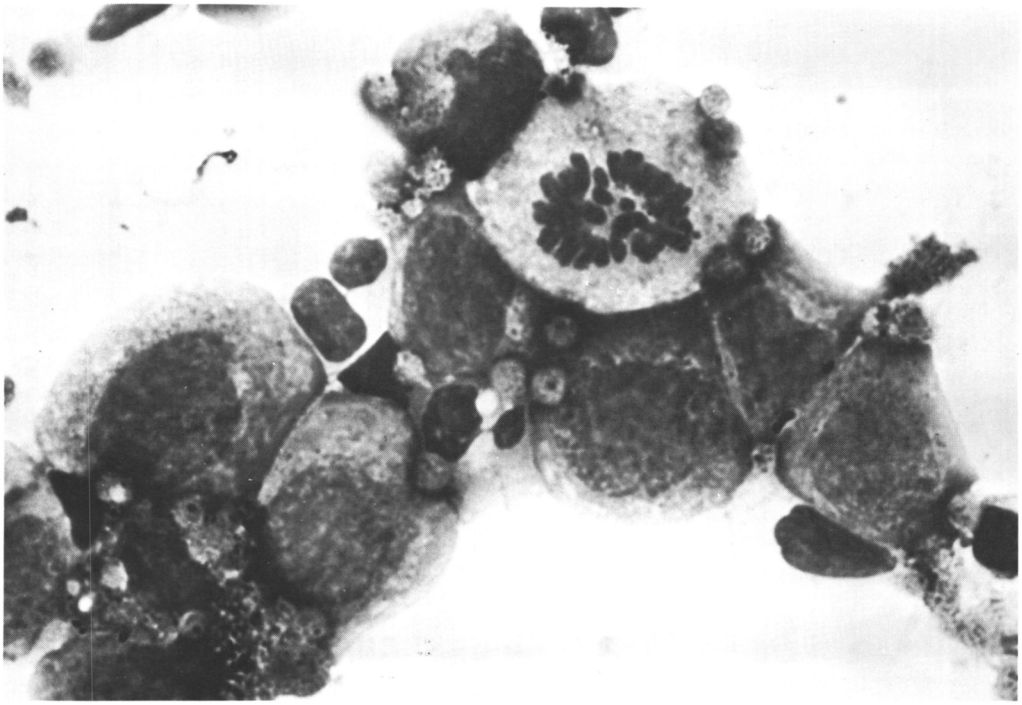

FIG. 2. Immature cells in culture at Day 5 (Giemsa stain).

Discussion. The adult spleen is generally regarded as predominantly a lymphoid organ composed of cells involved in immune reactions and as a reticuloendothelial organ whose mononuclear phagocytes are involved in the trapping and destruction of microorganisms and senescent blood cells. The spleen is also an important reservoir of hematopoietic activity. The hematopoietic potential of the spleen is usually expressed in fetal and perinatal life (1, 2) and during times of increased need for blood cells (hemorrhage or infection). In the adult mouse, the spleen contains pluripotent stem cells (CFU-S) (1, 3) as well as committed progenitors of erythroid cells (11) and granulocytes and monocytes. Committed granulopoietic stem cells are present in relatively low numbers and appear to be resting rather than in the proliferative cell cycle (2). In contrast, bone marrow contains a heterogeneous population of granulocytic precursors with concatenated proliferative and maturational activity.

The present studies demonstrate that splenic granulocytic and mononuclear phagocyte precursors can be induced by CSA *in vitro* to enter the active proliferative cycle, to generate additional committed stem cells (CFU-C), and to mature to nonproliferating

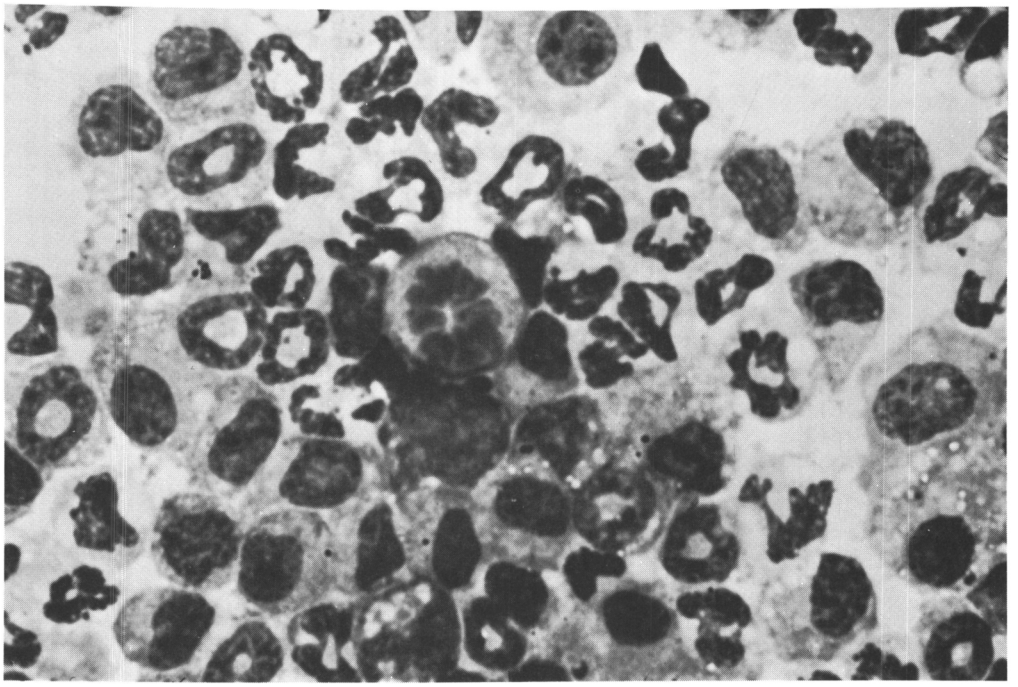

FIG. 3. Mature granulocytes and macrophages predominate in culture at Day 10 (Giemsa stain).

TABLE III. CFU-C IN LIQUID CULTURES OF NORMAL SPLEEN CELLS (FIVE EXPERIMENTS)

Time (days)	+CSA	−CSA
0	63 ± 13	63 ± 13
1-2	166 ± 32	70 ± 10
3-4	1272 ± 495	63 ± 2
5-6	3797 ± 1286	229 ± 68
7-10	4564 ± 707	913 ± 118
11-12	258 ± 117	197 ± 72

end cells. Cultures not containing CSA showed little proliferative activity and poor maintenance of cell viability. Under the influence of CSA, there was a rapid generation of CFU-C which resulted in a mean 70-fold increase over the number present at the initiation of culture. This increase in CFU-C was associated with a rise in [^{3}H]TdR labeling index from 2 to 47% after 5–6 days *in vitro*. Subsequently, viable cell counts increased and the labeling index fell with the appearance of mature granulocytes and macrophages. The very high labeling indices at Day 5 suggest that all or nearly all the proliferating cells are in cycle, representing a growth fraction near unity.

Various substances and environmental factors are known to stimulate DNA synthesis in the pluripotent stem cell (12, 13), and endotoxin specifically has been shown to cause a prominent increase in the number

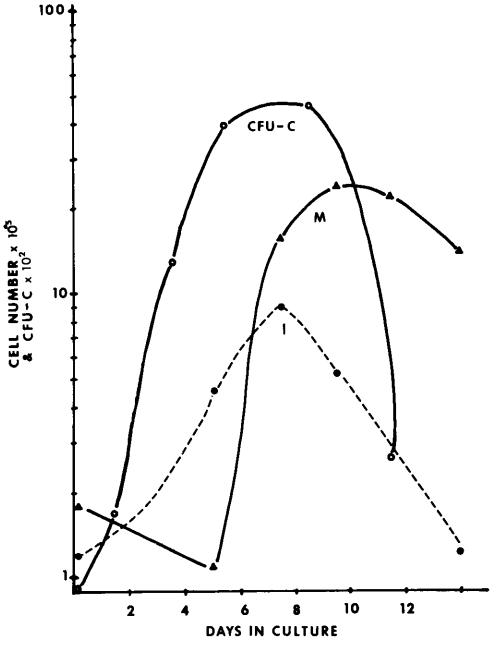

FIG. 4. Pattern of generation of CFU-C, immature cells (I) and mature (M) granulocytes and macrophages in CSA-stimulated cultures.

of such cells in the mouse spleen (14). The present observations are consistent with the hypothesis that CSA can be a physiologic inducer of committed granulopoietic stem cell proliferation in the spleen.

The orderly and uniform pattern of CFU-C generation, appearance of blast cells, and increased [^{3}H]TdR labeling index suggest that there is a synchronization of sequential proliferative and maturational activity. The liquid spleen cell culture system may thus provide a relatively simple means for studying the phenomena associated with stem cell activation and differentiation *in vitro*.

Summary. Murine spleen cells were cultured *in vitro* to study the induction of committed granulopoietic stem cell (CFU-C) proliferation and maturation. Marbrook-type diffusion cultures were established with and without the addition of colony-stimulating activity (CSA) and harvested at intervals up to 14 days for viable and differential cell counts, [^{3}H]TdR autoradiography, and quantitation of CFU-C by the agar plate method. Without CSA there was poor cell viability and little proliferative capacity. In CSA-stimulated cultures there was a prominent rise in viable cell counts and [^{3}H]TdR labeling indices rose from a mean of 2% at 0 time to 47% after 5 days *in vitro*. CFU-C increased by 70-fold in these cultures. Peak numbers of CFU-C, immature cells, and [^{3}H]TdR-labeled cells occurred at about 7 days. Thereafter, mature granulocytes and macrophages predominated in culture. Because the liquid spleen cell culture system begins in a resting state and undergoes a wave of proliferative activity in response to CSA, it can provide a useful model system for studying phenomena associated with stem cell activation and differentiation *in vitro*.

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