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Growth of Normal Hemopoietic Cells in Cultures of Bone Marrow from Leukemic Mice*

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Abstract—*Bone marrow from mice with spontaneous leukemia was studied in long-term culture in the presence of* 10^{-7} M *hydrocortisone. These conditions resulted in a complete loss of leukemia cells from marrows which initially showed* 95% *or greater replacement with lymphoblasts. The culture conditions were also found to favor the growth of hemopoietic stem cells and the surviving cultured cells produced sufficient numbers of these cells to protect lethally irradiated* (950 rads) *syngeneic mice. The irradiated recipients of the cultured marrow did not develop leukemia during the* 180 *days of observation post-irradiation, indicating the absence of leukemia cells in the inocula. A loss of leukemia cells* in vitro *occurred in cultures with and without the addition of hydrocortisone to the medium. Hydrocortisone was shown to have a stimulating effect on maintenance of hemopoietic stem cells, granulocyte progenitors and granulopoiesis in these cultures.*

INTRODUCTION

BONE marrow transplantation has now become a viable modality for the treatment of leukemia in man [1]. The necessity for HLA-matched sibling donors has been shown, and thus the availability of donors is limited [2]. Even with HLA-matched sibling donors graft vs host reaction is a serious problem. The results with identical twins have shown that a syngeneic transplant is more efficacious than a HLA-matched allotransplant [3]. Autologous transplantation, i.e. removal of marrow, administering high dose irradiation and chemotherapy, and rescuing the patient with his own marrow, has limited usefulness in leukemia because the marrow is the site of the disease. Therefore, a means of completely separating normal stem cells from leukemic cells is needed. In previous studies carried out in this laboratory we noted that marrow from AKR mice with advanced spontaneous thymic lymphoma was replaced with 90–100% lymphoblast-like cells. However, when this marrow was placed in the GM–CFUc assay in soft agar, many normal GM–CFUc were found. Lymphoma cells from the thymus and lymph nodes did not form colonies in the GM–CFUc assay [4]. This finding suggested that hemopoietic progenitors and lymphoma cells had different requirements for *in vitro* growth. We report here, using this murine model of marrow replacement with lymphoma cells, a successful method for effecting an *in vitro* separation of hemopoietic progenitors from lymphoma cells. It was done by culturing the leukemic marrow using methods which allow long-term survival of hemopoietic stem cells of normal bone marrow [5, 6]. These *in vitro* conditions resulted in loss of leukemia cells within one week and appearance of normal hemopoietic stem cells which persisted for as long as seven weeks (undergoing granulopoiesis *in vitro*). Cells recovered from these cultures after 4–6 weeks were able to protect lethally irradiated normal mice.

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MATERIALS AND METHODS

Mice

AKR mice from the inbred strain maintained in this laboratory were used. These animals have a high natural incidence of spontaneous T cell lymphoma which, although it begins in the thymus, completely replaces the bone marrow

in advanced disease. The mice used for the spleen colony assay and as hosts in the radiation protection experiments were specific-pathogen-free (SPF) AKR mice from our laboratory colony which is kept under strict isolation and fed autoclaved pellets and water. The animals were moved to a conventional environment after irradiation and marrow inoculation but kept in filter-top cages with autoclaved food and water for the duration of the study.

Marrow culture

This was done using a modification of previously described methods. In most of the experiments the single inoculum method described by Greenberger *et al.* [6] was used. One series of experiments utilized the dual inoculum method as originally described by Dexter *et al.* [5]. All normal mouse marrows were from 8 to 12-week-old mice. Leukemic mice were 6–12 months of age. Normal mice and mice with advanced leukemia were killed by cervical dislocation. In the *single inoculum method* the marrows were removed by flushing the contents of a femur and tibia into a 25 cm^2 flask with 10 ml of Fischer's medium with 25% horse serum (Flow Laboratories), penicillin and streptomycin, 0.5 ml per 100 ml medium, and hydrocortisone sodium phosphate (Merck, Sharp and Dohme, West Point, PA), 0.0484 μg/ml (10^{-7} M). The marrow from the opposite femur and tibia was used in a duplicate flask. Cytocentrifuge preparations (stained with Wright's stain) were used to evaluate cell morphology. The cultures were placed in a 5% CO_2 atmosphere at 33°C. At weekly intervals half of the medium with nonadherent cells was removed and the cultures were fed with fresh medium. At week 4 the horse serum was replaced with 25% fetal calf serum. Cell counts and morphologic evaluations were made on the cells which were removed each week. Assays for totipotent hemopoietic stem cells (CFUs) and granulocyte–monocyte progenitors (GM–CFUc) were done at selected weeks during the study.

In the *dual inoculum method* normal marrow from a femur and tibia was flushed into a flask with 10 ml of Fischer's medium with 25% horse serum, penicillin and streptomycin. The cells were cultured for 3 weeks with weekly removal of 1/2 of the medium plus nonadherent cells and addition of an equal volume of fresh medium. At the end of three weeks an adherent stromal layer had formed and few nonadherent cells were present. At this time all of the medium was removed and 10^7 bone marrow cells in 10 ml of fresh medium were added. The cultures were continued by removing half of the medium plus nonadherent cells at weekly intervals and feeding with an equal volume of Fischer's medium with 25% horse serum. The incubator conditions wer 5% CO_2 and 33°C temperature.

Stem cell and progenitor assays

The CFUs assay was that of Till and McCulloch [7]. SPF AKR mice were given 950 rads of [^{60}Co] and inoculated intravenously within 4 hr with 5×10^5 cells. The recipient mice were killed 9 days later. The spleens were removed, fixed in Bouin's fluid and the surface colonies counted.

GM–CFUc were assayed using 0.3% agar, 5×10^4 marrow cells per plate and conditioned medium from a continuous cell line of AKR thymic epithelium (TEPI) as a source of colony-stimulating activity (CSA) [8]. Control plates were prepared omitting CSA from the agar–cell-medium mixture. Duplicate CSA-containing and control plates were made for each assay. Colonies of > 50 cells were counted after incubation at 37°C for 7 days.

Irradiation and protection

Two to three-month-old SPF AKR mice were used. They were given 950 rads of midline absorbed tissue dose [^{60}Co] total body irradiation. The animals were 150 cm from the source and the dose rate was 86 rads per minute. This was followed with an inoculum of cells from the marrow culture at week 5; the cells were inoculated 2 hr after irradiation. The cell number inoculated depended upon the number of cells in the culture. The irradiated animals were then given cells from the same culture on two successive weeks. Therefore each mouse had a total of three inocula of cultured marrow cells—2 hr, 1 week and 2 weeks after 950 rads of total body irradiation. The mice were then observed for 180 days. The protective capacity of normal marrow was tested by a single intravenous inoculation of 5×10^6 cells 2 hr after 950 rads. The lethality of this irradiation dose was documented by treating 25 specific-pathogen-free AKR mice with 950 rads. All animals died 14–18 days post-irradiation. These animals, as well as the animals of the experiment reported, were moved to a conventional environment with filter-top cages and sterile food, water and shavings after the irradiation was given.

Lymphoma cell line

A line of lymphoma cells from the bone

marrow of a mouse with spontaneous lymphoma was established in Fischer's medium with 25% horse serum. The cells grew in suspension in the absence of an adherent layer and were maintained at 37°C with 5% CO_2 by splitting the cultures every 3 or 4 days. The cells were lymphoblasts and produced lymphoma when inoculated into syngeneic mice.

RESULTS

Eleven normal marrows and 14 marrows from mice with spontaneous leukemia were cultured for 7 weeks using the single-inoculum method with hydrocortisone. The leukemic marrows showed 95–100% lymphoblasts with 0–5% mature granulocytes prior to culture. The striking observation was that all of the leukemic marrow cultures lost their blast cells after the first week *in vitro* and maintained granulopoiesis for the observation period of 7 weeks (Tables 1, 2). There was no difference in the morphologic characteristics of the leukemic and normal marrow cultures. Both formed an underlayer of attached macrophages and fibroblasts with giant fat cells and hemopoietic islands, as described in other published studies

Table 1. *Cell counts and progenitor cell assays of marrows from normal and leukemic AKR mice*

	Normal			Leukemia		
Weeks cultured	Cells per culture $\times 10^6$ (± S.D.)	CFUs per culture	CFUc per culture	Cells per culture $\times 10^6$ (± S.D.)	CFUs per culture	CFUc per culture
1	4.4 ± 2.0			3.6 ± 2.9		
2	3.2 ± 5.0			0.55 ± 0.6		
3	2.5 ± 2.3			1.70 ± 2.5		
4	10.5 ± 6.8			6.70 ± 6.0		
5	12.3 ± 4.9			8.00 ± 4.5		
6	6.5 ± 2.5		14,061 ±9,977	5.30 ± 3.7		5,342 ±2,909
7	5.4 ± 3.0	170 ±52.4		4.00 ± 2.0	20 114	

The marrows were obtained from eleven 8 to 12-week-old normal AKR mice and 14 AKR mice (6–12 months of age) with spontaneous lymphoma. They were cultured in Fischer's medium with 10^{-7} hydrocortisone and antibiotics. Incubation was at 33°C in 5% CO_2. After week 4 fetal calf serum replaced the horse serum. All of the leukemic marrows had 95–100% lymphoblasts when the cultures were established. The total number of cells per culture was determined by multiplying the number of cells per ml in the nonadherent population removed each week by 10 (the volume of the culture was 10 ml). The CFUc and CFUs were measured at weeks 6 and 7 because this was just after the peak rise in granulocyte production (weeks 4–6).

Table 2. *Representative cell counts and morphologic evaluation of marrow cultures from a normal and leukemic AKR mouse*

	Weeks cultured	Cells per culture $\times 10^6$	% Immature granulocytes/ % mature granulocytes	CFUs per culture	GM-CFUc per culture
Normal	2	2.4	15/66		
	4	15.8	27/72		
	5	18.7	16/81		
	6	7.8	19/81		7950
	7	6.4	51/71	120	
Leukemic	2	0.1	28/14		
	4	3.2	19/80		
	5	10.4	12/86		
	6	11.9	12/88		7850
	7	6.2	2/86	20	

The marrows were flushed from a tibia and femur into 10 ml of Fischer's medium with 25% horse serum, 10^{-7} hydrocortisone and antibiotics. Incubation was at 33°C in 5% CO_2. Each week 5 ml of cells plus medium was removed and fresh medium added. Counts, cytocentrifuge slides for morphology and progenitor cell assays were performed on the nonadherent cells. The total number of cells per culture were determined by multiplying the number of cells per ml in the nonadherent population removed each week by 10 (the volume of the culture was 10 ml). No leukemic cells were seen in the nonadherent population after the first week in culture.

of long-term marrow cultures [5]. The nonadherent cells from both leukemic and normal cultures were composed of granulocytes at various stages of maturation. As shown in Table 2, there was considerable variation in the numbers of nonadherent cells removed from the cultures at the time of feeding. The leukemic cultures usually had fewer nonadherent cells than those from normal marrow. Also, fewer CFUs and GM CFUc were found.

In a second series of experiments marrows from two mice with spontaneous lymphoma were cultured using the dual-inoculum method without hydrocortisone. Bone marrows containing 92% and 95% lymphoblasts were compared with two normal marrows. In each instance the cultures were established by adding 10^7 cells to separate flasks containing a prepared stromal layer from normal AKR marrow. The leukemic marrow cultures showed loss of leukemic cells after the first week of culture. During the next 3 weeks low numbers of granulocytes were seen; however, by week 4 the cultures were composed exclusively of macrophages. The normal cell cultures produced greater numbers of cells which were composed almost completely of immature and mature granulocytes for the 7-week study period.

Having shown in the first series of experiments that the single-inoculum method gave both loss of leukemic cells and good stem cell survival, experiments were carried out to determine if the cultures derived from the leukemic marrow using this method had sufficient numbers of stem cells to protect lethally irradiated mice. In observing these mice after irradiation and marrow graft we could also determine if the cell inocula contained viable lymphoma cells. The peak cell counts in the cultures were found at weeks 4, 5 and 6 (Table 1). Therefore, cells for radiation-protection studies were harvested from these time periods. Recipient SPF AKR mice were given 950 rads of [^{60}Co] irradiation followed within several hours by intravenous inoculation of cells from the cultures at week 4. These recipients also received cells from the same cultures at weekly intervals for the next two weeks. This procedure was utilized to provide adequate numbers of stem cells to populate the radiation-depleted bone marrow. Four animals were used in these experiments because the cultures did not provide enough cells for inoculation of large numbers of mice. The results are presented on Table 3 and show that the cells from the cultures derived from leukemic marrows could protect lethally irradiated mice in that all treated animals survived for 180 days. Irradiated non-inoculated controls died within 2 weeks of treatment. There was no evidence of lymphoma in the mice receiving the cultured marrow cells when they were sacrificed at 180 days after irradiation and marrow graft. A single intravenous inoculum of 5×10^6 fresh normal AKR bone marrow cells protected 100% of ten 950 rad-treated mice of this same age. These animals also did not develop lymphoma before 180 days.

The following experiments were carried out to illustrate the growth potential of leukemia/lymphoma cells in this system. Four animals were given 950 rads and 10^7 cells from uncultured leukemic marrows. They all died 18 days after irradiation and their spleens were diffusely enlarged and replaced by lymphoma

Table 3. Radioprotective capacity of cells from long-term cultures established from leukemic marrows

Mouse No.	Number of cells (CFUs) injected Week 0	Week 1	Week 2	Survival (days post irradiation)
1	7×10^6 (42 CFUs)	5.5×10^6	1×10^6	180
2, 3	1.4×10^7	9×10^6	4.9×10^6	180
4	4.3×10^6 (80 CFUs)	3×10^6	2.2×10^6	180
5, 6	None	None	None	14

The recipients were 60 to 70-day-old AKR mice. All animals were given 950 rads of [^{60}Co] irradiation at week 0. The first cell inoculation was four hours after irradiation. The cells were injected intravenously using the lateral tail vein. The cells were obtained at weeks 4, 5 and 6 from the nonadherent cell populations of long-term cultures established from bone marrows of AKR mice with spontaneous leukemia.

cells. In an additional study an intravenous inoculation of 10^2 cells from four different spontaneous AKR thymic lymphomas was given to ten 2-month-old AKR mice. All of these animals developed generalized lymphoma 25–30 days after inoculation. These two studies demonstrate the ability of viable lymphoma cells to regularly produce disease in this animal model. They indicate that lack of lymphoma development by 180 days in the irradiated animals given the cultured marrow reflects absence of lymphoma cells in the inoculum.

Hydrocortisone has been shown to stimulate hemopoiesis in long-term culture of normal mouse marrow [9, 10]. However, steroids are known to induce remission with a rapid loss of blast cells in lymphoblastic leukemia [11] and have been shown to be toxic to mouse lymphoma cells *in vitro* [12]. Therefore, to evaluate the hydrocortisone effect in our system the following experiments were done. Marrow from a single animal with spontaneous lymphoma was cultured with and without medium containing hydrocortisone. Marrow composed of 95% lymphoblasts from each tibia and femur was inoculated into two flasks. Fischer's medium, horse serum and antibiotics with and without 10^{-7} M hydrocortisone were used. The results are presented on Table 4. Blast cells disappeared by the first week in both cultures. The culture without hydrocortisone steadily declined in numbers of nonadherent cells and granulopoiesis did not take place. The flask with hydrocortisone showed, as in the previous cultures, a rise in cell number at week 5 which was the result of the production of granulocytes within the culture. Cultures from a second leukemic mouse marrow duplicated these findings. Additional evidence for lack of hydrocortisone effect on lymphoma cells was provided by study of a lymphoma cell line derived from bone marrow of a leukemic mouse. These lymphoma cells have been growing continuously in suspension for over 1 yr. Addition of 10^{-7} M hydrocortisone to the medium did not alter their growth properties.

DISCUSSION

These experiments show that the conditions provided by the long-term bone marrow culture technology do not support lymphoma stem cell growth. The lymphoma studied here was a spontaneous T cell lymphoma which began in the thymus and invaded the bone marrow as the disease progressed. We chose this model rather than a transplanted lymphoma or a lymphoma/leukemia induced by laboratory strains of murine leukemia viruses because we thought it better reflected the situation as it occurs in human lymphoma/leukemia. Lymphoma cells are lost in cultures utilizing either the dual or the single inoculum methodology. Our findings indicate that the single inoculum system with added hydrocortisone also favors the growth of functional stromal support cells and hemopoietic stem cells in the leukemic marrows. Thus, using this culture system normal hemopoietic stem cells in numbers sufficient to protect lethally irradiated animals can be obtained. The removal of the leukemic cells from these marrow cultures is complete. This is demonstrated by the observation that the irradiated recipients did not develop lymphoma after receiving cells from the cultured marrow. If lymphoma cells

Table 4. The effect of hydrocortisone on the leukemic marrow cultures

	Hydrocortisone				No Hydrocortisone			
		Differential Count				Differential Count		
Week	Cells per culture $\times 10^6$	Granulocytes Immature	Mature	Macrophages	Cells per culture $\times 10^6$	Granulocytes Immature	Mature	Macrophages
1	2.3	13	84	3	1.9	24	67	9
2	0.3	6	30	64	0.5	4	22	74
3	0.2	32	50	18	0.2	2	5	93
4	4.8	23	75	2	0.2	1	22	77
5	11.8	8	92	0	0.3	4	19	82
6	1.8	25	75	0	0.02	0	0	100

A single AKR mouse with spontaneous leukemia was used as a donor. The marrow containing 95% lymphoblasts was placed in two flasks; one with Fischer's medium and 25% horse serum with 10^{-7} hydrocortisone, and the other with medium and serum without added steroid. The cultures were fed by demidepopulation at weekly intervals and cell counts made on the supernatant fluids.

had been present they would have been expected to produce disease within 3–4 weeks after the transplant. Confirmation of this statement was provided by the experiments in which viable primary explants of lymphoma cells were inoculated into both irradiated and normal AKR mice. With long-transplanted AKR or L 1210 leukemias an inoculation of small numbers of cells in growth phase produces generalized disease within an even shorter period of time.

In the present experiments, using the culture method with hydrocostisone, it was shown that marrows from mice with advanced disease not only contained stem cells which could give rise to granulocyte progenitors and granulocytes *in vitro* but also provided a 'stromal' layer which could promote a maintenance of hemopoiesis similar to that observed in long-term marrow cultures from normal mice. When 'stroma' from a normal bone marrow was charged with leukemic marrow cells using medium without hydrocortisone, the malignant cells did not grow but granulopoiesis was supported for only a short period of time. In the single-inoculum system without hydrocortisone the loss of hemopoietic progenitors was more rapid. These findings indicate that normal hemopoietic cells from leukemic marrows do not survive well in the absence of steroid. Since leukemic cells were lost under all culture conditions we believe the hydrocortisone effect is primarily that of stimulating growth of hemopoietic stem cells, GM–CFUc and granulopoiesis. This effect has been reported with long-term cultures of normal mouse bone marrow [9, 10]. It could be argued that hydrocortisone present in the horse serum might have suppressed lymphoma cell growth. The demonstration that a lymphoma cell line could be established in Fischer's medium with 25% horse serum and that this cell line could also grow in the presence of added steroid does not support this argument.

In summary, we have reported a simple *in vitro* system which can select for a normal stem cell population in bone marrow invaded by lymphoma cells. This observation of an *in vitro* 'cure' of leukemia could have potential usefulness in providing normal hemopoietic stem cells for treatment of human leukemia with high dose chemotherapy and irradiation followed by transplantation of the autologous cells.

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