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Utilization of Mouse Stem Cell-Depleted Marrow in the Study of Diffusion Chamber Myelopoiesis (41147)

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Abstract. Mouse brain antisera with anti-CFU-S activity was utilized to evaluate the role of the pluripotent stem cell (CFU-S) in a diffusion chamber (DC) myelopoiesis. Antisera incubation with marrow resulted in a 93 ± 2% reduction in detectable CFU-S in the standard spleen colony assay and a variable reduction in the granulocyte–monocyte progenitor cell (CFU-C). Marrow virtually devoid of detectable CFU-S and containing variable CFU-C relative to control demonstrated equal or greater numbers of CFU-S, CFU-C, and differentiated progeny at varying time intervals after DC implantation. These results may indicate that neither the CFU-S nor the CFU-C is the progenitor cell for DC growth. Alternatively, CFU-S antibody may not kill CFU-S *in vitro* or in the *in vivo* DC system but may opsonize the cell prior to eventual reticuloendothelial destruction. The latter concept is supported by reversal of antisera effect on CFU-S recovery after *in vitro* exposure of antisera-treated cells to pronase.

Previous investigators (1–4) have demonstrated the utility of Millipore diffusion chambers (DC) in the study of granulopoiesis. The implantation of DC containing hematopoietic cell suspensions into the peritoneal cavity of host mice has permitted the quantitative *in vivo* evaluation of murine myelopoiesis. The progenitor cell responsible for DC proliferation, however, remains to be elucidated. To evaluate the role of the pluripotent stem cell (CFU-S) in DC myelopoiesis, we have utilized mouse brain antisera with anti-CFU-S activity to study the growth of marrow cells depleted of CFU-S by prior antisera exposure. We were particularly interested in examining stem cell self-renewal in DC following implantation with antisera-treated marrow and to determine how stem cell self-renewal and differentiation in DC would be affected by the addition of antisera-treated marrow suspensions containing very low numbers of assayable CFU-S. Concomitantly, we have examined the capacity of CFU-S antisera resistant stem cells to grow in DC and have evaluated the effect of CFU-S antisera on the granulocyte-monocyte progenitor cell (CFU-C).

Methods. *Stem cell assay.* Granulocyte–monocyte progenitor cells (CFU-C) were assayed by a modification of the double-layer soft agar technique (5, 6), using serum from endotoxin-injected mice (7) or murine lung-conditioned media (8) as the source of colony-stimulating activity. Pluripotent stem cells (CFU-S) were assayed in mice which received 950 R from a cesium-137 source (118 R/min). Marrow cells from CD_1 mice was assayed in irradiated mice from the same strain.

Diffusion chambers. Female CD_1 or CF_1 mice exposed to 1000 R whole body radiation from a cesium-137 source (118/min) were used as host mice for the DC cultures which were prepared as previously described (4). Untreated CD_1 marrow cells and cells previously exposed to antisera and complement or absorbed control sera and complement were grown in DC at an initial concentration of 250,000 cells per DC (3, 4). DC were removed after 7, 8, or 16 days of growth and the cells harvested for cell counts, differentials, and stem cell as-

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says. DC were transferred to secondary sets of irradiated host mice on Day 8, for the 16-day time point. A 1- to 1½-hr incubation in 0.5% Pronase and 5% Ficoll at 25° was utilized to lyse the clot which forms in DC. Cell suspensions were washed three times prior to stem cell assay.

Antisera preparation. Rabbit anti-mouse brain sera was prepared in accordance with the procedure originally described by Golub (10). Normal rabbit sera from uninjected rabbits served as the source of control sera. Control sera resulted in a 44 ± 9% reduction in CFU-C and a 37 ± 3% reduction in CFU-S relative to control marrow incubation without sera. Prior to assay, both normal rabbit (control) and mouse brain antisera (CFU-S antisera) were routinely absorbed with syngeneic murine bone marrow and red blood cells as described by Monette *et al.* (11). Different lots of antisera were utilized for each subsequent stem cell assay. Additional tissue absorptions in some experiments were performed as follows: (i) Spleen cell suspensions (5×10^7 cells) were washed in MEM and centrifuged. One and one-half milliliter of either CFU-S antisera or control sera, previously absorbed with bone marrow and red blood cells, was added to the cell pellet and incubated for 1 hr at 37° followed by 2 hr at 4°. Utilizing fresh tissue each time, splenic absorptions were performed in some experiments with control and CFU-S antisera previously absorbed with bone marrow.

Cell incubations. Pooled CD_1 marrow cells were washed in MEM and 10^7 cells resuspended in 0.175 ml. Either absorbed control sera or CFU-S antisera was then added in 0.3-ml volumes and the suspensions incubated at 4° for 30 min. Either 0.05 ml of fresh mouse serum or rabbit serum was then added as a source of complement, and the suspension incubated at 37° for 1 hr. Cells were then washed three times and either assayed for stem cells or grown in diffusion chambers (DC). For some experiments, complement was added to control and CFU-S antisera-treated marrow as above, and the suspensions were then incubated with 0.5% Pronase for an additional 1−1½ hr at 25° prior to assay.

Results. The effect of CFU-S antisera incubation with CD_1 bone marrow on subsequent CFU-S and CFU-C detection is demonstrated in Table I. CFU-S reduction ranged from 83 to 97% (93 ± 2%). The addition of exogenous complement did not appear necessary to demonstrate the activity of antisera against CFU-S. Antisera activity against CFU-C was more variable with values ranging from 14 to 214% of normal serum controls. The variation in CFU-C detection appeared to be explained by differences in antisera lot activity; CFU-C reduction was not dependent upon the presence of a complement source with marrow incubation and was independent of tissue absorption with red cells and bone marrow. Serial splenic absorptions may have partially reduced antisera activity against CFU-C.

To examine the effect of antisera marrow incubation on cell proliferation and stem cell renewal, CD_1 marrow cells treated with complement and either normal or anti-CFU-S sera were placed in diffusion chambers (DC), implanted into irradiated CD_1 or CF_1 host mice, and subsequently removed after 7, 8, or 16 days of growth. Cell suspensions thus obtained were compared to CD_1 marrow similarly grown in DC without exposure to either serum or complement. All suspensions were examined for total cell number, differentials, and stem cell recovery. As shown in Table II, total cell yield from DC implanted with antisera-treated marrow equaled or exceeded cell yield from normal sera DC at 7, 8, and 16 days of growth. Figure 1 indicates, similarly, no difference between antisera and normal sera DC suspensions when analyzed according to total granulocyte and macrophage content 8 and 16 days following implantation. Proliferating and nonproliferating granulocytes were also similar for both antisera and normal sera DC suspensions at 8 and 16 days (data not shown).

Table III indicates that, despite the low numbers of assayable CFU-S in antisera-treated marrow suspensions initially implanted in DC (Day 0), CFU-S recovery from antisera DC at 7, 8, and 16 days equaled or exceeded the number of CFU-S obtained from normal serum DC. Similarly, recovery of CFU-C from antisera DC par-

TABLE I. EFFECT OF CFU-S ANTISERA INCUBATION WITH CD_1 MARROW ON SUBSEQUENT CFU-C AND CFU-S DETECTION

Experiment	*n*	CFU-C/10^5 cells (mean ± SE)	Percentage reduction	*n*	CFU-S/10^5 cells (mean ± SE)	Percentage reduction
I[a,b]						
CON[c]	4	155 ± 17		7	12.4 ± 1.3	
EXP[d]	4	55 ± 6.7	65	5	0.86 ± 0.5	93
II[a,b]						
CON	8	98 ± 4		9	11.1 ± 1.6	
EXP	8	38 ± 4	61	9	0.3 ± 0.3	97
III[b,e]						
CON	10	134 ± 6		6	11.1 ± 0.8	
EXP	10	92 ± 6	31	6	0.3 ± 0.1	97
IV[e]						
CON	10	101 ± 8	—	5	5.1 ± 1.9	
EXP	10	123 ± 18		4	0.2 ± 0.1	96
V[b,f]						
CON	10	65 ± 3	—	7	4.6 ± 1.3	
EXP	10	139 ± 7		8	0.8 ± 0.3	83
VI[b,f]						
CON	5	102 ± 5.0		7	12.0 ± 3.9	
EXP	5	14 ± 1.6	86	7	0.6 ± 0.2	95
VII[b,f]						
CON	5	133 ± 4.5		10	31.4 ± 1.8	
EXP	5	148 ± 4.0	—	10	3.1 ± 0.8	90

[a] Single absorption each with bone marrow and RBC.
[b] Fresh mouse serum at 1:10 dilution utilized as complement source.
[c] Marrow incubated with control sera.
[d] Marrow incubated with mouse brain antisera.
[e] Single absorptions each with bone marrow and RBC plus serial splenic absorptions.
[f] Single absorption with bone marrow and triple RBC absorption.

alleled that found in both untreated DC suspensions and in suspensions incubated with normal serum and complement.

We next examined the effect of exposing antibody-treated marrow cells to proteolytic digestion with 0.5% Pronase prior to stem cell assay. Table IV illustrates the effect of Pronase incubation on subsequent recovery of antisera-treated CFU-S. *In vitro* exposure to Pronase reversed the anti-

TABLE II. EFFECT OF CFU-S ANTISERA ON TOTAL CELL RECOVERY FROM DIFFUSION CHAMBERS (DC)

Days of DC growth	Total cell/DC			
	No sera ($\times 10^5$)	EXP + C′[a] ($\times 10^5$)	CON + C′ ($\times 10^5$)	*n*
0 (Input)	2.50	2.50	2.50	
7	3.43	6.22	3.43	1
8	2.80 ± 0.08	3.43 ± 0.8	3.66 ± 0.6	2
16	5.94 ± 2.30	10.73 ± 2.80	10.50 ± 2.51	2

Note. These data are expressed as the mean pooled counts ± 1 SEM from separate experiments.
[a] EXP, Marrow incubated with mouse brain antisera; CON, marrow incubated with control sera. Fresh mouse sera was used as a source of complement (C′).

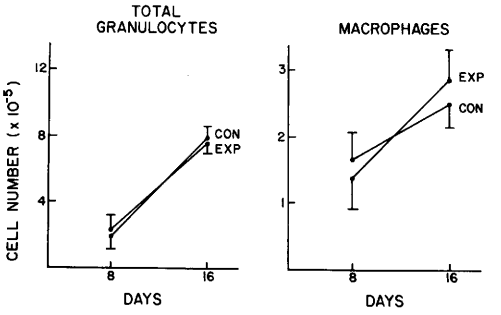

FIG. 1. Diffusion chamber granulocyte and macrophage recovery after exposure to CFU-S antisera (EXP) or control sera (CON). The data are expressed as mean pooled counts ± 1 SEM and are from the same experiments presented in Table II.

sera effect on CFU-S recovery. Similar Pronase reversal was found in two further experiments (data not shown).

Discussion. Rabbit anti-mouse brain antisera (CFU-S antisera), in addition to selecting for specific neuronal cell surface antigens (12), appears to have activity against several separate hematopoietic cell lines. Golub and co-workers (13) have demonstrated activity of antisera against thymocytes and T-reactive lymphocytes. This activity can be selectively removed by thymocyte absorption. Activity against B-cell antigens (14–16) and murine erythrocytes (17) has also been described. The profound effect of antisera on subsequent detection of the murine pluripotent stem cell (CFU-S) was first noted by Golub in 1972 (10). To further characterize the growth kinetics of antisera-treated marrow and the role of the CFU-S in diffusion chamber (DC) myelopoiesis, we examined the growth and differentiation of murine marrow in DC following cell incubation with antisera.

The rapid recovery of stem cells and differentiated progeny in DC following marrow incubation with antisera is particularly striking. Marrow that was virtually devoid of detectable CFU-S and contained variable CFU-C relative to control marrow exposed to normal sera demonstrated equal or greater numbers of CFU-S, CFU-C, macrophages, and proliferating and nonproliferating granulocytes at varying time intervals after DC implantation. Stem cell self-renewal (Table III) and differentiation (Table II and Fig. 1) were equivalent to control DC myelopoiesis, in DC containing antisera-treated marrow with consistently low CFU-S input values. Several possible interpretations of these data exist. First, such rapid recovery of differentiated cell progeny might indicate that the CFU-C rather than the CFU-S is the more appropriate progenitor cell for DC cell growth. However, no correlation appeared to exist between the varying CFU-C cell input in different experiments and eventual DC differentiation and cell production. In addition, such an explanation would not account for the rapid recovery of the pluripotent stem cell, presumably a progenitor of the CFU-C. Alternatively, neither the CFU-S nor the CFU-C may be the requisite progenitor cell for DC growth. The CFU-D described by Jacobsen (18) may, for example, be the more appropriate assayable stem cell for growth in DC.

TABLE III. EFFECT OF CFU-S ANTISERA ON STEM CELL RECOVERY IN DIFFUSION CHAMBERS (DC)

Days of growth	CFU-S/DC			CFU-C/DC			
	No sera	CON + C′[a]	EXP + C′	No sera	CON + C′	EXP + C′	*n*
0	36 ± 5.4	21.2 ± 6.5	0.93 ± 0.22	460 ± 110	247 ± 52	233 ± 75	3
7	270 ± 3.9	36.4 ± 7.1	38.6 ± 3.7	460 ± 14	553 ± 24	504 ± 22	1
8	50 ± 18	63 ± 4	73 ± 14	576 ± 128	786 ± 276	710 ± 70	2
16	—	34.8 ± 3	88 ± 54	—	1059 ± 158	1622 ± 699	2

Note. Day 0 represents numbers of stem cells in DC implanted into irradiated CD_1 host mice. Values are expressed as mean number of stem cell per DC ± 1 SEM from one to three separate experiments. The number of DC per individual experiment harvested on Days 7, 8, and 16 ranged from 10 to 17, 10 to 14, and 8 to 16, respectively.

[a] CON, Marrow incubated with control sera; EXP, marrow incubated with mouse brain antisera. The source of complement (C′) in these experiments was fresh mouse sera.

TABLE IV. PRONASE REVERSAL OF CFU-S ANTISERA EFFECT ON MARROW CFU-S RECOVERY

	n	CFU-S/10^5 cells
CON + C′[a]	9	30.7 ± 2.2
EXP + C′	9	0.81 ± 0.3
CON + C′ and Pronase	8	28.8 ± 2.5
EXP + C′ and Pronase	10	34.5 ± 2.2
Noninjected control	4	0.25 ± 0.25

Note. n, Number of mice per group. Values are expressed as mean ± SEM.

[a] CON, Marrow incubated with control sera + C′; EXP, marrow incubated with mouse brain antisera + C′. Fresh rabbit sera used as a source of complement (C′).

Second, rapid CFU-S renewal may reflect an increased proliferative capacity of antisera-resistant CFU-S. Previous investigators (19–21) have demonstrated that the CFU-S compartment of cells is composed of a heterogenous population as determined by cell size, density, self-replicative potential, repopulating potential, and differentiation. Recent work by Monette *et al.* (22) suggests that extensively absorbed mouse brain antisera may segregate out a small subpopulation of antisera-resistant CFU-S with increased self-renewal potential.

A third explanation for the rapid CFU-S renewal observed in DC is that the antisera effect is on CFU-S detection in the spleen colony assay (i.e., an effect on the f fraction or stem cell seeding) and is separate from its effect on the *in vivo* repopulation potential of the pluripotent stem cell. Prior studies from our laboratory suggest, however, that antibody-coated stem cells do *not* preferentially seed the marrow (vs the spleen) (22). Finally, antisera exposure, rather than initiating *in vitro* complement-induced cytolysis, may mediate a killing effect requiring the *in vivo* participation of the host assay animal's reticuloendothelial system. Such an interpretation is supported by Alder *et al.* (23) who have demonstrated recovery of antibody-treated CFU-S injected into an irradiated assay mouse pretreated with potassium carrageenan, an agent presumed to inactivate the reticuloendothelial system. Support for such an opsonization-like mechanism is also derived from reversal of antisera effect with papain as demonstrated by Van den Engh and co-workers (24), as well as our own data on reversal of antisera-complement effect by pronase.

Van den Engh and Golub (25) utilizing antisera absorbed with erythrocytes, liver and thymus have suggested that granulocyte progenitor cells (CFU-C) are not affected by an antisera retaining potent activity against CFU-S, thereby implying antigenic variation in the process of hematopoietic stem cell differentiation. Subsequent work by these investigators, however, has indicated a certain variability in CFU-C effect, utilizing antisera that have all been similarly absorbed and retain potent anti-CFU-S activity (22). The antisera utilized in our experiments had a variable effect on CFU-C causing, in some instances, a marked inhibition of growth. The discrepancy between our results and those of prior investigators may be explained by variations in antisera preparation or differences in tissue absorptions; it is unlikely, however, that absorption differences in our studies account in total for the variable effect on CFU-C since identically absorbed antisera had widely discrepant effects on CFU-C detection (experiments V, VI, and VII, Table I). It is also possible that stem cell specificity of antisera varies with the lot utilized, independent of differences in preparation. Our data thus indicate the specificity of even highly absorbed mouse brain antisera for CFU-S should not be assumed without experimental verification.

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