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Marrow Colony-Forming Units: Age-Related Changes in Responses to Anti-θ-Sensitive Helper/Suppressor Stimuli (40985)[1]

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Abstract. Earlier studies with the anemic W/W^v mouse and healthy aged mice have revealed the presence in bone marrow of an anti-θ-sensitive regulatory cell (TSRC) with "helper" function. In experiments presented here, treatment of marrow cells from young mice with anti-θ serum or anti-Ly-1.2 serum and complement had no effect on the number of spleen colonies formed in irradiated recipients; similar treatment of marrow cells from old donors, however, resulted in a 40% reduction in colonies which was reversed if young thymus cells were added to the inoculum. When marrow cells from young or old mice injected 4 days before with SRBC were treated with anti-θ or anti-Ly-2.2 serum and complement, spleen colonies increased by about 50%; treatment of the same cells with anti-Ly-1.2 serum had no significant effect on colony formation. Marrow cells from immunized young and old donors treated with anti-θ serum and complement had no colony inhibitory activity when mixed with marrow from normal young donors; however, the same cells were highly inhibitory to CFU-S from old donors. These observations demonstrate the presence in mouse marrow of distinct TSRC populations with CFU-S helper and suppressor functions. Other findings suggest that in old mice one or several CFU regulatory mechanisms are disturbed and/or responses to them are defective.

It has been reported that mouse bone marrow, thymus and spleen normally contain a population of anti-θ-sensitive cells that are required for continuous differentiation of marrow stem cells along the erythroid pathway and that these cells are absent or defective in W/W^v anemic mice (1, 2). The "helper" function of this anti-θ-sensitive regulatory cell (TSRC) is not destroyed by irradiation, and initial cell-to-cell contact between the TSRC and the stem cell or colony-forming unit (CFU) appears to be necessary (2). Work from this laboratory also has demonstrated anti-θ-sensitive CFU helper activity in the mouse and has shown that *in situ,* marrow from healthy old animals is responding to a relative or absolute increase in TSRC helper activity (3).

Data to be presented here support and extend these findings and demonstrate anti-θ-sensitive CFU helper *and* suppressor functions which can be abrogated with anti-Ly-1.2 and anti-Ly-2.2 alloantisera, respectively. Other observations suggest that in old mice one or several CFU regulatory mechanisms are disturbed and/or the responses to them are defective.

Materials and methods. Mice. Bone marrow donors were 3- to 36-month-old male B6AF$_1$ or female B6D2F$_1$ mice; recipients were 3- to 4-month-old male B6AF$_1$ or female B6D2F$_1$ mice. The animals were purchased from the Jackson Laboratory, Bar Harbor, Maine. They were housed in a conventional open colony. In several experiments the donors were injected intraperitoneally with normal saline or washed sheep red blood cells (SRBC) 3 or 4 days before use.

Bone marrow cells. Only healthy donors were used. Mice found to have enlarged spleens or other evidence of malignancy or infection were discarded. Cells were flushed from the femoral cavity with Hank's solution. The cells were washed and counted in a hemocytometer in duplicate. After appropriate adjustments in concentration the cells were injected iv in 0.2

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ml into the lateral tail vein of irradiated recipients.

Treatment of marrow cells with antisera. Cells from young and old donors (5×10^5/ml) were incubated at room temperature for 30 min with AKR anti-C3H thymus serum (anti-θ), mock anti-θ serum (both from Bionetics, Kensington, Md.) at dilutions of 1:40 or 1:20 or anti-Ly-1.2 or anti-Ly-2.2 serum (generously supplied by Dr. Edward A. Boyse, Memorial Sloan–Kettering Cancer Center, New York) or mock anti-θ serum at dilutions of 1:100 or 1:200. The cells were washed once, and guinea pig complement (1:5; anti-θ serum) or freshly thawed rabbit serum (1:5; anti-Ly serum) absorbed with washed mouse spleen cells was added. The cells were incubated for 30 min, washed three times, suspended in the original volume of medium, and injected into syngeneic recipients(10^5 cells per host). In several experiments, cells from normal and presensitized young or old syngeneic donors were mixed 4:1, and 10^5 cells were injected. In another experiment 10^6 thymus cells from young donors were injected together with 10^5 antiserum treated old marrow cells.

Colony-forming unit assay (4). Recipients were given 850 rad of whole-body X radiation (250 kVp, 15 mA, HVL 0.5-mm Cu, target to source, 50 cm; less than 0.1 macroscopic colony per uninjected control) on the day of cell transfer. The irradiated mice were housed five per cage, and they were given water containing neomycin. Eight or nine days after being injected iv with 10^5 syngeneic cells the mice were killed, and the spleens were fixed in Bouin's solution. Surface nodules (spleen colonies) were counted under 4 × magnification. All donors were studied individually, and there were at least five recipients in each group.

Results. Effect of anti-θ serum and complement on spleen colony formation by marrow cells from normal mice (Table I). The generation of spleen colonies by young marrow cells was unaffected by treatment with anti-θ serum and complement; however, similar treatment of marrow from old

TABLE I. EFFECT OF ANTI-θ SERUM AND COMPLEMENT ON SPLEEN COLONY FORMATION BY MARROW CELLS FROM YOUNG AND OLD B6AF$_1$ MICE

Experiment	Age of donor (months)	Spleen colonies ± SD		
		Marrow treatment		
		Mock θ + C′	θ + C′	θ + C′/mock θ + C′
18	3	10.4 ± 1.5	9.8 ± 1.6	0.94
	33	12.6 ± 1.5	9.0 ± 1.8	0.71
20	3	14.4 ± 3.4	15.8 ± 2.1	1.10
	33	14.2 ± 3.0	7.8 ± 2.2	0.55
21	3	15.8 ± 1.6	17.6 ± 2.2	1.11
	33	15.4 ± 4.2	8.4 ± 2.1	0.54
23	3	12.0 ± 2.6	11.6 ± 3.2	0.97
	34	12.1 ± 2.9	8.4 ± 4.1	0.69
27	3	9.8 ± 4.8	13.0 ± 3.2	1.33
	36	11.0 ± 2.5	6.1 ± 2.0	0.54
36	3	17.4 ± 4.2	18.0 ± 4.5	1.03
	36	15.1 ± 2.0	9.0 ± 2.9	0.60
40	6	18.0 ± 2.3	15.3 ± 1.5	0.85
	26	15.4 ± 4.0	10.0 ± 1.9	0.65
41	8	20.8 ± 2.8	20.0 ± 3.4	0.96
	26	19.2 ± 3.1	12.0 ± 1.8	0.62
Mean	Young			1.03 ± 0.14[a]
	Old			0.61 ± 0.07[a]

[a] $P < 0.002$, Student's t test.

TABLE II. EFFECT ON SPLEEN COLONY FORMATION OF ADDING YOUNG THYMUS CELLS (Tx) TO OLD B6AF$_1$ MARROW WHICH HAD BEEN TREATED WITH ANTI-θ SERUM AND COMPLEMENT

Age of donor (months)	Treatment of donor cells	Spleen colonies ± SD		
		10^5 BM only	10^5 BM + 10^6 Tx	BM + Tx/BM
33	None	12.4 ± 3.3	10.2 ± 2.3	0.82
33	None	13.6 ± 3.4	17.2 ± 3.5	1.26
36	None	24.2 ± 2.5	21.1 ± 3.6	0.87
36	Mock θ + C′	11.0 ± 2.5	10.9 ± 3.4	0.99
36	Mock θ + C′	15.0 ± 2.0	13.8 ± 3.6	0.92
36	anti-θ + C′	6.0 ± 2.0	9.8 ± 3.6	1.60
36	anti-θ + C′	9.0 ± 0.6	13.0 ± 2.9	1.44

mice resulted in a 40% reduction in macroscopic colonies. The latter effect was almost completely reversed when 10^6 young thymus cells were added to the anti-θ serum-treated old marrow (Table II); the addition of thymus cells to similarly treated young marrow produced no significant change in colony formation (3).

Effect of anti-θ serum and complement on spleen colony formation by marrow cells from preimmunized mice (Table III). Young and old B6AF$_1$ and B6D2F$_1$ mice were given SRBC ip 3 or 4 days before sacrifice, and their marrow cells were treated with mock anti-θ or anti-θ serum and complement. It was found that treatment of the marrow cells taken from mice 3 days after injection of SRBC with anti-θ serum had no

TABLE III. EFFECT OF ANTI-θ SERUM AND COMPLEMENT ON SPLEEN COLONY FORMATION BY MARROW CELLS FROM YOUNG AND OLD B6AF$_1$ AND B6D2F$_1$ MICE GIVEN SRBC INTRAPERITONEALLY 3 OR 4 DAYS BEFORE SACRIFICE

Experiment (strain)	Age of donor (months)	Day SRBC given	Spleen colonies ± SD		
			Marrow treatment		
			Mock θ + C′	θ + C′	θ + C′/mock
18 (B6AF$_1$)	6	−3	11.0 ± 0.3	14.0 ± 1.8	1.3
	33		12.6 ± 1.5	9.0 ± 1.6	0.8
20 (B6AF$_1$)	3	−3	15.8 ± 1.6	17.6 ± 2.2	1.1
23 (B6AF$_1$)	3	−3	19.8 ± 4.2	15.5 ± 3.1	0.8
	34		12.0 ± 2.9	8.4 ± 4.1	0.7
41 (B6AF$_1$)	8	−3	21.8 ± 1.9	15.3 ± 7.5	0.7
	26		16.8 ± 1.9	15.8 ± 1.8	0.9
Mean	Young	−3			1.0 ± 0.3[a]
	Old				0.8 ± 0.1[b]
37 (B6D2F$_1$)	3	−4	15.7 ± 5.5	27.8 ± 2.7	1.8
38 (B6AF$_1$)	3	−4	10.4 ± 1.8	15.8 ± 1.6	1.5
	26		6.7 ± 1.5	10.4 ± 2.5	1.9
39 (B6AF$_1$)	3	−4	4.8 ± 3.5	11.6 ± 0.9	2.4
	26		12.2 ± 2.4	15.0 ± 5.9	1.2
Mean	Young	−4			1.9 ± 0.5[a]
	Old				1.6 ± 0.5[b]

[a] $P < 0.01$: young donors injected with SRBC on Day −3 vs Day −4.
[b] $P < 0.01$: old donors injected with SRBC on Day −3 vs Day −4.

significant effect upon the number of colonies found; however, by the fourth day the same treatment resulted in a 1.6- to 1.9-fold increase in spleen colonies. This suggests that marrow cells from young and old mice are at least partially regulated by TSRC suppressor, as well as helper, cells which can be generated in response to the injection of xenogeneic RBC.

Effects of anti-Ly-1.2 and anti-Ly-2.2 sera and complement on spleen colony formation (Table IV). As was noted with anti-θ serum the generation of spleen colonies by young marrow cells from saline-injected donors was unaffected by treatment with anti-Ly-1.2 serum and complement but similar treatment of marrow from old mice resulted in a significant reduction in detectable spleen nodules. Similarly, it was found that anti-Ly-2.2 treatment of young and old marrow cells from mice injected 4 days previously with SRBC resulted in about a 50% increase in colonies; anti-Ly-1.2 serum decreased colony formation slightly but not significantly.

Effects of anti-θ serum-treated marrow cells from SRBC injected donors on spleen colony formation by marrow cells from normal young and old donors (Table V). Marrow cells from young and old mice injected ip 4 days before with SRBC were treated with mock anti-θ or anti-θ serum and complement and mixed 1:4 with marrow cells from saline-injected young and old donors. It was found that treatment of the "inhibitory cells" with anti-θ serum almost completely abolished the CFU suppressor activity if the washed cells were mixed with marrow from young donors; however, the same cells (young and old) remained highly inhibitory to CFU from old donors.

Discussion. The regulation of hematopoietic stem cells is complex; both hormonal and cellular systems have been shown to be involved in the differentiation and proliferation of marrow constituents. Macrophages and lymphocytes appear to be among the significant contributors to the regulation of CFU (5). It has been shown that (a) thymocytes enhance the formation of spleen colonies by radiation-damaged CFU and by parental CFU transplanted into F_1 recipients (6, 7), (b) activated T cells elaborate a factor which increases *in vivo* colony formation (8), (c) lymphocytes have both suppressor (9) and enhancing (10) effects on erythroid differentiation, (d) bone marrow from normal littermates which has been treated with anti-θ or anti-Ly-1 serum and complement continues to produce normal numbers of spleen colonies in anemic W/W^v mice but no longer corrects the anemia (the anemia is corrected if viable syngeneic thymocytes are added to the marrow inoculum or if the recipient is treated with thymosin after transplantation) (1, 11, 12), (e) marrow from old mice is responding to a relative or absolute increase in TSRC helper activity (3), and (f) θ^- marrow cells of several types have helper and suppressor effects on the proliferation and differentiation of marrow components (5, 13–16).

TABLE IV. EFFECTS OF ANTI-Ly-1.2 AND ANTI-Ly-2.2 SERA AND COMPLEMENT ON SPLEEN COLONY FORMATION BY MARROW CELLS FROM YOUNG AND OLD B6D2F$_1$ FEMALE MICE INJECTED 4 DAYS BEFORE WITH SALINE OR SRBC

Experiment No.	Age of donors (months)	Spleen colonies ± SD				
		Saline-injected donors		SRBC-injected donors		
		Mock anti-θ	Anti-Ly-1.2	Mock anti-θ	Anti-Ly-1.2	Anti-Ly-2.2
45	3	8.2 ± 3.4	9.6 ± 2.6	9.2 ± 1.6[a]	8.8 ± 1.8	13.2 ± 3.8[a]
	27	7.3 ± 1.2[b]	4.2 ± 0.8[b]	6.2 ± 2.7[a]	5.4 ± 2.6	9.7 ± 2.3[a]
47	3	11.4 ± 2.5	10.6 ± 2.2	11.8 ± 3.8[a]	9.0 ± 0.8	16.2 ± 2.8[a]
	27	9.0 ± 0.5[b]	4.1 ± 1.4[b]	14.3 ± 2.2[a]	12.3 ± 2.4	19.4 ± 3.6[a]

[a] $P < 0.05$: SRBC-injected donors, mock anti-θ vs anti-Ly-2.2 treatment.
[b] $P < 0.05$: saline-injected donors, Mock anti-θ vs anti-Ly-1.2 treatment.

TABLE V. INHIBITION OF SPLEEN COLONY FORMATION BY MARROW CELLS FROM YOUNG AND OLD DONORS BY MARROW FROM SRBC-INJECTED MICE TREATED *IN VITRO* WITH MOCK ANTI-θ OR ANTI-θ SERUM AND COMPLEMENT[a]

Experiment (strain)	Age of donor of normal cells (months)	Age of donor of inhibitory cells (months)	Percentage inhibition	
			Treatment of inhibitory cells	
			Mock θ + C′	θ + C′
37 ($B6D2F_1$)	4	4	39	8
	4	25	34	4
38 ($B6AF_1$)	4	4	31	13
	4	25	61	21
39 ($B6AF_1$)	4	4	31	2
	4	26	13	3
Mean		Young	33.6 ± 4.6	7.7 ± 5.5
		Old	36.0 ± 24.0	9.3 ± 10.1
37 ($B6D2F_1$)	25	4	37	44
	25	25	53	32
38 ($B6AF_1$)	25	4	33	35
	25	25	49	58
39 ($B6AF_1$)	26	4	16	30
	26	26	32	39
Mean		Young	28.6 ± 11.1	36.3 ± 7.1
		Old	44.6 ± 11.2	43.0 ± 13.4

[a] Young or old mice were injected ip with 0.2 ml SRBC 4 days before they were killed. Marrow cells were obtained and treated with Mock anti-θ or anti-θ serum and complement. They were then mixed 1:4 with marrow cells from normal young or old mice and 10^5 cells were injected iv into lethally irradiated young hosts. Eight to nine days later spleen nodules were counted. Percentage inhibition was calculated as:

$$\frac{\text{CFU-S}/10^5 \text{ cells (4 parts normal + 1 part inhibitory)}}{4/5 \text{ CFU-S}/10^5 \text{ normal} + 1/5 \text{ CFU-S}/10^5 \text{ inhibitory}} \times 100.$$

In the experiments reported here it was found as has been reported previously that treatment of marrow cells from normal young mice with anti-θ or anti-Ly-1 sera and complement has no significant effect on colony formation (11, 17). However, similar treatment of marrow from old mice reduced colony formation by up to 40%, and this could be reversed by the addition of 10^6 young thymocytes to the inoculum. These observations suggest that θ^+ cells either play a small role or no role in the regulation of colony formation in young animals, but that in old mice CFU are responding to a primary or secondary increase in θ^+ helper activity.

When marrow cells from young and old mice injected 3 days before with SRBC were studied, it was found that the number of spleen colonies formed per 10^5 cells transferred and their reponses after treatment with anti-θ serum and complement were not significantly different from those noted with marrow cells from noninjected young and old mice. However, by the next day, treatment of marrow cells from young and old SRBC-injected mice with anti-θ or anti-Ly-2 serum and complement resulted in a significant increase in colony formation even though the number of colonies formed per 10^5 untreated cells again was not significantly different from that noted with marrow from age-matched noninjected donors. This suggests that following the injection of SRBC the increase in θ^+ inhibitory activity is balanced by an increase in θ^- helper activity. The addition of marrow from young or old mice injected 4 days before with SRBC to marrow from noninjected young and old donors resulted in significant inhibition of colony formation which was abrogated when the inhibitory cells were treated

TABLE VI. A MODEL OF AGE-RELATED CHANGES IN THE REGULATION OF MURINE MARROW CFU BY MARROW CELLS

Source of bone marrow (functional state)	Net effect of θ^- CFU-regulating systems	Net effect of θ^+ CFU-regulating system(s)	Net effect of θ^- suppressor of helper T cell	Net change observed in spleen colonies/ 10^5 cells	Effect on spleen colonies of elimination of θ^+ cells
Young (resting)	BALANCED[a]	balanced	NS[b]	NS	NS
Young (SRBC −4 days)	HELPER	SUPPRESSOR	suppressor	NS	Increased
Old (resting)	SUPPRESSOR	HELPER	NS	NS	Decreased
Old (SRBC −4 days)	HELPER	SUPPRESSOR	suppressor	NS	Increased

[a] Capital letters signify that the regulatory system has a major effect on CFU. Lowercase letters indicate that the system has a relatively minor direct effect on CFU.
[b] Not significant.

with anti-θ serum and complement and then mixed with cells from normal young donors. However, in apparent conflict with these results, the same cell preparations remained inhibitory to colony formation by marrow from normal old donors. (The reader is referred to (13) for a review of the complex interactions of T-cell regulatory circuits with immunological and other cell systems.)

In an effort to explain the observations presented here, the broad outline of a cellular CFU regulatory mechanism has been constructed and is presented in Table VI. It has been assumed that (i) in the "resting" state and, within limits, in the perturbed state there are pressures to maintain colony-forming potential at a constant concentration, (ii) the injection of SRBC induces an increase in a population(s) of suppressor cells which lacks significant quantities of surface θ and which expresses suppressor activity by blocking the action of helper T cells and other θ^- CFU regulators (although the existence of a cell with these functions is, in part, hypothetical, there is evidence which suggests that such a cell(s) may be present in marrow (14–16)), (iii) hormones and the microenvironment contribute importantly to CFU regulation, and (iv) in the young adult mouse θ^+ cells play a minor role in the regulation of CFU.

Under these conditions, it is postulated that in the resting state the marrow in young mice is regulated by the actions of θ^- systems and that the contribution of the θ^+ system is minor but balanced as to helper and suppressor activity. The elimination of θ^+ or Ly-1$^+$ cells in this case would not be expected to produce significant changes in colony formation. In the old mouse, however, for reasons not clear, θ^+ helper activity is increased in the resting state, and this is balanced by an increase in θ^- suppressor activity which is distinct from that expressed through inhibition of θ^+ helper cells. Elimination of θ^+ cells here would result in a net increase in suppressor activity and a decrease in spleen colony formation.

The injection of young and old mice with SRBC results in a phasic increase in θ^+ suppressor activity which appears to be balanced by the sum of an increase in θ^- helper activity and θ^- suppressor activity which is expressed in part by blocking θ^+ helper cells. Anti-θ and anti-Ly-2 sera eliminate θ^+ suppressor activity with a resultant relative increase in helper activity from θ^- systems, and colony formation is increased. The addition of these untreated inhibitory cells to young marrow results in decreased colony formation as a result of the action of the induced θ^+ suppressor cells; inhibition of colony formation by marrow from old mice is expressed through θ^+ cells and the action of the induced θ^- suppressor on the expanded population of θ^+ helper cells. It follows from this that the addition of anti-θ serum treated marrow cells from SRBC-injected young and old mice

would have no significant effect on colony formation by marrow from young mice because the elimination of θ^+ suppressor cells would result in a very small net increase in helper activity; however, the same preparation would continue to inhibit colony formation by old marrow primarily because of the interaction of the induced θ^- suppressor cells with the θ^+ helper cells in resting old marrow.

In summary, the studies presented here demonstrate the presence in mouse marrow of distinct anti-θ-sensitive cells with CFU helper and suppressor functions. The observation that helper function is abrogated by anti-Ly-1 serum and suppressor activity by anti-Ly-2 serum suggests that these regulatory cells may be identical to or subsets of the T cells which regulate lymphocyte functions. Other findings show that in old mice one or several of the CFU regulatory mechanisms are disturbed and/or responses to them are defective.

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