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Enhanced Lymphoid and Decreased Myeloid Reconstituting Ability of Stem Cells From Long-Term Cultures of Mouse Bone Marrow

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Mature, functional lymphocytes rapidly disappear from long-term cultures of mouse bone marrow cells and never reappear. One reason for the loss of B lymphocytes is that the optimal culture conditions for maintenance of myeloid stem cells are suboptimal for lymphocyte survival. However, despite the absence of functional lymphocytes, stem cells from such cultures retain the ability to reconstitute irradiated mice with mitogen-responsive B and T lymphocytes. In fact, in vitro grown stem cells repopulate the lymphoid system better than the myeloid system; the defective myeloid potential does not result from the absence in the cultures of Thy–1 bearing regulatory cells (TSRC). Although the cultures lack mature lymphocytes, they contain putative T cell precursors detectable with an in vitro colony-forming assay (CFU-T). In vitro maintenance of CFU-T requires an appropriate adherent monolayer. Monolyaters from congenitally anemic mice of genotype Sl/Sl^d fail to support either myeloid precursors or CFU-T.

Key words: pluripotent stem cells, restricted stem cells, CFU-S, T lymphocytes, B lymphocytes

Dexter and his colleagues have described an in vitro tissue culture system in which hemopoietic stem cells can proliferate and differentiate for many months [1, 2]. In many respects, this culture system provides a good in vitro model for studies on the regulation of the complex pathways of hemopoietic differentiation. The unique feature of these cultures is that they require for their maintenance an adherent layer of cells on which several types of nonadherent hemopoietic cells differentiate. Several observations suggest that the adherent layer mimics the hemopoietic microenvironment [3]. Although the cultures contain many types of differentiated cells, they fail to produce lymphoid cells which are known to be among the progeny of pluripotent hemopoietic stem cells. The absence of lymphocytes represents a major defect in Dexter cultures and limits their overall usefulness as a model for hemopoietic differentiation. We [4] and others [5] have demonstrated that when cultured cells are grafted into irradiated recipients, the cultured

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stem cells produce functional B and T lymphocytes. These results clearly show that the stem cells in culture retain the potential to produce lymphocytes and that their absence in vitro represents a defect in the culture system rather than the selection of stem cells restricted only to myeloid differentiation [6]. The results presented in this paper extend this observation and show that the stem cells from long-term cultures have more potential for lymphoid differentiation than for myeloid differentiation, that the cultures contain putative lymphoid progenitors detectable by an in vitro colony-forming assay, and that the optimal conditions for long-term cultures are not suitable for differentiation of B lymphocytes.

MATERIALS AND METHODS

All of the mice for the experiments described below were obtained either from BioBreeding Laboratories, Ottawa, Canada or the Jackson Laboratory, Bar Harbor, Maine. Because all of the techniques and assays used in this study are described in detail elsewhere, only brief summaries are given here. To initiate Dexter cultures, the marrow plug from a single femur was flushed into a 25-cm^2 flask containing α-MEM and 20% horse serum [4]; 1 week later, 10^6 bone marrow cells were added to each flask. Medium was changed weekly; some cultures contained 10^{-7}M hydrocortisone [7]. Multipotent stem cells were assayed by their ability to form colonies in the spleens of irradiated recipients (CFU-S) [8]. Granulocyte-macrophage colony-forming cells (CFU-GM) were induced to form colonies in methylcellulose [9] using L-cell conditioned medium as a stimulator [10].

Jacobs and Miller have developed an assay which detects precursors of Thy.1 positive cells (CFU-T) [11]. These colonies are induced in methylcellulose by a conditioned medium from PHA-stimulated human peripheral blood leukocytes [12]. Densely packed, floating colonies containing several hundred cells are counted at day 6 of incubation. Few, if any, other types of colonies are induced by this conditioned medium and they are easily distinguished from CFU-T on the basis of colony morphology.

To study the effects of various media on B lymphocyte activity [13], fresh bone marrow cells were stimulated with LPS and the number of antibody-producing cells were counted on day 6, the time of the peak response. Total IgM-secreting cells were assayed using sheep erythrocytes coated with protein A [14].

RESULTS

Reconstitution of Irradiated Mice

Because typical functional lymphocytes have never been observed in long-term cultures of mouse bone marrow, it was possible that the culture conditions selected for stem cells committed only to myeloid differentiation [6] and that pluripotent or lymphoid-committed stem cells died in vitro. Two groups have presented evidence against this possibility. Schrader and Schrader have shown that bone marrow cultured for several weeks maintains its ability to reconstitute the lymphoid system of irradiated recipients [5]. They used histocompatibility antigens as markers to demonstrate the origin of lymphocytes. We have reported similar experiments using chromosome markers to identify lymphocytes derived from cultured stem cells [4, 15]. In these experiments, cultures were established from CBA.T6T6 bone marrow cells and at several times during the culture period, cells were harvested and injected into irradiated CBA mice. Two months

TABLE I. Distribution of T6 Marker in Bone Marrow Cells and Lymphoid Blasts

Mouse	Tissue culture (weeks)	Marked metaphases (%)		
		Bone marrow cells	Con A blasts	LPS blasts
1	4	<10%	48%	58%
2	4	<10%	52%	48%
3	4	48%	56%	44%
4	4	< 4%	38%	36%
5	5	< 4%	11%	20%
6	5	< 4%	3%	6%
7	5	4%	16%	16%
8	5	8%	44%	36%

later, splenic lymphocytes were stimulated with LPS and Con A to examine the dividing B and T lymphocytes, respectively, for the presence of the T6 marker. Because there is a strong correlation between the proportion of spontaneously dividing cells in bone marrow with a specific marker and the proportion of CFU-S with that marker [6,16], only spontaneously dividing cells in bone marrow were analyzed in this study. A typical result similar to those presented previously [15] is shown in Table I. Despite the fact that the cultures contained numerous multipotent stem cells identified by their ability to form spleen colonies, few of the irradiated recipients showed long-term maintenance of myeloid function. In Table I, only one of eight recipients had detectable numbers of T6-marked cells in the bone marrow. In contrast, there was a high frequency of T6-marked cells in both the B and T lymphoid blasts, indicating that stem cells from long-term cultures have the potential for lymphoid reconstitution.

To interpret the results in Table I in terms of differentiative potential of the cultured stem cells, one must first rule out the possibility that the differences are related to regulation of differentiation. There are several observations indicating that Thy-1-bearing cells play some role in the regulation of myeloid differentiation. The experiments most relevant for our data are those of Sharkis et al [17]. These investigators have shown that the maintenance of myeloid stem cells in vivo requires the presence of an anti-Thy 1 sensitive regulatory cell (TSRC) in bone marrow. When this cell is removed from bone marrow by treatment of a cell suspension with anti-Thy 1 antibody and complement, the resulting stem cell suspension is unable to reconstitute the myeloid system in genetically anemic mice despite the fact that the treatment has no effect on the number of multipotent spleen colony-forming cells. Sharkis et al [17] reported that addition of 10^7 syngeneic thymocytes reconstitutes the ability of anti-Thy-1-treated stem cells to cure genetically anemic mice.

On the basis of these observations, one could argue that long-term cultures of bone marrow cells are deficient in the TSRC required for the long-term maintenance of myeloid cells. To test this possibility, we injected irradiated recipients with T6-marked bone marrow cells from long-term cultures and with thymocytes from CBA mice. The data in Table II show that addition of thymocytes does not increase the ability of cultured stem cells to repopulate the myeloid system of irradiated mice. In all recipients there was extensive reconstitution of the lymphoid system by the cultured stem cells, but in only one mouse was there significant contribution to the myeloid system by stem cells from the cultures.

TABLE II. Effect of Thymocytes on Myeloid and Lymphoid Differentiation by Cultured Stem Cells

Culture (weeks)	Thymocytes injected	Percent mitoses with T6 marker[a]		
		BM	Con A blasts	LPS blasts
3	None	0	60	ND
	10^7	0	24	ND
	10^7	0	52	ND
6	None	0	56	90
	None	0	20	14
	10^7	50	77	95
	10^7	0	36	40
11	None	0	48	60
	None	0	56	44
	10^7	0	40	68
	10^7	0	64	92

[a]Irradiated CBA recipients were given 2×10^6 CBA.T6T6 bone marrow cells from cultures incubated for 3, 6 and 11 weeks; the mice also received 10^7 thymocytes from CBA donors. The recipients were studied 2 months after grafting. The cultures contained 10^{-7}M hydrocortisone.

The cultures used to obtain the data in Table II contained 10^{-7}M hydrocortisone. Addition of this hormone greatly increases the functional lifetime of the cultures [7]. In other experiments without hydrocortisone, reconstituting ability was lost after 5 or 6 weeks (Table I). Cultures with hydrocortisone retain their lymphoid reconstituting ability for at least 11 weeks in culture.

Progenitors of Thy-1 Positive Cells in Long-Term Cultures

Cells from normal bone marrow and from the spleens of nude mice contain CFU-T activity. In both tissues, the colony-forming cells are Thy-1 negative, but the cells within the colony are Thy-1 positive [11]. We have monitored long-term cultures for CFU-T and have detected activity similar to that observed in nude spleen and normal bone marrow [18]. To study the possible relationship between CFU-T and myeloid stem cells, we studied the maintenance of myeloid cells and CFU-T in cultures derived from mice of genotype $S1/S1^d$. These mice have a congenital anemia resulting from a defective hemopoietic microenvironment [19, 20]. However, their lymphoid system is normal [21]. When bone marrow cells from $S1/S1^d$ mice are placed in tissue culture, they cannot be maintained because the adherent layer produced by such cells is defective. When stem cells from $S1/S1^d$ mice are placed on monolayers derived from mice with a normal allele, the stem cells from $S1/S1^d$ are maintained and function normally [3]. These observations indicate that the interaction between the adherent and nonadherent cells in long-term cultures may be a model for the interaction between hemopoietic stem cells and the hemopoietic microenvironment in vivo. Therefore, one might expect that bone marrow stem cells placed on monolayers prepared from $S1/S1^d$ mice would maintain lymphoid progenitors but that the myeloid cells would disappear. The data in Table III show that monolayers from $S1/S1^d$ mice fail to maintain both myeloid progenitors (CFU-GM) and

TABLE III. Failure of S1/S1^d Monolayers to Support Hemopoiesis

Monolayer[a]	Nonadherent cells	Activity/culture after 6 weeks		
		No. cells	CFU-GM	CFU-T
+/+	S1/S1^d	34×10^5	14,000	1,300
S1/S1^d	S1/S1^d	4.6×10^5	390	64

[a]Monolayers were established by incubating bone marrow cells for 3 weeks as described in the text. At that time, the adherent cells were trypsinized and transferred to other flasks. One week later, the monolayers were recharged with 10^6 bone marrow cells from S1/S1^d donors. The medium was changed weekly; the activities shown were measured 6 weeks after the cultures were recharged with fresh S1/S1^d bone marrow cells.

the putative lymphoid precursors (CFU-T). These results provide indirect evidence that CFU-T and GM-CFU are both derived from stem cells whose maintenance in vitro is dependent on a normal adherent layer. These data do not give any information on whether or not both activities arise from a common stem cell.

B Lymphocytes Fail to Differentiate in Medium Optimized for Maintenance of Long-Term Bone Marrow Cultures

Since Dexter cultures probably do not mimic the microenvironment of the thymus, it is not surprising that such cultures lack T cells. However, the absence of B cells is puzzling because they are normally produced in bone marrow [22]. One possible explanation for the disappearance of functional B lymphocytes in long-term cultures is that the conditions for maintaining bone marrow cultures are not optimal for the proliferation and differentiation of B lymphocytes. To test the ability of B lymphocytes to function in the medium used for long-term cultures, we incubated fresh bone marrow cells in the same medium used for the long-term cultures and in medium supplemented with fetal calf serum which will support the differentiation of marrow B cells [13]. The cultures were stimulated with LPS and plaque-forming cells were measured on day 6, the time of the peak response in the FCS-containing cultures. Table IV shows the results of this experiment; all of the results have been normalized to the plaque-forming cell response obtained in 10% fetal calf serum. It is clear that the optimal conditions for long-term culture, namely the presence of horse serum and hydrocortisone, completely inhibits the plaque-forming cell response by B lymphocytes from bone marrow. In fact, addition of small amounts of hydrocortisone to cultures containing fetal calf serum is slightly inhibitory as is the addition of even 1% horse serum to cultures with fetal calf serum. These results indicate that the development of a culture system suitable for the differentiation of B lymphocytes will require replacement of horse serum with something less toxic, preferably fetal calf serum and perhaps a reduction in the concentration of hydrocortisone.

DISCUSSION

The above results show that stem cells from long-term cultures can reconstitute the lymphoid system of irradiated mice but that such stem cells are deficient in their ability to maintain myeloid function in irradiated recipients. Furthermore, this deficiency seems

TABLE IV. Effect of Horse Serum and Hydrocortisone on the LPS Response of Bone Marrow B Cells

	Relative LPS response			
	Hydrocortisone concentration			
Serum[a]	0	10^{-6}M	10^{-7}M	10^{-8}M
10% FCS	1.00[b]	0.075	0.29	0.69
15% FCS	1.72	0.24	0.41	0.93
10% HoS	<0.001	<0.001	<0.001	<0.001
10% FCS + 5% HoS	0.27	0.002	0.014	0.084
10% FCS + 1% HoS	0.63	0.032	0.084	0.068

[a]FCS, fetal calf serum; HoS, horse serum.

[b]Control response at day 6: 7000 PFC/10^5 cells cultured. All results were normalized to this response.

to be an intrinsic property of the cultured stem cells and is not due to the loss of TSRC from the cultures. Other implications of the lymphoid potential of cultured stem cells have been discussed previously [4, 15, 18, 23].

One proposed application of long-term cultures is as a source of stem cells for bone marrow transplantation. The rapid disappearance of mature lymphoid cells in such cultures markedly reduces the risk of graft vs. host disease in allografts [24]. However, the defective myeloid potential of cultured stem cells limits their usefulness in allogeneic bone marrow transplantation. Cultured stem cells may be appropriate for use in reconstitution of congenital immune deficiency, but they will be ineffective for aplastic anemia or for reconstitution of leukemic patients given supra lethal anti-leukemia therapy.

Another implication of the separation of myeloid and lymphoid reconstituting abilities is that the cultures do not maintain pluripotent stem cells capable of both myeloid and lymphoid differentiation [6]. The above data on reconstitution show that such cells seem to be absent from most cultures as early as 3 weeks after initiation of the cultures. It is not clear from our experiments whether the pluripotent stem cells die in vitro or are gradually diluted out because they do not self-renew in vitro.

The results obtained with the adherent layers from S1/S1^d donors are difficult to reconcile with the in vivo phenotype of S1/S1^d mice. In vivo, these mice have defective myeloid function [19] but their immune system functions normally [21]. These observations contrast with the in vitro experiments where both myeloid progenitors (CFU-GM) and lymphoid progenitors (CFU-T) are lost in cultures growing on adherent layers from S1/S1^d mice. There are several possible explanations for the discrepancy between the in vivo and in vitro results. First, since the culture conditions seen are toxic for B lymphocytes (Table IV), we would not detect B lymphocytes even if they were produced in long-term cultures. Second, both myeloid and lymphoid stem cells may require the function provided by the adherent layer. According to this model, the inefficiency of the adherent layer established from S1/S1^d donors will affect both myeloid and lymphoid function. Third, CFU-T may belong in the myeloid rather than the lymphoid pathway. For example, we cannot rule out the possibility that the cells in such colonies are Thy-1-bearing

macrophages. Until it is possible to quantify the lymphoid stem cells in vitro and to determine the function of CFU-T, it will not be possible to determine which of these mechanisms accounts for the growth deficiencies on adherent layers prepared from $S1/S1^d$ mice.

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