Leukemic Inhibition of Normal Hematopoiesis*

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Introduction

The effects of human leukemic blasts on normal in vitro granulocyte-macrophage colony-forming cells (CFU-C) [2,19] have been studied with conflicting results [4,5,8,9,11,13,18,25]. In some studies inhibition of normal human CFU-C by leukemic blasts was seen [4,5,13,18] while other studies failed to show inhibition [8,9,25]. These discordant results may, in part, result from histocompatibility differences as suggested by Bull et al. [4].

We have utilized a syngeneic murine model, the C1498 acute myeloid leukemia of C57B1/6J mice [6,7] to further study the effects of leukemic blasts on normal hemopoietic stem cells. In these studies we have employed coculture of leukemic blasts both in-vitro [2,19] and in-vivo diffusion chamber (DC) cultures [1,22]. In preliminary studies we have also evaluated interactions of human leukemic blasts with normal human CFU-C.

Methods

Female CF1 mice were used as host mice for diffusion chamber (DC) cultures and female C57BL/6J mice were used as sources for normal marrow cells and as tumor bearers for the C1498 acute myeloid leukemia.

Stem Cell Assays

Murine granulocyte-macrophage progenitor cells (CFU-C) were assayed by a modification of a double layer soft agar technique [3,21] utilizing sera from endotoxin injected mice [20] or murine lung conditioned media [23] as sources of colony stimulating activity (CSA). Pluripotent stem cells (CFU-S) were assayed according to the method of Till and McCulloch [12,27] except that assay mice received 950 R from a cesium 137 source (118 R per minute). C57BL/6J cells were assayed in irradiated C57BL/6J mice. Burst-forming unit erythroid (BFU-E) and colony forming unit erythroid (CFU-E) stem cells were assayed by a plasma clot technique as previously described [28]. Human CFU-C were cultured by the method of Robinson and Pike [26].

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In Vivo Diffusion Chamber

Normal C57BL/6J marrow, C1498 cells or mixtures of the two were grown in diffusion chambers [1,22] implanted into unirradiated or irradiated (950 to 1000 R) CF_1 host mice and differentiated and stem cell recovery assessed after 2–14 days. Normal marrow cells were implanted at $0{,}25-0{,}50\times 10^6$ cells per diffusion chamber and C1498 cells at the same levels; normal marrow-C1498 mixtures consisted of $0{,}05\times 10^6$ C1498 cells plus $0{,}45-0{,}50\times 10^6$ marrow or $0{,}125-0{,}25\times 10^6$ C1498 cells plus $0{,}25\times 10^6$ marrow cells. The effect of diffusible factors from C1498 cells was assessed by growing the leukemic cells adjacent to normal marrow cells, but separated from them by a cell impermeable 0,22 micron millipore filter in double diffusion chambers. In each experiment normal marrow was implanted into one of the chambers of the double diffusion chamber and the opposite chamber was implanted with either normal marrow or C1498 leukemic cells at the same concentration or with a higher concentration of normal cells; the recovery of differentiated and stem cells from the chambers with normal marrow grown adjacent to these groups was then assessed. The test chambers were implanted with $0{,}25-0{,}5\times 10^6$ normal marrow cells while the adjacent chambers were implanted with either $0{,}25-0{,}5\times 10^6$ normal marrow, $0{,}25-0{,}5\times 10^6$ C1498 cells (C1498) or $2{,}5\times 10^6$ normal marrow cells. Cocultures of human leukemic blasts (previously cryopreserved at −196°C in 10% DMSO) with normal marrow from HLA-MLC compatible siblings were carried out in a similar manner.

Cell Separation Experiments

C1498 cells were separated by unit gravity velocity sedimentation by the method of Miller and Phillips [16]. Cells were pooled and different pools assessed for their effect on normal marrow growth when mixed directly with normal marrow cells in diffusion chambers.

Probability figures were calculated using Student's T Test.

Results

The C1498 cells were unresponsive to CSA in in-vitro soft agar culture. In repeated experiments neither cluster (3–50 cells) nor colony formation was noted after 8–10 days growth. Mixing of from 0,25 to $0{,}5\times 10^5$ normal marrow cells with varying concentrations of leukemic cells in in-vitro agar cultures inhibited CFU-C growth (Fig. 1). Similarly direct mixtures of normal marrow and C1498 blasts in DC cultures inhibited CFU-C recovery (Fig. 2) but normal differentiated cell recovery was only inhibited with higher input concentrations (33–50%) of C1498 cells ($61 \pm 9\%$ of control over 5–12 days of culture, $p < 0{,}01$). This inhibition correlated in general with total C1498 cell recovery from DC (Fig. 3).

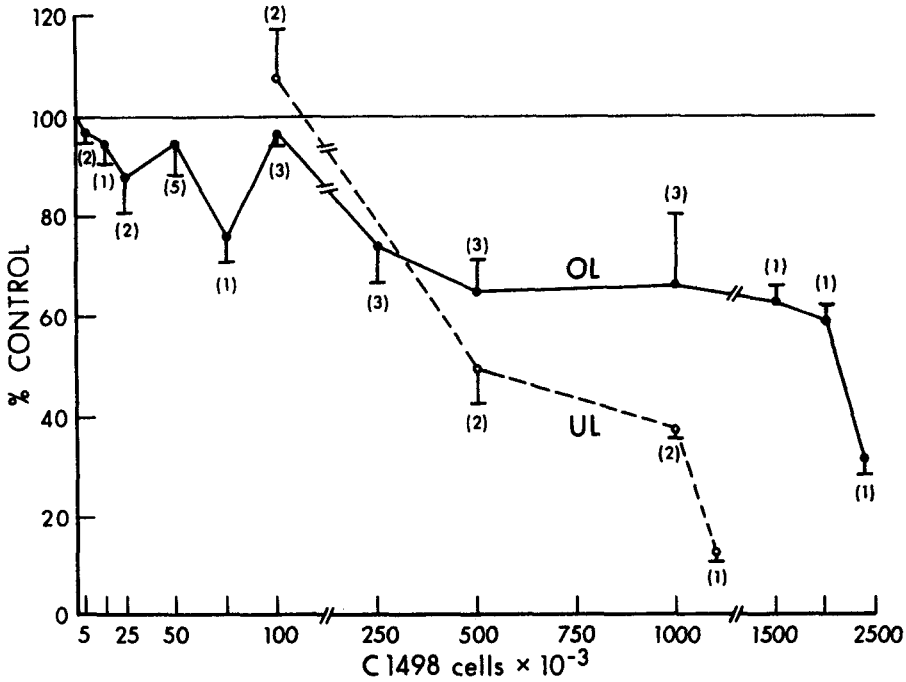

Fig. 1. The effect of C1498 cells on normal C57BL/6J marrow CFU-C in in-vitro soft agar cultures. Varying numbers of C1498 cells were added to cultures of from 0.25–0.50 × 10^5 normal marrow cells and CFU-C growth compared to cultures without added leukemic cells. C1498 cells were either mixed directly with normal marrow cells in the overlayer (OL) or with the source of CSF in the underlayer (UL) of the double layer agar cultures. The results are expressed as a per cent of control ± one standard error of the mean (SEM). The numbers in parenthesis represent the number of separate experiments for each concentration of C1498 cells. These data are derived from a total of 11 separate experiments. (Reprinted from Quesenberry et al. by permission from N. Engl. J. Med., **24**.)

Similar diffusion chamber mixing experiments were carried out with subpopulations of C1498 cells separated by unit gravity velocity sedimentation and the degree of inhibition compared to that seen with unseparated leukemic cells (Table 1). Inhibition was most marked with the larger more rapidly growing C1498 cells (pools 1–2) as compared either to unseparated or smaller C1498 cells (pools 3–4) mixed at the same per cent with normal marrow. There was a general but not exact correlation of CFU-C inhibition with total numbers of C1498 cells derived from the various pools (Fig. 4).

Transmembrane Experiments

The effect of either C1498 cells or normal marrow cells cultured transmembrane from normal C57BL/6J marrow cells on CFU-C and CFU-S recovery after varying periods of double diffusion chamber growth is presented in Fig. 5.

The CFU-S, and to a lesser extent the CFU-C, were inhibited when normal marrow cells were cultured adjacent to C1498 cells. The mean recovery of CFU-S grown across from C1498 cells in diffusion chambers for 5–14 days was 45 ± 7% of control ($p < 0{,}002$) and that of CFU-C was 72 ± 7% of control

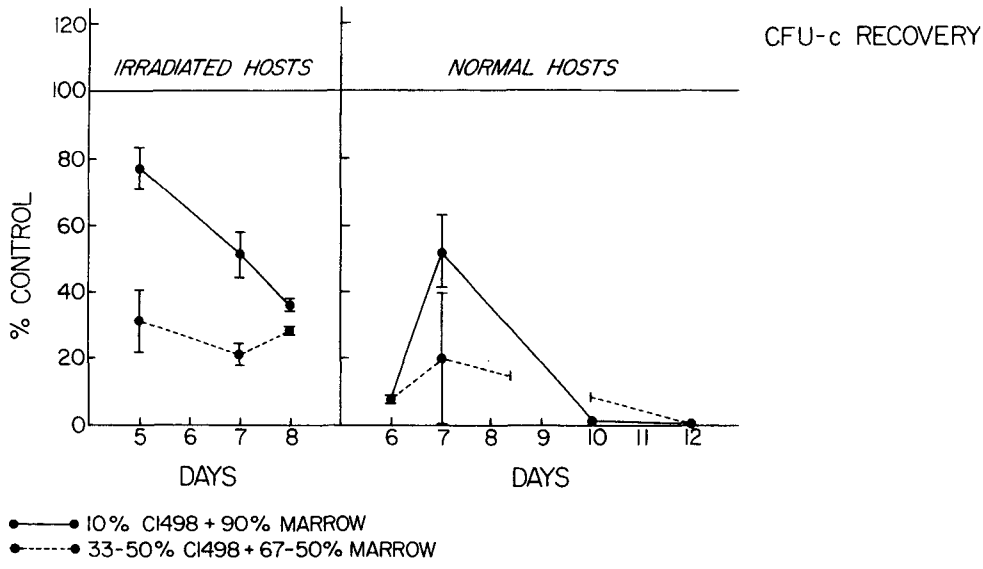

Fig. 2. The effect of C1498 cells mixed with C57BL/6J cells on CFU-C recovery from DC cultures. The number of CFU-C recovered from DC with 10–50% C1498 cells mixed with normal marrow cells at varying times after surgical implantation expressed as a per cent of control ± one SEM. The number of cells plated for the in vitro assay of CFU-C varied from experiment to experiment; in 13 of 15 experiments the numbers of C1498 cells plated were not sufficient to account for the observed degree of inhibition. In 7 of 15 experiments the number of C1498 cells plated in-vitro was below 118947, a number unlikely to give significant in-vitro inhibition (see Fig. 1). These data are from 15 separate experiments (4 separate surgical implantations); the data at day 5 are the mean of 2 separate experiments for each concentration of C1498 cells. A total of 195 DC were utilized in these experiments

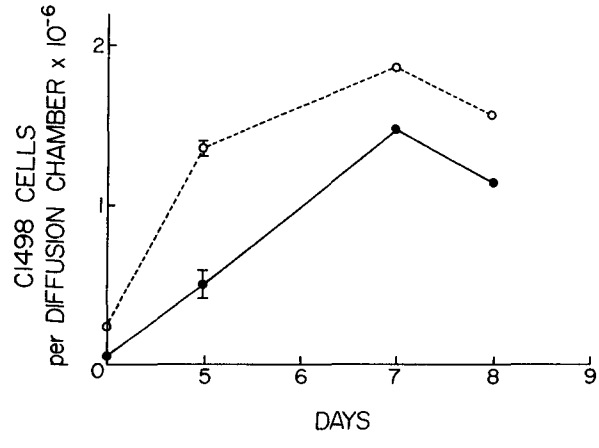

Fig. 3. Blast recovery per chamber from chambers with 10% (solid line) or 50% (dashed line) C1498 cells mixed with normal marrow. These studies utilized irradiated CF_1 hosts and are from the same experiments presented in panel 1 (Fig. 2). The data are presented as number of C1498 cells per chamber ± 1 SEM. (Reprinted from Quesenberry et al. by permission from N. Engl. J. Med., **24**)

($p < 0{,}02$). Stem cell recovery was not effected by a 10 fold higher input of normal marrow in the opposite chamber.

In general, chambers implanted with C1498 cells or high concentration normal marrow had equivalent cell yields. Chambers implanted with $0{,}25 \times 10^6$ C1498 cells yielded 1,0, 1,8, 3,0 and 5,0 million cells on days 5, 7, 8 and 14, respectively, while chambers implanted with $2{,}5 \times 10^6$ normal marrow cells yielded 2,3, 3,4 and 1,3 million cells on days, 7, 8 and 14 of growth, respectively. There was no demonstrable inhibitory effect of C1498 cells on total cell yield from adjacent chambers implanted with $0{,}25 \times 10^6$ normal marrow (mean 104 ± 19% of control, 5–14 days growth) but there was a suggestion of augmented recovery of cells from normal marrow grown opposite high concentration marrow (mean 154 ± 28% of control, 7–14 days of growth). There

Table 1. The effect of C1498 cells separated by velocity sedimentation on normal marrow CFU-C recovery from diffusion chamber mixtures (per cent of control)

Exp.	Days diffusion chamber growth	% Unseparated C1498 cells	% Separated C1498 cells			
		3%			3%	
			Pools 1	2	3	4
1	5	133 ± 6	89 ± 3	122 ± 7	186 ± 3	178 ± 8
	7	79 ± 7	69 ± 2	70 ± 5	94 ± 6	—
					5%	
2	5	71 ± 2	39 ± 2	50 ± 7	60 ± 4	65 ± 1
	7	51 ± 7	10 ± 1	22 ± 1	68 ± 1	129 ± 3

The results are expressed as a per cent of control ± 1 SEM. A total of 160 and 144 diffusion chambers were evaluated in exp. 1 and 2, respectively. The mean number of control CFU-C implanted into $0{,}45 \times 10^6$ diffusion chambers in these 2 experiments was 1324, while the mean number recovered on days 5 and 7 of diffusion chamber growth was 1044 and 1665, respectively, the range of sedimentation velocities in exp. 1 and 2 for pools 1, 2, 3 and 4 were 10,1–6,9, 6,9–4,7, 4,5–2,8 and 2,7–0,73 mm/hr, respectively

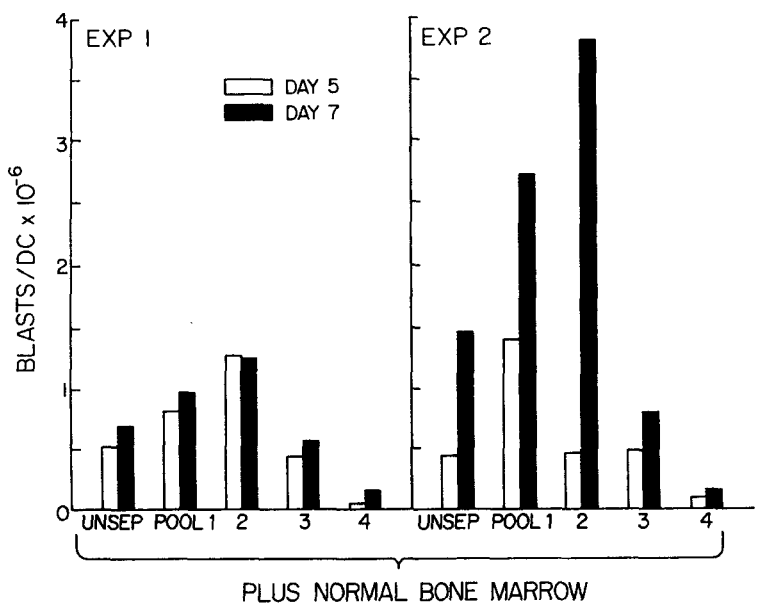

Fig. 4. The number of C1498 blasts per diffusion chamber from unseparated and separated C1498 cell-normal marrow mixtures after 5 to 7 days of diffusion chamber cultures. These results are from the same experiments presented in Table 1. (Reprinted from Quesenberry et al. by permission from N. Engl. J. Med., **24**)

was also no significant shift in differentiation when normal marrow was grown adjacent to the various groups. The over-all (5–14 days) mean percentage of granulocytes in the normal marrow groups ranged from 63,6 to 66,1 and that of macrophages from 32,6 to 35,2.

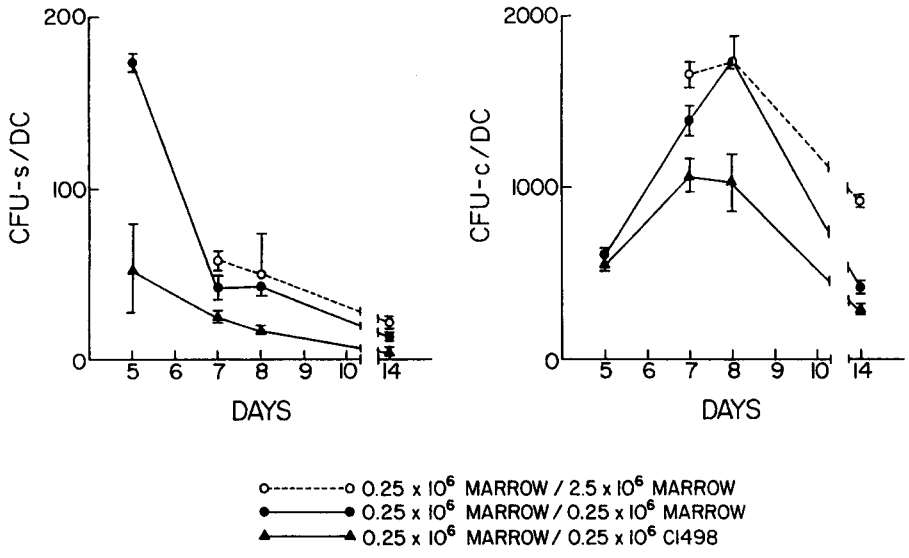

Fig. 5. Transmembrane effects of C1498 cells or high concentrations of C57BL/6J cells on CFU-S and CFU-C recovery in double diffusion chamber cultures. C57BL/6J marrow cells implanted at a concentration of $0{,}25 \times 10^6$ cells per chamber (test chamber) were grown adjacent to C1498 cells implanted at the same cell level or C57BL/6J marrow implanted at $0{,}25 \times 10^6$ or $2{,}5 \times 10^6$ cells per diffusion chamber and the recovery of CFU-C and CFU-S assessed from the test chambers at varying times after implantation. These data are from 5 separate experiments for C1498 and 4 for $2{,}5 \times 10^6$ NL marrow cell studies (5 and 4 separate surgical implantations, respectively) in which cells from 84 control and 75 experimental double diffusion chambers were evaluated. The data at day 8 represents the mean from 2 separate experiments. Host CF_1 mice were pre-irradiated with 1000 R on the day of surgery. Data are expressed as stem cell per diffusion chamber ± one SEM. (Reprinted from Quesenberry et al. by permission from N. Engl. J. Med., **24**)

In preliminary experiments in-vitro coculture of C1498 cells in plasma clot cultures with normal C57BL/6J marrow resulted in inhibition of BFU-E but not CFU-E growth (Table 2).

Table 2. C1498 leukemic cell inhibition of normal C57BL marrow erythroid stem cells

Group	% Control	
	CFU-E/10^6 cells	BFU-E/10^6 cells
Normal: C1498 (1:2)	99 ± 5	102 ± 5
Normal: C1498 (1:10)	106 ± 8	61 ± 12

Coculture of blasts from a patient with acute myelomonocytic leukemia with HLA-mixed leukocyte culture compatible sibling marrow cells in DC cultures in irradiated mice resulted in inhibition of normal CFU-C growth (Fig. 6). The effects of subpopulations of blasts separated by velocity sedimentation was also assessed and it appeared that the smallest slowly sedimenting cells were most inhibitory.

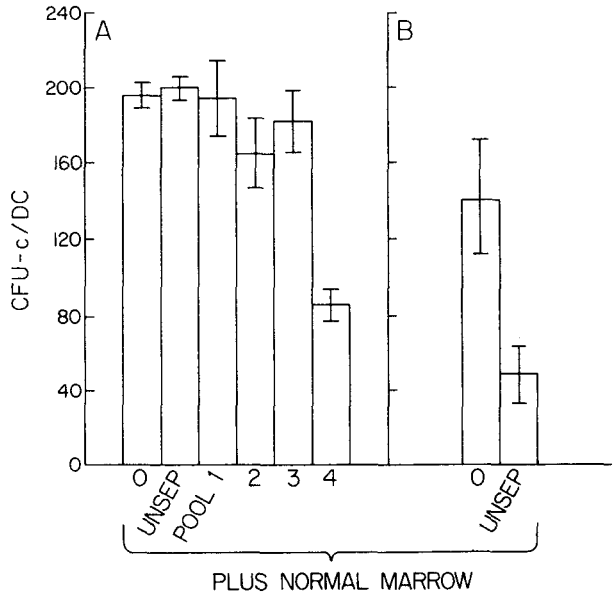

Fig. 6. Inhibition of histocompatible (HLA-MLC) normal Human CFU-C by marrow cells from a patient with acute myelomonocytic leukemia (AMML). Panel A shows the number of normal human CFU-C recovered after 4 days of diffusion chamber culture when unseparated AMML cells or AMML cells separated into four pools by velocity sedimentation were mixed directly with a marrow from an HLA-MLC compatible sibling in diffusion chambers. These AMML cells did not form colonies. 0.05×10^6 unseparated AMML or pool 2 and 3 cells or 0.02×10^6 pool 1 and 4 cells were mixed with 0.45×10^6 normal marrow cells in DC and CFU-C recovery compared to that seen with 0.45×10^6 normal marrow cells in DC alone (0 group). Inhibition of CFU-C recovery by pool 4 cells is apparant. Pools 1–4 had sedimentation velocities of 10.68–7.74, 7.17–5.02, 4.49–1.99 and 1.52–0.1 mm/HR. respectively. Results are expressed as the number of CFU-C per DC ± one SEM. Panel B shows the number of normal CFU-C recovered after 4 days of DC culture when 0.25×10^6 unseparated AMML cells were mixed in DC with 0.25×10^6 normal sibling marrow cells (unseparated group) or when 0.25×10^6 normal marrow cells were cultured alone in DC

Discussion

The present experiments indicate that C1498 leukemic blasts inhibit normal murine marrow hematopoietic stem cells. Inhibition of normal CFU-C and BFU-E (but not CFU-E) was demonstrable when C1498 cells were mixed with C57BL/6J marrow cells in in-vitro cultures (Fig. 1 and Table 2).

A decreased recovery of CFU-C was also noted when leukemic cells were mixed with normal marrow cells in in-vitro diffusion chamber cultures (Fig. 2), and experiments utilizing the double diffusion chamber technique, in which the C1498 and normal marrow cells are separated by a cell impermeable membrane, showed that both CFU-C and CFU-S were inhibited (Fig. 5) and that cell contact was not necessary for this inhibition to occur.

Inhibitory effects on differentiated cell production were only demonstrable with relatively high leukemic-normal cell input ratios (33–50% C1498 cells) and were not seen with a lower ratio (10% C1498 cells) or in the transmembrane experiments. These effects on differentiated cell production in diffusion chamber cocultures of C1498 and normal marrow are similar to those reported by Miller et al. [14]. Our results differ from those of Miller et al.

[14, 15] in that we found inhibition at the CFU-C, as well as the CFU-S level, and demonstrated that cell contact was not necessary for this inhibition.

Cocultures of subpopulations of C1498 cells separated by unit gravity velocity sedimentation and normal marrow indicated that the large rapidly growing leukemic cells (pool 1) were most inhibitory with little or no inhibition observable with the smallest slower growing leukemic cells (pools 3 and 4) (Table 1). Although there was not an exact correlation with total numbers of chamber leukemic cells, the striking differences in the inhibitory effects of the larger and smaller C1498 cells seem most likely to be based predominantly on differences in growth rate and final diffusion chamber leukemic cell concentration, rather than any other unique characteristic of the separated leukemic cell subpopulation.

The double diffusion chamber studies on the transmembrane effects of C1498 cells on normal marrow stem cells indicate that a substance released by the leukemic cells mediates stem cell inhibition. The cell recovery after 7–14 days of in-vivo culture from diffusion chambers implanted with $2{,}5 \times 10^6$ normal marrow cells approximated that from chambers with C1498 cells implanted at $\frac{1}{10}$th that level, suggesting that there was more cell death in the chambers with high concentrations of normal marrow, yet there was no demonstrable inhibition by these cells. These results suggest that the inhibition seen with C1498 cells was not due simply to cellular degradation products. The inhibitory substance which is released by C1498 cells could be unique to leukemic blasts, a by-product of malignant cells in general or simply relate to rapidly proliferating cells.

In toto, the present data indicate that murine leukemic blasts inhibit normal marrow stem cells by elaborating a diffusible inhibitory substance. Consistent with these observations are the studies of Handler et al. [10] showing that rat myeloid leukemic cells can condition media with substances inhibitory to normal CFU-C. Furthermore, in the present studies this inhibition appears to be most marked at the pluripotent stem cell level with lesser degrees of inhibition seen with increasing degrees of differentiation within the myeloid pathway. These data are consistent with the concept that leukemic inhibition acts primarily on CFU-S with effects on CFU-C, BFU-E and differentiated granulocytes possibly being of a secondary nature.

Preliminary studies from our laboratory indicate that human HLA-mixed lymphocyte compatible leukemic blasts can inhibit normal CFU-C recovery from diffusion chamber cultures and that it may be possible to isolate a subpopulation of inhibitory cells from marrow cells from patients with acute myelomonocytic leukemia (Fig. 6). These studies, along with the studies on human CFU-C inhibition noted above [4, 5, 13, 18] and Jacobson's studies on human leukemic inhibition of normal human marrow stem cells in plasma clot diffusion chamber cultures [11] suggest that it may be possible to extrapolate the present results to the mechanisms underlying the myelosuppression in humans with acute leukemia.

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