Stem Cell Stimulatory Properties *in Vitro* of an Agar Colony-Stimulating Factor (35322)

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The development of the nutrient agar technique for assaying that class of hemopoietic progenitor cells which when suitably stimulated give rise to colonies composed of granulocytes and macrophage cells (1, 2) has introduced new possibilities for the study of granulopoiesis. We (3, 4) have demonstrated that the agar colony system monitors the committed myeloid stem cell compartment; and more recently we (5, 6) have reported the presence of a factor in the serum of irradiated leg shielded mice (ILS) or whole body irradiated mice (WBI) with colony-stimulating activity. That this factor might be an important humoral regulator of granulopoiesis is suggested by the observations that its level in irradiated mice correlates in time and magnitude with the neutropenia that occurs after irradiation (5), with the granulocytic hyperplasia that occurs in response to neutropenia (5, 7) and with the enlargement of the committed myeloid stem cell compartment that also develops in response to neutropenia (6).

The *in vitro* effect of active serum on the colony-forming cell (CFC) essentially involves stimulation of this cell to differentiate along the granulocytic pathway. In this paper we present evidence that serum from WBI mice has an additional effect on the CFC compartment when the latter is studied in a bone marrow suspension maintained in a short-term tissue culture.

Materials and Methods. The medium used for these experiments was essentially as described by Metcalf and Foster (8). It is termed E2020 and is supplemented with trypticase soy broth (3 g/100 ml) 20% (v/v), DEAE-dextran in a final concentration 75 μg/ml, and L-asparagine, final concentration 20 μg/ml. To produce E1010 the above medium is mixed in equal proportions with sterile distilled water.

To obtain WBI serum, exbreeder mice of the CF_{1s} strain were irradiated (total dose 800 R, 62 R/min, 250 kV, 12 mA with 1.0 mm Cu and 1.0 mm Al filtration) and on the fifth postirradiation day the mice were bled by cardiac puncture. The blood was allowed to clot and the clot to separate at 37°. The serum was then sterilized by Millipore filtration through a 0.45-μ membrane and stored at —40° prior to use.

Bone marrow cell suspensions were prepared as previously described (4). To obtain enough cells for each experiment it was usually necessary to expel the bone marrow cells from the tibiae of at least 20 mice. These cells were pooled as a suspension in E1010. 1.7-ml aliquots of this cell suspension were mixed in 35 × 10-mm plastic petri dishes with 0.3 ml of WBI or normal serum. In one experiment, 0.1-, 0.3-, and 0.5-ml volumes of WBI serum were added to the bone marrow cell suspension. The plastic petri dishes containing the above mixtures were incubated at 37° in a humidified atmosphere continuously flushed with 7.5% CO_2 in air.

For the assay of *in vitro* colony-forming cells (CFC) or spleen colony-forming units (CFU) in the incubation mixtures, representative dishes were removed from the incubator at various time intervals after the commencement of incubation, and the cell suspensions in the dishes evenly dispersed by gentle pipetting with a Pasteur pipette. Nucleated cells were counted in these resultant suspensions and aliquots of the suspensions were plated in E2020-agar. Conditioned medium was used as a colony-stimulating factor and was prepared as previously described (4). Each suspension was usually assayed at

TABLE I. The Incubation of 1.7 ml of CF_{1s} Bone Marrow Cell Suspension and 0.3 ml of serum from Whole Body Irradiated Mice (WBI) or 0.3 ml of Normal Serum (NS) (CFC/plate).

Time of incubation (hr)	Expt. no.: 117		124		137		141	
	WBI	NS	WBI	NS	WBI	NS	WBI	NS
Original	9020 ± 740	9020 ± 740	7660 ± 800	7660 ± 800	14,100 ± 850	14,100 ± 850	12,200 ± 640	12,200 ± 640
0.25			6500 ± 350	7750 ± 440				
2	7030 ± 230	6930 ± 150						
4	8270 ± 360	7820 ± 380	8260 ± 690	7700 ± 390	11,100 ± 340	12,600 ± 450	12,480 ± 400	13,370 ± 450
10	9350 ± 350	8040 ± 320						
24	8400 ± 480	8600 ± 250	7300 ± 120	6370 ± 100	12,790 ± 320	8800 ± 490	11,440 ± 470	8320 ± 310
48	9310 ± 210	4200 ± 280	9890 ± 450	5100 ± 320	15,120 ± 270	6940 ± 270	12,990 ± 350	3990 ± 310
72			6140 ± 180	3840 ± 90	11,390 ± 260	1730 ± 300	7400 ± 490	2130 ± 190
96							2730 ± 80	210 ± 180
125			4920 ± 140	1330 ± 20				
150			890 ± 70	190 ± 30				

two cell dose levels, 0.5 and 1.0 × 10^5 cells/plate, and 5 plates were used for each dose level. Aliquots from the same suspensions were transplanted into irradiated syngeneic hosts (total dose 800 R) and the CFU were assayed as previously described (9). The conditions of incubation for the nutrient agar plates were as described above. Both agar and spleen colonies were scored at day 8 by two observers.

The total number of CFC or CFU were derived from the number of agar or spleen colonies produced by a known aliquot of cells from the incubation mixture and from the total numbers of cells in the incubation mixture.

Results. The effect of 0.3 ml of WBI or normal serum was studied in 4 experiments and the results are shown in Table I. In 1 of the 4 the effect of various doses of WBI serum was also studied and the results are shown in Fig. 1. In each of the 4 experiments the total number of CFC per plate remained at or near its original value for at least 48 hr in the presence of WBI serum; thereafter the number gradually began to decline. When the marrow was incubated with normal serum, however, the number of CFC per plate had usually fallen to some 50% of its

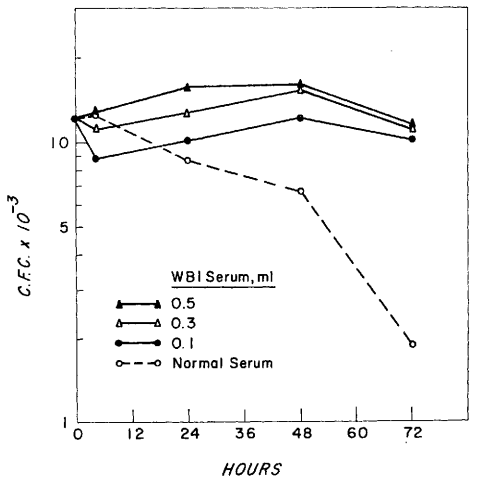

FIG. 1. The total number of CFC in a short-term tissue culture of normal murine bone marrow incubated in the presence of WBI serum at various doses (0.1, 0.3, and 0.5 ml/plate) and normal serum (0.3 ml/plate) are plotted against time of incubation.

TABLE II. The Incubation of 1.7 ml of CF_{18} Bone Marrow Cell Suspension and 0.3 ml of Serum from Whole Body Irradiated Mice (WBI) or 0.3 ml of Normal Serum (NS) (CFU/plate).

Time of incubation (hr)	Expt. no.: 137		141	
	WBI	NS	WBI	NS
4	2560 ± 200	2570 ± 430	3560 ± 620	3260 ± 460
24	1100 ± 200	1100 ± 150	940 ± 150	450 ± 60
48	690 ± 120	100 ± 20	770 ± 140	110 ± 80
72	100 ± 20	10 ± 1	280 ± 40	80 ± 40

original value by 48 hr; thereafter their number further decreased and was always at a much lower level than the corresponding value obtained with WBI serum. The effect of WBI serum appeared to be dose related, as shown in Fig. 1.

The total numbers of CFU per plate in the presence of WBI serum and normal serum at various times during the incubation process are shown in Table II. With continuing incubation, the total numbers of CFU per plate were higher at any point in time in the presence of WBI serum than with normal serum. The CFU began to decrease much earlier than did the CFC but the decline in CFU was more rapid in the presence of normal than WBI serum.

Discussion. One may only speculate as to the mechanisms responsible for these findings. The net numbers of CFC or CFU in the incubation mixture at any point in time reflect the birth rate as compared with the death rate. In the case of the latter, actual death of cells of efflux into a more differentiated compartment are included. The net birth rate within a compartment will reflect influx from a precursor compartment together with new cell production as the result of mitotic activity. Normal serum from this strain of mice contains very little myeloid colony-stimulating ability as judged by the soft agar technique (5). From this one may conclude that with normal serum the decrease in the CFC in the short-term cultures described herein was not importantly affected by differentiation or efflux from this compartment but rather reflected predominantly cell death. The more rapid decline in CFU in the presence of normal serum may have reflected differentiation in an effort to maintain the more differentiated CFC compartment, although it seems likely that cell death also contributed importantly to the decline.

WBI serum in contrast to normal serum, will effect differentiation of committed cells to form myeloid colonies. Hence efflux from this compartment in the short-term cultures may have significantly affected the resulting numbers of CFU. The total numbers of CFC, however, were maintained over a period of 48 hr. The evidence that WBI serum contains a specific regulator of granulopoiesis makes it unlikely that this CFC-maintaining effect is nonspecific. A specific effect could be due to increased levels of a stimulator or decreased levels of an inhibitor but the fact that the differentiating effect of WBI serum is probably due to a stimulator (5) suggests that the CFC-maintaining effect is also. The observations that CFC proliferate more rapidly in irradiated animals (6, 10) in which the serum level of colony-stimulating activity is high, suggest that the action of WBI serum in the present study was to stimulate mitotic activity within the CFC compartment. Whether this was a direct effect or resulted from differentiation of cells by the serum factor which in turn triggered remaining cells to enter cycle awaits further studies. Perhaps there are two substances in WBI serum, one a differentiating and the other a mitotic stimulant. On the other hand WBI serum may contain a single substance influencing both proliferation and differentiation of CFC in a manner analogous to the influence of erythropoietin on erythropoietin-sensitive cells (11, 12). The eventual decline in CFC in the WBI incubation mixtures may have resulted from the consumption or inactivation of the stimulating factor by these cells. This notion receives some support from studies in which we have observed that WBI

serum loses its colony-stimulating activity after short-term incubation with high concentrations of bone marrow cells (13). The slower decline of CFU in the presence of WBI serum appears to be better explained by a lower rate of efflux into the CFC compartment rather than a stimulatory property of WBI serum on the CFU. The latter cannot be excluded but the maintenance of the more differentiated CFC would appear to result in a lower demand. The eventual decline of CFU is thought to represent primarily death of cells.

Although there was a dose relationship in respect to the CFC and WBI serum the magnitude was not as great as one might have anticipated. This may reflect a higher rate of differentiation in the presence of more concentrated serum and as a result a more modest increase in the numbers of CFC.

Summary. Bone marrow was incubated over a short period *in vitro* in the presence of WBI and normal serum. The number of cells capable of forming colonies in soft agar was maintained over a 48-hr period in the presence of WBI but not normal serum. Furthermore the decline in transplantable CFU was slower in the presence of WBI serum. Possible mechanisms for these results are discussed although firm conclusions cannot be drawn.

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