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DIFFERENTIATION OF THY 1-BEARING CELLS FROM PROGENITORS IN LONG-TERM BONE MARROW CULTURES[1]

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Lone-term marrow cultures, established according to the technique described by Dexter, contain a colony-forming cell (CFU-T) that produces Thy 1-positive progeny after stimulation with PHA-induced conditioned medium from human leukocytes. When 10^{-7} M hydrocortisone is added to the medium for the long-term cultures, CFU-T can be maintained *in vitro* for at least 16 weeks. Cells from individual colonies lack precursors for cytotoxic lymphocytes but can provide nonspecific help required for the generation of cytotoxic lymphocytes.

Many classes of myeloid and lymphoid progenitors can form clones of mature cells in semisolid media (1). Although these techniques give information on the regulation and differentiation potential of particular precursors, there is need for *in vitro* culture techniques that allow the differentiation of mature functional cells from pluripotent stem cells in an environment closely approximating *in vivo* conditions. Dexter *et al.* (2) have described a tissue culture system that supports the growth of pluripotent stem cells (3) for many weeks. Dexter *et al.* (4) and Williams *et al.* (5) have observed granulopoiesis and erythropoiesis, but not lymphopoiesis in these cultures. In a previous study, we found that cells from Dexter cultures retain their potential for lymphoid differentiation and will produce functional lymphocytes in irradiated recipients (6, 7). Similar observations have been made by Schrader and Schrader (8) who have also shown (9) that some cells *in vitro* contain the enzyme terminal deoxynucleotidyl transferase, a possible marker for immature lymphoid precursors (10). In the present, study we demonstrate the presence of putative T lymphocyte colony-forming cells (CFU-T)[5] (11) in long-term cultures of mouse bone marrow cells.

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[5] Abbreviations used in this paper: CFU-T, cell or cells producing in *in vitro* colonies of Thy 1-bearing cells; CFU-S, multipotential stem cells detected by their ability to form colonies in the spleens of irradiated mice; CFU-C, cells producing *in vitro* colonies of granulocytes and macrophages; CLP, the precursors of cytotoxic T lymphocytes; A-MEM, α-minimal essential medium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

MATERIALS AND METHODS

Mice. Female F_1 hybrids resulting from crossing strains C57BL/6 and C3H (BCF1) were obtained from BioBreeding Laboratories, Ottawa, Ontario; AKR and DBA/2 mice from The Jackson Laboratory, Bar Harbor, Maine; and B6.Thy 1.1 mice from Cedarlane Laboratories, London, Ontario. RNC mice, both normals and nudes, were bred in the animal facility at the Ontario Cancer Institute. The properties of these mice have been described by Croy and Osoba (12).

Long-term cultures. Long-term bone marrow cultures were established by using the method described by Dexter *et al.* (4) and incorporating modifications by Williams *et al.* (5) and ourselves (7). Adherent layers were established by aspirating the contents of one femur directly into a 25 cm^2 tissue culture flask (Falcon No. 3013) containing 8 ml of α-minimal essential medium (A-MEM; K. C. Biologicals, Lenexa, Kan.) supplemented with 20% (v/v) horse serum (Woodlyn Farms, Guelph, Ontario). In some experiments, 10^{-7} M hydrocortisone was added to the cultures to increase their longevity (13). After incubation for 1 week, all of the growth medium and nonadherent cells were removed and replaced by 8 ml of fresh growth medium containing 10^6 fresh bone marrow cells, prepared as a single cell suspension (the "recharge" cell population). All cultures were maintained at 33°C in a humidified atmosphere of 5% CO_2 and air. At weekly intervals the cultures were fed by removing one-half the growth medium, along with one-half the nonadherent cells, and replacing it with fresh medium. The harvested cells were centrifuged at 275 × G for 15 min, resuspended in the appropriate medium, and used for the assays described below.

Colony assays. a) CFU-S.[5] These were measured as described by Till and McCulloch (3). Spleen colonies were counted 9 to 11 days after injection of the cells into irradiated recipients.

b) CFU-C.[5] Restricted granulocyte/macrophage progenitors (1) were assayed by immobilizing 5×10^4 cells (suspended in A-MEM) in 0.8% (w/v) methyl cellulose (Dow Chemical Co., Midland, Mich.) and A-MEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and 5×10^{-5} M 2-mercaptoethanol. Twenty percent L cell-conditioned medium was used as a stimulator (14). Cells were plated into 35-mm plastic Petri plates (Lux Scientific Corp., Thousand Oaks, Calif.) in 1-ml volumes. Colonies of greater than 20 cells were counted after 15 days of incubation at 37°C in a humidified atmosphere of 8% CO_2 and air.

c) CFU-T. As described by Jacobs and Miller (11), the desired number of cells was placed in 35-mm dishes in 1 ml of A-MEM containing 0.8% (w/v) methylcellulose, 20% (v/v) FCS (Lot No. 4055979, Flow Laboratories, Rockville, Md.), and 40% (v/v) PHA-leukocyte-conditioned medium (15) and 5×10^{-5} M 2-mercaptoethanol. Only certain batches of FCS and conditioned medium will support colony formation by CFU-T. Col-

onies, which contained at least 300 cells, were counted after 5 days' incubation at 37°C in a humidified atmosphere of 5% CO_2 and air. Typically, colonies contained 300 to 1000 cells.

d) Precursors of cytotoxic T lymphocytes (CLP) and accessory cells. The limiting assay described by Teh *et al.* (16) was used. The essential components of this assay are a source of CLP, accessory cells, and stimulator cells. Standard cultures contained 3×10^3 normal RNC lymph node cells as responders, 10^5 RNC nude spleen cells as accessory cells, and 3×10^5 irradiated (1500 R) DBA spleen cells as stimulators. The cells were incubated in 0.2 ml of culture medium and then tested for cytotoxicity on day 5 by using ^{51}Cr-labeled P815 tumor cells and a ^{51}Cr-release assay as described previously (16). To test CFU-T for CLP, the lymph node cells were replaced by a single colony picked from cultures at 6 days with a Pasteur pipet. To test for accessory cells, the nude spleen cells were replaced by a single colony (11).

Antisera. Rabbit anti-mouse brain Thy-1 antiserum was provided by Dr. M. Letarte (Ontario Cancer Institute). The properties of this antiserum that was prepared by immunization with purified brain Thy-1 antigen have been described (17). We used the antiserum at a dilution of 1/75; this concentration together with 1/15 C is cytotoxic for 98% of thymocytes. Lyophilized ascitic fluid from the F7D5 hybridoma secreting anti-Thy 1.2 antibody was kindly provided by Dr. P. Lake (Tumor Immunology Unit, University College London, London, England). The cytotoxic titer of F7D5 for thymocytes varied from one reconstituted sample to another. The batch (No. 2) used to characterize the specificity of F7D5 had a 50% cytotoxicity titer for thymocytes of 1/5000. Other batches (No. 3 and No. 4) used to test colonies had titers of up to 1/40,000. All batches were prepared from the same pool of ascitic fluid. All dilutions of antiserum were made with A-MEM containing 10% heat-inactivated FCS and 10 m*M* HEPES (see abbreviations).

The specificity of the F7D5 hybridoma was tested by absorption with thymocytes from different mouse strains. For absorption, the reconstituted antiserum was diluted 1/40, and thymocytes were added to give a concentration of 2×10^8 cells/ml. This mixture was incubated overnight at 4°C; the thymocytes were removed by centrifugation, and aliquots of the absorbed antibody were stored at −70°C.

Treatment of cells with antisera. Individual colonies were picked and treated in microtest plates (H 3034, Falcon Plastics, Oxnard, Calif.) according to the method of Jacobs and Miller (11). Rabbit C (Cedarlane Laboratories Ltd., London, Ontario) was used at dilutions of 1/45 or 1/15. Viability was determined by eosin dye exclusion.

For treatment of CFU-T, cells from long-term cultures were suspended at 2×10^6 cells/ml in F7D5 antiserum at a dilution of 1/2000. The suspension was incubated for 30 min at 0°C, washed once, resuspended in 1/15 rabbit C, and incubated for 45 min at 37°C. The suspension was then washed twice and plated to test colony formation.

RESULTS

Serial measurements of CFU-S, CFU-C, and CFU-T were made in several experiments, three of which are summarized in Table I. Several conclusions can be made from these experiments. CFU-S and CFU-C (data not shown) can be maintained for at least 6 weeks. The observation that the number of colony-forming units remains high despite the weekly removal of one-half of the cells indicates that these progenitors are being continuously produced in the cultures. When 10^{-7} M hydrocortisone is added (expt. 3), these activities are detectable for at least 16 weeks. CFU-T were detected at all times studied. Because 5×10^5 cells gave the optimal efficiency for CFU-T in earlier studies of spleen cell suspensions from nude mice (11), we initially used this concentration (expts. 1 and 2). However, it appears to be too high for cultured bone marrow cells, which give higher plating efficiency at lower concentrations (expt. 3).

The colonies stimulated by PHA-induced conditioned medium have the typical appearance of T lymphocyte colonies (11); they float, are densely packed, and are composed of peroxidase-negative, mononuclear cells (data not shown). To test for presence of the Thy 1 antigen, the cells from individual colonies were treated with antibody and C as described above. Table II shows the results of testing individual colonies from four different experiments. With both the hybridoma antibody

TABLE I

CFU-S, and CFU-T in long-term cultures of bone marrow

Expt. No.	Weeks After Recharge[a]	Nucleated Cells per Flask ($\times 10^{-5}$)	CFU-S/ Flask	CFU-T	
				No. cells plated	No. colonies
1	2	26	123	5×10^5	244
	3	17	156	5×10^5	177
	4	62	334	5×10^5	224
	5	51	228	5×10^5	287
	6	42	71	5×10^5	38
2	2	19	56	5×10^5	83
	3	14	75	5×10^5	163
	4	58	321	5×10^5	120
	5	72	198	5×10^5	254
3[b]	12	33	131	3×10^5	55
				3×10^4	23
	14	28	325	5×10^5	111
	16	20	295	4×10^5	132
				1×10^5	58
				5×10^4	14

[a] Recharge denotes addition of 1×10^6 fresh bone marrow cells 1 week after establishing adherent layers.

[b] Cultured in medium containing 10^{-7} M hydrocortisone.

TABLE II

Treatment of individual colonies with anti-T cell antisera

Expt. No.	Weeks After Recharge	% Viability[b]			
		Antiserum[a] + C	(Mean ± S.E.M.)	C alone	(Mean ± S.E.M.)
1	2	36, 45, 61, 42, 54, 47, 22, 41, 59, 47, 39	(45 ± 3)	86, 86, 77, 71, 67	(77 ± 4)
	3	35, 59, 15, 55, 33, 29, 48	(39 ± 6)	70, 79, 63, 81	(73 ± 4)
4	4	11, 30, 45, 13	(24 ± 8)	92, 76, 82	(83 ± 5)
5	3	0, 14, 17	(10 ± 5)	97, 97, 98, 44	(84 ± 13)
6	5	45, 5, 53	(34 ± 15)	100, 94, 93	(96 ± 2)

[a] In experiments 1, 4, and 5, rabbit anti-mouse brain Thy 1 was used at a dilution of 1/75. In experiment 6, hybridoma anti-Thy 1.2 (F7D5) was used at a dilution of 1/20,000. With this batch of hybridoma antiserum, a dilution of 1/20,000 killed 70% of mouse thymocytes. In experiment 1, rabbit C was used at a dilution of 1/45; in other experiments the final dilution was 1/15.

[b] Each number represents the percent viability of cells from a single colony. The mean ± S.E.M. is given in parentheses.

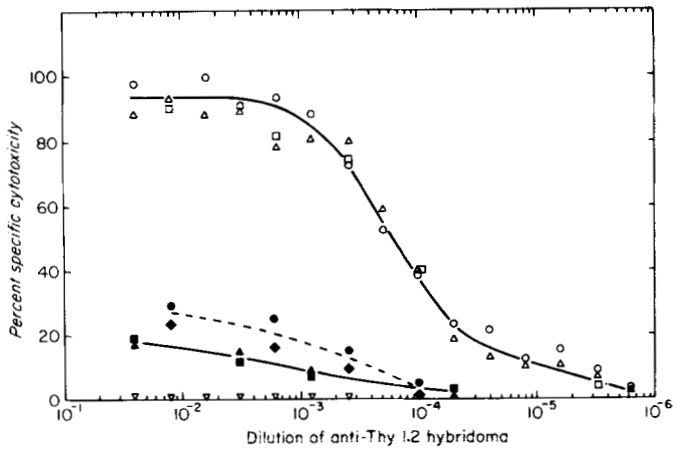

Figure 1. Varying dilutions of the F7D5 hybridoma were tested for cytotoxicity against BCF_1 thymocytes (*open symbols*) or against cells from pooled colonies grown from BCF1 bone marrow cells maintained in Dexter cultures (*closed symbols*) for 6 weeks (●, ◆) or 19 weeks (■, ▲). The serum was used either unabsorbed (□, ■) or absorbed with B6 Thy 1.1 thymocytes (○, ●), AKR thymocytes (△, ▲, ◆), or BCF_1 thymocytes (▽). The rabbit C alone gave between 5 and 15% cytotoxicity; this background lysis was subtracted from all the cytotoxicity values to give the value of specific cytotoxicity on the oridinate. All of the specificity tests were done with batch 2.

and the rabbit anti-mouse brain Thy 1, there was significant killing of cells in the colonies; the increased killing observed in experiments 4, 5, and 6 compared with experiment 1 probably resulted from the use of a higher concentration of C in the latter experiments. Cells from individual CFU-C colonies showed no specific killing with either antiserum; the colonies were tested between days 5 and 7, at a time when the colonies are a mixture of macrophages and granulocytes.

Although a variable proportion of cells in CFU-T are sensitive to anti-Thy 1 and C, all of the cytotoxicity appears to be the result of anti-Thy 1 activity in the two sera used. The rabbit anti-Thy 1 has been characterized by Letarte and Meghji (17). Absorption tests with the F7D5 hybridoma anti-Thy 1.2 showed that all of the detectable cytotoxicity was the result of anti-Thy 1.2 activity (Fig. 1). Only absorption with BCF1 thymocytes removed the cytotoxicity of the hybridoma for BCF1 targets. Absorption with B6-Thy 1.1 thymocytes or with AKR thymocytes had no effect on the cytotoxicity of the hybridoma for BCF1 thymocytes or for colonies obtained from long-term cultures of BCF1 bone marrow. Table III shows the results of other tests of the absorbed antisera on pooled colonies from CFU-T or CFU-C. Despite the low proportion of the cells from CFU-T killed in these experiments, the absorption tests indicate that the killing is the result of anti-Thy 1.2 activity. Only absorption with cells bearing the Thy 1.2 specificity removed the cytotoxic activity against pooled colonies derived from CFU-T.

In three experiments, we measured the effects of F7D5 antiserum and C on CFU-T; Table IV gives the results of a typical experiment. None of the experiments showed significant killing of CFU-T by this treatment.

The functional activities contained in colonies from both normal spleen[6] and from nude spleen (11) have been studied. Both contain accessory cell activity, but only normal spleen T cell colonies contain CLP. The Thy+ colonies derived from Dexter cultures were tested for CLP and for accessory cells as described in *Materials and Methods*. The results of these

[6] Ching, L. M. and R. G. Miller. Manuscript in preparation.

TABLE III

Effects of absorbed antisera on pooled colonies from CFU-T

Expt. No.	Type of Pooled Colonies[a]	Antiserum	Dilution	Absorption	% Cytotoxicity	
					Antiserum + C	C alone
8	CFU-T	F7D5 (batch 1)	1/40	None	50	15
	CFU-T	F7D5 (batch 1)	1/40	AKR thymus	44	15
9	CFU-T	F7D5 (batch 1)	1/40	None	33	15
	CFU-T	F7D5 (batch 1)	1/40	AKR thymus	33	15
10	CFU-T	F7D5 (batch 2)	1/200	None	48	5
	CFU-T	F7D5 (batch 2)	1/20,000	None	62	5
		Rab anti-Thy 1	1/45	None	50	5
11	CFU-T	F7D5 (batch 4)	1/80	None	78[b]	18
	CFU-T	F7D5 (batch 4)	1/80	AKR thymus	39	15
		F7D5 (batch 4)	1/80	B6. Thy 1.1	46	19
	CFU-C	F7D5 (batch 4)	1/80	None	21	19
12	CFU-T	F7D5	1/80	None	39	19
	CFU-T	F7D5	1/80	BCF1-1[c]	20	19
	CFU-T	F7D5	1/80	BCF1-2[c]	17	19

[a] Colonies were grown from Dexter cultures between weeks 8 and 18; all cultures contained 10^{-7} M hydrocortisone.

[b] The absorbed sera for this experiment were frozen and thawed one more time than the serum used for the control. Freezing and thawing of the F7D5 reagent always results in loss of cytotoxic activity.

[c] Two different samples of absorbed antibody were tested.

TABLE IV

Treatment of CFU-T with F7D5 hybridoma anti-Thy 1.2 antibody

Expt. No.	Weeks After Recharge[a]	No. of Cells Plated	No. of Colonies After Treatment With		% Killed
			Anti-Thy 1.2[b] + C	C Alone	
7	6	3×10^5	433	503	14
		1×10^5	192	178	0
		5×10^4	75	92	18

[a] Culture medium contained 10^{-7} M hydrocortisone.

[b] F7D5 was used at a dilution of 1/2,000. This concentration killed 95% of thymocytes.

experiments are summarized in Table V. No CLP activity was detected when individual colonies were used to replace the LN node cells in the assay for CLP (group 6). However, individual colonies have significant accessory cell activity and markedly enhance the cytotoxic response of lymph node CLP; e.g., compare the cytotoxic activity of group 5 with that of group 2.

DISCUSSION

Dexter *et al.* (4) previously found that long-term marrow cultures maintain granulocyte and erythrocyte precursors but that mature lymphoid cells were undetectable. We have previously shown (6, 7), as have Schrader and Schrader (8), that cultured cells can produce lymphoid progeny when they are grafted into irradiated recipients. The data in this paper provide direct evidence for the precursors of Thy 1-bearing cells in Dexter cultures. The results summarized in Tables II, III, and IV suggest that the colony-forming cell that is insensitive to anti-Thy 1 antibody and C differentiates into sensitive progeny after stimulation wtih PHA-induced conditioned medium. It is not clear whether resistance to this treatment indicates an absence of Thy 1 antigen on CFU-T or other factors, such as a

TABLE V

Functional activities of cells from individual colonies

Group No.	Responder Cells[a]	Accessory Cells[b]	Stimulator Cells[c]	Expt. 1[d] Positive/Total[e]	Cytotoxic Activity[f]	Expt. 2 Positive/Total	Cytotoxic Activity[f]
1	RNC LN	nu/nu Spleen	DBA Spleen	6/6	41.7	12/12	71.7
2	RNC LN	0	DBA Spleen	2/6	3.5	12/12	10.5
3	0	nu/nu Spleen	DBA Spleen	0/6	0	0/12	−0.6
4	0	0	DBA Spleen	0/6	−2.1	0/12	−1.3
5	RNC LN	1 colony	DBA Spleen	11/12	10.5	18/18	24.6
6	1 Colony	nu/nu Spleen	DBA Spleen	1/12	1.2	0/18	−0.5
7	0	1 colony	DBA Spleen	0/12	−1.6	0/12	−1.7

[a] Each culture contained 3×10^3 lymph node cells from normal RNC donors or one T colony from a Dexter culture. The colonies were picked at 6 days.

[b] Accessory cells were either 10^5 spleen cells from nude RNC donors or single T colonies.

[c] 3×10^5 indicated (1500 rad) spleen cells.

[d] After 6 days of incubation, individual cultures were assayed for cytotoxicity to P815 targets. To maximize lysis, the assays were done in the presence of PHA.

[e] Number of cultures giving cytotoxic responses significantly greater than the spontaneous cultures.

[f] The cytotoxic activity is defined as $-100\ ln(1 - p)$ where p is the fractional specific ^{51}Cr release (18). This activity is directly proportional to the number of cytotoxic lymphocytes per culture and is approximately equal to the percent specific ^{51}Cr release for small values; an activity of 10 corresponds to 10% release, 30 to 26%, and 100 to 63%.

very low density of Thy 1 or reduced susceptibility to C-mediated lysis.

The *in vitro* differentiation of CFU-T during the colony assay may account for the marked variability among colonies in the proportion of cells killed by anti-Thy 1 and C (Table II). If, during colony formation, the cells differentiated from a state insensitive to lysis to a sensitive state, it is unlikely that all of the cells within a colony will differentiate at the same time. Therefore, at any given time one would expect to observe marked colony-to-colony variation in the frequency of cells sensitive to anti-Thy 1 and C.

Normal bone marrow contains CFU-T with properties similar to those detected in Dexter cultures (11). For this reason, it is difficult to interpret the significance of their continued presence in long-term cultures. CFU-T could be maintained by self-renewal of the CFU-T initially placed in culture or by differentiation from immature progenitors, perhaps stem cells. Experiments are currently in progress to distinguish between these alternative mechanisms.

Only preliminary attempts have been made to characterize the functional properties of cells derived from the CFU-T in Dexter cultures. The cells from a colony do not contain precursors for cytotoxic lymphocytes. However, a single colony can replace the requirement for nude spleen cells in a limiting dilution assay for cytotoxic lymphocytes (Table V).

The cells in the colonies derived from CFU-T from Dexter cultures may not be typical T cells. However, there are three criteria that we have used to classify them as T lymphocytes: 1) A significant proportion of the cells in each colony have the Thy 1 antigen. 2) The colonies have helper-like activity for CLP. 3) Morphologically, the cells in colonies look like typical lymphoblasts. For these reasons, it is likely that the cells in these colonies represent a subclass of T cells.

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