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Cell Interactions in the Bone Marrow Microenvironment: Role of Endogenous Colony-Stimulating Activity

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Adherent stromal cells from mouse bone marrow inhibited the formation of granulocyte/monocyte (G/M) colonies induced in vitro by colony-stimulating factor (CSF). This inhibition occurred both when crude conditioned media obtained from various sources were used to induce colony formation or when a pure CSF preparation from mouse lung origin was tested. The inhibition did not appear to be toxic in nature since despite the lack of colony formation, progenitor CFU-C proliferated in the presence of stromal cells. Medium conditioned by adherent stromal cells was devoid of inhibitory activity when incorporated into the culture medium used for G/M colony formation, indicating that the inhibitory activity may not be present in a soluble form. Inhibitors of prostaglandins did not affect G/M colony formation. In contrast, D-glucose and a number of other free monosaccharides but not pyruvate lactate or glycerol induced formation fo myeloid colonies in the presence of stromal cells. This did not require addition of exogenous CSF. Released factors concentrated from serum-free medium conditioned by stromal cells exhibited colony-stimulating activity provided that the medium contained a high glucose concentration during incubation. It is proposed that stromal cells produce a resident CSF that, in contrast to exogenous CSF species, is capable of inducing myelopoiesis within the bone marrow stroma.

Key words: myeloid progenitor cells, bone marrow, stroma, monosaccharides, colony-stimulating factor

Early observations by Pluznik and Sachs [1] and Bradley and Metcalf [2] and extended studies that followed these observations (reviewed by Burgess and Metcalf [3]), supported the notion that colony-stimulating factor (CSF) is a regulator of myelopoiesis. Clonal proliferation of myeloid cells in vitro is indeed strictly dependent upon this factor. Nevertheless, progenitor cells tend to differentiate terminally

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in culture [4]. It is unlikely that this represents the course of events taking place in vivo. The culture system developed by Dexter et al [5] allow long-term hematopoiesis. In the presence of adherent stromal cells from mouse bone marrow, production of mature cells occurred without exhaustion of the stem cell pool [5–10]. It is intriguing that no CSF activity could be detected in those cultures [6] and that addition of pure CSF from the L-cell line did not affect stem cell maintenance, suggesting that within the bone marrow environment the activity of colony-stimulating factor is restricted [11].

We found that adherent stromal cells from the bone marrow inhibit the formation of granulocyte/monocyte [G/M] colonies induced by CSF from embryo fibroblasts, but promoted the proliferation of progenitor CFU-C [12]. It was therefore proposed that the bone marrow stroma enables accumulation of myeloid progenitors by restraining their differentiation. It is noteworthy that stromal cells from mouse bone marrow also supported the in vitro growth of lymphoblastoid cell lines exhibiting immature properties, while the growth of other cell lines expressing differentiated functions was inhibited [13–15].

The mechanism by which stromal cells modulate differentiation and proliferation is unresolved. However, we have previously shown that the inhibition of G/M colony formation, the proliferation of myeloid progenitors, and the inhibition of growth of the myeloma cell line MPC-11 require that the target cells be in the close vicinity of the stromal cells [12, 15]. A variety of cell interactions possibly requiring the activity of glycosylated factors can be competitively inhibited by specific monosaccharides and oligo-saccharides. These involve natural cytotoxic activity [16], erythrocyte rosette formation [17], and suppressor cell functions [18]. Such interactions may also be involved in the inhibition of G/M colony formation since certain monosacharides partially relieved this inhibition [Zipori, Sasson, and Frenkel, submitted for publication]. The interactions between stromal cells and CFU-C and the effect of free monosaccharides is further discussed in this communication. It is shown that free monosaccharides affect myelopoiesis in the presence of stromal cells by inducing endogenous CSF production.

MATERIALS AND METHODS

Materials

The following monosaccharides were used: D-glucose, D-galactose, D-mannose, D-fructose, L-fucose, L-rhamnose. The activity of these sugars was compared with that of sodium pyruvate, sodium lactate, and glycerol. D-Glucose and D-galactose were obtained from BDH biochemicals and the other materials including indomethacin and flufenamic acid from Sigma.

Colony-Stimulating Factor (CSF)

Media conditioned by mouse embryo fibroblasts (EF), L-cells, endotoxin-treated mouse lung (ML), and WEHI-3 cells were used as sources of CSF. Fibroblasts were obtained from 16-day gestation embryos and were passaged two to four times in vitro before the conditioned medium (CM) was collected, passed through a millipore filter (0.45 μ), and frozen. Plateau colony formation occurred with 1:3 dilution of the conditioned medium. The murine L-cell and WEHI-3 lines were continuously grown in culture, and conditioned media were collected and frozen when

cells reached a concentration of about 8×10^5 cells/ml. These conditioned media induced plateau number of colonies in 1:5 and 1:10 dilution, respectively. Mouse lung conditioned medium was obtained as previously described by Burgess et al (19). Purified mouse lung CSF used in the experiments reported here is a gift of Dr. A.W. Burgess. A 1:4,000 dilution of this preparation induced the formation of 80 $\pm$ 11 colonies/1 $\times 10^5$ bone marrow cells.

Preparation of Bone Marrow Stromal Cell Conditioned Medium

Media conditioned by adherent layers of mouse stromal cells, formed as indicated below, were collected at various time intervals following culture and were tested for their capacity to inhibit and/or induce G/M colony formation. Since no such activities were present in the crude conditioned media, the following attempts were aimed at concentrating putative factors. Adherent bone marrow cells were incubated for 24 h in serum-free medium containing either glucose or pyruvate. The medium was then collected and dialyzed 48–72 h against a 30-fold larger volume of distilled water. The material retained in the dialysis membrane was then freeze dried, reconstituted in phosphate buffer (pH 7.4) to obtain a final 20-fold concentration, and frozen in $-20°C$. Similar procedure was followed with L-cells.

Bone Marrow Cells

Male, BALB/c mice from the breeding center of the Weizmann Institute of Science, Rehovot, were used. Bone marrow cells seeded for the formation of adherent layers were obtained from 4-week-old mice and secondary bone marrow inocula (see below) were obtained from 6-week-old-mice. Cell suspensions were adjusted to the desired concentration by dilution in Dulbecco's modified Eagle medium (DMEM) (Gibco, Cat. No. 430-1600) supplemented with 10–20% fetal calf serum (FCS).

Adherent Layers of Stromal Cells From Mouse Bone Marrow

Bone marrow cell suspensions in various concentrations ranging between 1×10^5 to 8×10^6/plate in DMEM supplemented with 20% FCS were inoculated into 35-mm plastic Petri dishes (Nunc) (2.0 ml/plate). Cultures were incubated at 37° C in a humidified atmosphere of 10% CO_2 in air and were refed weekly by removal of half of the medium and addition of fresh medium. At the end of the second week, adherent cell layers were formed. Detailed description of the cellular composition of these adherent layers was previously reported [13]. Fibroblastoid cells that are the predominant cell type in these cultures form large colonies surrounded by phagocytic cells.

Granulocyte/Macrophage (G/M) Colonies

Bone marrow cell suspensions were adjusted to a concentration of 10^5 cells/ml in DMEM supplemented with 20% FCS, the indicated CSF preparation and 0.8% (W/V) methlycellulose. Additional ingredients such as sugars or inhibitors of prostaglandins were added as specifically indicated. One ml aliquots of these suspensions

were inoculated into 35 mm plastic petri dishes or on top of preformed adherent cell layers (triplicate cultures) and were then incubated for 8 days under the above conditions. G/M colonies were counted by use of an inverted microscope.

Recovery of CFU-C From Short-Term, Liquid, Bone Marrow Cultures

Bone marrow cells (10^5/plate) suspended in DMEM supplemented with 20% FCS and the indicated concentration of CSF or similar cell suspensions containing no CSF were seeded on top of preformed adherent cell layers (1 ml/plate) or in control cultures containing no adherent cells. Following three and seven days incubation, 0.6-ml aliquots from duplicate cultures were collected separately, diluted in a final volume of semisolid medium comprising DMEM, supplemented with 20% FCS, 25% L-cell conditioned medium, and 0.8% (w/v) methylcellulose; 3.0 ml of the suspensions were then inoculated into 50-mm plastic Petri dishes (Nunc). G/M colonies were counted following eight days incubation.

RESULTS

Bone marrow cells in semisolid methylcellulose medium containing EF-CM or L-cell CM were plated either on top of preformed layers of bone marrow stromal cells or in their absence (control). The adherent cells inhibited the formation of G/M colonies (Fig. 1). Similar results were obtained with three different CM preparations of each type that were tested in both plateau and suboptimal concentrations (results not shown). CSF preparations from various sources including purified CSF from endotoxin-treated mouse lung were then tested and were all incapable of inducing a significant number of G/M colonies in the presence of adherent bone marrow cells (Table I). Evidently, this inhibition could result from toxicity of the confluent adherent cell layers to the cultured CFU-C. The following experiment was therefore designed to determine the in vitro survival of progenitor CFU-C in the presence of adherent bone marrow cells.

Figure 2A and B demonstrate the recovery of CFU-C in the presence or absence of EF-CM as a function of time and concentration of cells in the adherent layers. Significant survival of CFU-C at day-7 culture (Fig 2B) occurred in the presence of confluent adherent layers whether or not CSF was added to the culture. The addition of EF-CM in the absence or with low numbers of adherent cells caused an increase in CFU-C number at day-3 culture (Fig. 2A), followed by disappearance of the progenitors at day-7 culture (Fig. 2B). Similar experiments were then performed with L-cell CM. It appeared that the day-7 survival of CFU-C induced by the adherent cells alone, was prevented by L-cell CM (Fig. 2C and D). This conditioned medium was then added to the cultures in a concentration that induced colony formation up to only 50% of the plateau value and did not cause an increase in CFU-C number at day-4 culture. Under such conditions, CFU-C survival occurred in a similar manner as with plateau concentration of EF-CM (Fig. 2E and F).

Adherent bone marrow cells could have inactivated the added CSF and thereby would not allow colony formation. However, L-cell CM preincubated four days in the presence of adherent cells (formed by 6×10^6 bone marrow cells/plate) retained its capacity to induce G/M colonies; 115 ± 10 G/M colonies

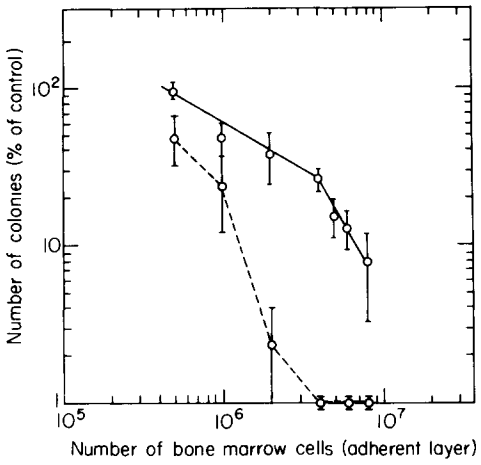

Fig. 1. Inhibition of G/M colony formations by adherent stromal cells: number of G/M colonies induced by CSF from embryo fibroblasts (____) or from L-cells (----) as a function of the number of bone marrow cells seeded for formation the adherent layers of stromal cells ± standard error (results of five separate experiments were averaged).

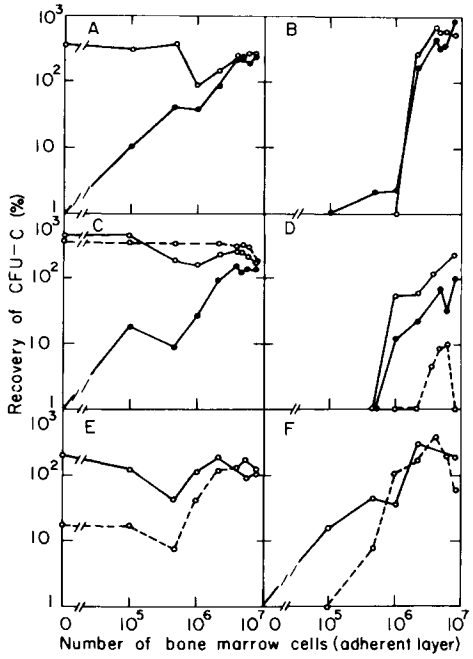

Fig. 2. Recovery of CFU-C from liquid cultures at 3 (A,C,E) and 7 (B,D,F) day incubation as a function of the concentration of bone marrow cells seeded for the formation of the adherent layers. Cultures were performed either in the absence (●) or presence (○) of CSF. EF-CSF (— ○ —) was included as 35% of the medium (A-F) and L-cell CSF (--○--) as 25% (C, D) or 5% (E, F) of the medium. AB, CD, and EF each represent an individual experiment.

were formed by 10^5 bone marrow cells stimulated by L-cells CM preincubated with adherent cells as compared with 95 ± 7 colonies induced by L-cell CM preincubated under control conditions. This finding also implied that medium conditioned by adherent stromal cells may be nontoxic to CFU-C. The medium was then collected from adherent cell cultures after seven days incubation and was incorporated as 35% of the medium used for the formation of G/M colonies. The number of colonies produced was not significantly lower than that of the control (Table II). The possible involvement of prostaglandins was examined by the use of specific inhibitors. Neither indomethacin nor flufenamic acid in concentration of 0.5–2.0 μg/ml could modify the effect of adherent stromal cells on G/M colony formation (Table II). On the other hand, colony formation occurred in the presence of stromal cells following the addition of a number of free monosaccharides (Table III). D-Glucose, D-mannose, and D-fructose induced equal number of colonies, whereas D-galactose was less efficient, and L-fucose and L-rhamnose were completely inactive. Pyruvate, lactate, ai l gylcerol could not mimic the effect of the free sugars (Table III). The formation of the sugar-induced colonies occurred in the absence of externally added CSF. Since the monosaccharides themselves are incapable of inducing colony formation in the absence of stromal cells, it was implied that the sugars trigger production of CSF. Adherent layers of bone marrow cells were therefore cultured either in the presence of serum-free medium containing D-glucose or in glucose deprived medium supplemented with pyruvate as an energy source. Following 24-h incubation, the medium was collected, and factors released by the stromal cells were concentrated as detailed in the Methods section. These concentrates were then tested for their capacity to stimulate colony formation by bone marrow cells. Pyruvate-containing medium was completely devoid of colony-stimulating capacity, and low-glucose medium had only marginal activity, whereas up to 80 colonies per 10^5 bone marrow cells were induced by high-glucose medium (Table IV). Such strict glucose dependence of CSF production was not observed in the case of L-cells (Table V).

DISCUSSION

Colony-stimulating factor from a variety of sources failed to induce G/M colony formation in the presence of adherent layers of bone marrow cells. Concomitantly with this inhibition, proliferation of progenitor CFU-C occurred. A comparison of the dose response curves (Fig. 1 and Fig. 2A and B) shows that optimal recovery of CFU-C occurs when the inhibition of colony formation is most efficient, indicating that these two phenomena may be mutually dependent. It is further indicated that the inhibition of colony formation involves differentiation restraint rather than toxicity of the stromal cells to myeloid progenitors. The elimination of CFU-C in day-7 cultures containing relatively high concentrations of L-cell CM was probably due to the presence of contaminating factors in the conditioned medium. It has been previously shown by others that L-cell CM prevents stem cell proliferation in long-term bone marrow cultures [11, 20], whereas pure L-cell CSF did not [11]. It is noteworthy that the inhibition of G/M colony formation exerted by stromal cells occurred whether the CSF used was from crude conditioned media or a purified CSF preparation (Table I). One of the characteristic features of L-cell CSF is the capacity to promote production of prostaglandins, which in turn may inhibit formation of myeloid colonies [21–23]. The ques-

TABLE I. Inhibition of CSF Activity by Adherent Cells From Mouse Bone Marrow

CSA[a]	Number of G/M colonies/10^5 bone marrow cells ± SE[b]	
	With adherent cells	Control
EF-CM	0	118 ± 13
L-Cell CM	0	115 ± 9
WEHI-3 CM	8 ± 3	150 ± 12
ML-CM	0	111 ± 7
ML-CSF (pure)	0	80 ± 11

[a]Colony-stimulating activity: see also Methods section.
[b]Bone marrow cells were seeded in semi-solid medium either in control cultures or on top of adherent cell layers preformed by seeding 6×10^6 bone marrow cells per plate. Results of a single experiment are presented. Figures are number of day-8 G/M colonies ± standard error. Three separate experiments were performed, each with some of the CSF preparations and similar results were obtained.

TABLE II. The Effect of Indomethacin, Flufenamic Acid, and Medium Conditioned by Adherent Cells, on G/M Colony Formation

Additions	Number of G/M colonies/10^5 bone marrow cells ± SE[a]	
	With adherent cells	Control
None	0	108 ± 7
Indomethacin I[b]	0	113 ± 6
II[c]	0	91 ± 6
Flufenamic acid[b]	0	120 ± 9
Medium conditioned by adherent cells[d]	–	90 ± 8

[a]Bone marrow cells (10^5/plate) were seeded on top of preformed adherent cell layers (8×10^6 bone marrow cell/plate) or, in control cultures, in semi-solid medium containing 25% L-cell CSF. Figures are numbers of G/M colonies counted following eight days incubation ± standard error, averaged from two separate experiments performed.
[b]Indomethacin and flufenamic acid were added to the semi-solid medium containing bone marrow cells in concentration of 0.5, 1.0, or 2.0 μg/ml. Results obtained with these concentrations were not significantly different and are therefore averaged.
[c]Indomethacin was added to the adherent cell layers 96 h (1 μg/ml) and 48 h (0.5 μg/ml) before addition of the bone marrow cell suspension, and at time-0 culture (1 μg/ml).
[d]Medium conditioned for seven days by stromal cells was incorporated as 35% of bone marrow cultures stimulated with CSF.

TABLE III. Induction of Myeloid Colonies in the Presence of Adherent Bone Marrow Cells

Additions	Number of colonies/10^5 bone marrow cells ± SE[a]
None	0
D-Glucose	49 ± 8
D-Fructose	42 ± 7
D-Mannose	42 ± 9
D-Galactose	18 ± 3
L-fucose	2 ± 1
L-Rhamnose	0
Glycerol	0
Pyruvate	0
Lactate	0

[a]Bone marrow cells were seeded in the presence of 17 mM of each of the various sugars (in addition to 5.6 mM glucose present in the basic medium) on top of adherent cell layers formed by 6×10^6 bone marrow cells/plate or with 10–20 mM pyruvate or lactate. No CSF was added to these cultures. Number of colonies was determined after eight days incubation. In control cultures containing no adherent cells, the various additions did not affect G/M colony formation nor were they capable of inducing colony formation by themselves (results not shown).

TABLE IV. Effect of Glucose Concentration in the Culture Medium on the Production of Colony-Stimulating Factor by Adherent Bone Marrow Cells*

Conditioned medium (CM) (% in culture)	Glucose 22,6 mM	Glucose 5,6 mM	Pyruvate 10 mM
10	0	0	0
25	0	0	0
35	30 ± 3	0	0
100	62 ± 5	0	0
200	70 ± 5	10 ± 3	0
300	81 ± 7	18 ± 5	0

*Adherent layers of bone marrow stromal cells formed by 6×10^6 cells per plate were incubated 24 h in serum-free medium containing the indicated concentration of glucose or pyruvate in place of glucose. The medium was then collected, concentrated as detailed in the Methods section, and incorporated in the indicated concentration into cultures containing bone marrow cells in semi-solid medium. The figures represent number of day-8 colonies ± standard error. Separate experiments with three different stromal cell CM were performed and the results were averaged.

TABLE V. Effect of Glucose Concentration in the Culture Medium on the Production of Colony-Stimulating Factor by L-cells*

Conditioned medium (CM) (% in culture)	Glucose 22,6 mM	Glucose 5,6 mM	Pyruvate 10 mM
12.5	47 ± 3	59 ± 7	57 ±4
25.0	51 ± 5	63 ± 3	69 ±7
35.0	61 ± 7	74 ± 3	69 ±5

*L-cells were cultured in medium containing the different sugar concentrations for two weeks prior to their transfer to serum-free medium as detailed in Table IV.

tion was then raised as to whether the inhibition of myeloid differentiation exerted by stromal cells was due to the activity of prostaglandins; however, both indomethacin and flufenamic acid were incapable of abolishing the inhibition. It may be argued that these agents could not suppress the high concentration of prostaglandins present in the system. This possibility seems unlikely in view of the lack of inhibitory activity in conditioned media (Table II). We recently examined the actual content of prostaglandins using a radioimmunoassay and did not observe any differences between control cultures and those containing stromal bone marrow cells [Zipori and Strulovici, unpublished observations]. The molecular nature of the stromal-cell-derived inhibitor(s) of myeloid differentiation is unknown. Indirect evidence such as the relief of inhibition by an agar layer separating the CFU-C from the stromal cells [12] and by specific methyl glycosides [Zipori, Sasson and Frankel, submitted for publication] may suggest the participation of glycosylated surface receptors.

The inhibition of G/M colony formation by adherent stromal cells could also be relieved by the free monosaccharides, D-glucose, D-mannose, and D-fructose. These sugars are metabolized by bone marrow cells, and their effects on myeloid differentiation could therefore be attributed to supply of energy or intermediates of metabolism. This did not appear to be the case since pyruvate lactate and glycerol could not replace the free sugars. It cannot be excluded that gluconeogenesis is inefficient in these bone marrow cultures resulting in deficiency in the hexose backbone pool available for cells cultured in medium in which

pyruvate replaced glucose. Pouysségur et al [24] observed loss of high-molecular-weight protein in chick embryo fibroblasts incubated without glucose. Similar results were obtained with virus-infected fibroblasts and with the myeloma tumor MOPC-46 [26]. Stark and Heath [26] suggested that glucose controls protein glycosylation. This and the observation that the formation of glucose induced colonies did not require addition of exogenous CSF, suggested that free monosaccharides added in high concentrations to cultures of stromal cells, trigger the production of an active factor required for colony formation. Indeed, serum-free medium conditioned by stromal cells exhibited colony-stimulating activity, provided that it contained high glucose concentration during incubation. It is noteworthy that this glucose dependence of CSF production was not observed in L-cells. Moreover, the titer of CSF obtained from L-cells, as well as from the other sources used here, was up to one decade higher than that present in medium conditioned by stromal cells (Table IV).

CSF production by non-hemopoietic cells from the bone marrow has been previously reported [27]. On the other hand, no stimulating activity was observed by Dexter et al [6] and by Bentley and Foidart [28] in long-term stromal cell

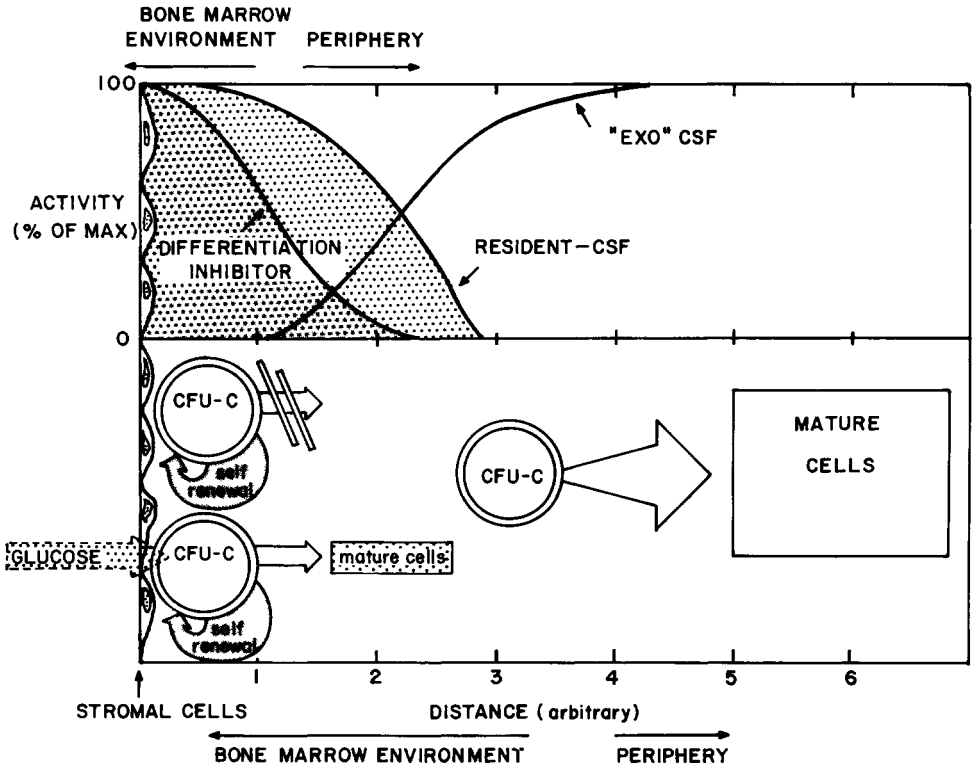

Fig. 3. An hypothetical model of cell interactions in the bone marrow microenvironment. The upper chart indicates schematically that the activities of the inhibitor and the stimulator (glucose-dependent, resident CSF) of differentiation produced by stromal cells are declining as a function of distance from the stroma, whereas exogenous CSF is blocked in the vicinity of stromal cells. In the lower chart, the bared arrows represent differentiation or self-renewal, as indicated, and their relative size signifies the amount of cells produced (additional information is presented in the Discussion section).

cultures. Lord et al [29–31] and Frindel et al [32, 33] reported that the bone marrow tissue produces both an inducer and an inhibitor of DNA synthesis in hemopoietic stem cells. Similar factors were recently detected in long-term cultures of bone marrow cells [34]. It has not been determined so far whether these factors are responsible for the long-term survival of stem cells in culture. The lack of effect of these factors on myeloid progenitors [34] suggests that they may differ from the stromal cell derived activities described in this communication.

Based on the experimental culture system studied here, a hypothetical model of cell interactions occurring in the microenvironment of the bone marrow was constructed (Fig. 3). It demonstrates schematically that the ultimate fate of a given CFU-C is determined by its positioning relative to the stromal cells that form the marrow environment. In the close vicinity of stromal tissue, the activity of exogenous CSF (ie, CSF from sources other than the bone marrow) is restrained. This results in self-renewal and prolonged maintenance of progenitor CFU-C. The inhibition of differentiation may then be partially relieved by a resident CSF produced by the stromal cells themselves. The lack of inhibitory activity in conditioned media (Table II) and the low CSF titer produced by stromal cells (Table V) are depicted in the figure by the steep decline of activity as a function of distance from the bone marrow environment. The physiological significance of the effect of glucose starvation on the production of bone marrow resident CSF is doubtful. It can be envisaged though that within certain domains of the bone marrow tissue, cell density is high enough to create local glucose deficiency. It follows that the degree of differentiation restraint and thus, progenitor cell maintenance, be directly related to the increase in cell packing.

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