Brief Definitive Report

MAINTENANCE OF HEMOPOIETIC STEM CELLS AND PRODUCTION OF DIFFERENTIATED PROGENY IN ALLOGENEIC AND SEMIALLOGENEIC BONE MARROW CHIMERAS IN VITRO*

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A liquid culture system has been developed in which proliferation of hemopoietic stem cells (CFU-s) (1) and production of granulocyte precursor cells (CFU-c) (2, 3) can be maintained in vitro for several months (4, 5). In this system, mouse bone marrow-derived adherent cell cultures established for 3 wk are reinoculated with freshly isolated samples of bone marrow cells. The latter subsequently undergo proliferation for several months. This proliferation, differentiation, and maturation of hemopoietic cells is dependent upon the formation of a bone marrow-derived adherent population comprised of phagocytic mononuclear cells, "epithelioid" cells, and giant fat-containing cells which appear to provide an in vitro microenvironment necessary for pluripotential stem cell renewal and differentiation. Within this adherent layer extensive cellular interactions occur (6).

Thus far, the studies reported have been with syngeneic systems, i.e., those in which both the bone marrow-derived adherent layers and reinoculated bone marrow cells were from the same mouse strain. However, if this technique is to be applied to the culture of human marrow cells (or, indeed, other noninbred species), it may often be necessary to use an allogeneic culture system. For this reason, we have investigated the capacity of established marrow adherent layers to promote the prolonged growth of allogeneic hemopoietic cells.

Materials and Methods

Establishing the Cultures. To establish the adherent layers, the contents of a single mouse femur were flushed into glass culture bottles (United Glass, London, England) containing 10 ml of Fischer's medium (Grand Island Biological Corp., Grand Island, N. Y.) supplemented with 25% horse serum (Flow Laboratories, Inc., Rockville, Md.) and antibiotics as described previously (5). These cultures were maintained at 33°C in an atmosphere of 5% CO_2 in air and were fed at weekly intervals by removal of half the growth medium (which contains nonadherent cells) and addition of an equal volume of fresh medium. Over a 3-wk interval the adherent population of cells in these cultures became well established, but there was a rapid decline in the nonadherent cells and in the CFU-c population. Onto each of these adherent layers was added 10^7 syngeneic, semiallogeneic, or allogeneic bone marrow cells. The cultures were fed at weekly intervals as before, and the

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nonattaching cells present in the growth medium were assayed for hemopoietic precursor cells.

Animals. Mice used in these experiments were CBA/Cum (*H-2*^{k}), C57BL/6 (*H-2*^{b}), and (CBA/Cum × C57BL/6)F_1.

Precursor Cell Assays. The nonadherent cells removed were counted, centrifuged, and resuspended in Fischer's medium. After counting the cells again, appropriate dilutions were made and the cells assayed for pluripotential stem cells (CFU-s) using the method previously described (1). Granulocyte progenitor cells (CFU-c) were assayed in semisolid agar, McCoy's medium, using conditioned medium from WEHI-3 myelomonocytic leukemic cells as a source of colony-stimulating activity (7).

Results

Cell proliferation is obviously occurring in all groups, i.e., after the 50% depopulation by "feeding" weekly, the cell numbers recover (Table I). During this time there is little change in the numbers of adherent cells (reference 5, and unpublished observations). In all groups the nonattaching cells collected throughout the culture period consisted mainly (>75%) of granulocytes in all stages of maturation. The remainder of the population consisted of large phagocytic mononuclear cells. No lymphoid or erythroid cells were seen after the 1st wk in culture.

In the F_1 plus F_1 marrow cultures CFU-c were maintained for at least 5 wk. When the weekly depopulation due to feeding the cultures is taken into account, it is obvious there has been an appreciable production of CFU-c over several weeks. When an F_1 adherent layer was seeded with either of the parental (i.e., semiallogeneic) bone marrow cells, there was a similar production of CFU-c during 5 wk. C57BL/6 plus C57BL/6 marrow cultures also showed prolonged production of CFU-c, whereas CBA plus CBA bone marrow cells showed a progressive decrease in total CFU-c, indicating a rather limited proliferative capacity. Of particular interest was the finding that the allogeneic combinations (CBA plus C57BL/6 and the reciprocal C57BL/6 plus CBA) show a sustained production of CFU-c during the time period studied.

While no attempt was made to monitor progressive changes in CFU-s (pluripotent stem cell) in these cultures, assays of the nonattaching cells after 5 wk of culture showed appreciable maintenance of these cells (Table II), except in CBA plus CBA cultures in which no CFU-s were detectable. The absence of stem cells in these cultures was compatible with the CFU-c data which indicated a progressive rundown in proliferative ability.

That the CFU-s are not derived from the bone marrow cells used to establish the adherent layer was indicated by the results obtained from cultures of F_1 plus C57BL/6 and C57BL/6 plus CBA. In the former case, injection of the cells produced typical large colonies in C57BL/6 recipients, whereas only a few small colonies were formed in F_1 recipients. In the latter case (C57BL/6 plus CBA), the cultured cells produced colonies only in CBA mice. These results are compatible with those observed in vivo when certain semiallogeneic (parent → hybrid) and allogeneic marrow transplants are assayed for CFU-s formation or ^{125}I-5-iodo-2′-deoxyuridine incorporation (8, 9). Thus, the large number of colonies formed when cells from F_1 plus C57BL/6 cultures were injected into C57BL/6 mice and the lower numbers (and smaller colonies) seen when these cells were transplanted into F_1 mice indicated, therefore, that the CFU-s were of C57BL/6

TABLE I

Production of CFU-c on Syngeneic, Semiallogeneic, and Allogeneic-Adherent Layers

Adherent layer	Cells added	Total suspension cells/culture (×10⁵)					Total CFU-c/culture				
		Weeks cultured					Weeks cultured				
		1	2	3	4	5	1	2	3	4	5
F_1	F_1	33.0	28.0	46.0	44.0	27.0	44,700	22,700	41,500	42,200	12,700
F_1	C57BL/6	58.0	29.0	13.0	10.0	ND	20,900	11,000	34,200	24,000	ND
F_1	CBA	45.0	49.0	29.0	14.0	24.0	15,500	10,000	15,400	14,200	34,500
C57BL/6	C57BL/6	43.0	53.0	58.0	18.0	30.0	23,000	40,000	75,400	26,000	80,700
C57BL/6	CBA	65.0	58.0	22.0	24.0	42.0	35,750	29,000	16,800	13,200	28,400
CBA	CBA	17.0	33.0	19.0	20.0	29.0	10,000	8,000	5,500	1,800	1,250
CBA	C57BL/6	36.0	39.0	28.0	15.0	13.0	13,700	15,800	38,400	17,100	35,000

TABLE II

Measurement of CFU-s After 5 wk Culture

Adherent cells	Cells added	No. cells injected	Recipient mice	Spleen colonies
F_1	F_1	3.0×10^5	F_1	34 ± 2
F_1	C57BL/6	1.4×10^5	C57BL/6	26 ± 3
		1.4×10^5	F_1	4 ± 1 (small colonies)
F_1	CBA	1.0×10^5	CBA	13 ± 1
		1.25×10^5	F_1	15 ± 2
C57BL/6	C57BL/6*	1.0×10^5	C57BL/6	12 ± 2
		3.2×10^5	F_1	4 ± 1 (small colonies)
CBA	CBA	8×10^4	CBA	0
		8×10^4	F_1	0
C57BL/6	CBA	1.2×10^5	CBA	8 ± 2
		1.2×10^5	C57BL/6	0

* After 4 wk culture.

marrow origin. Furthermore, in the allogeneic combination (C57BL/6 plus CBA), the cultured cells only formed spleen colonies in CBA mice, indicating their origin in the cultured cells of this strain. In the culture combination, F_1 plus CBA, the cells formed colonies in both CBA and F_1 mice.

Discussion

These data demonstrate that bone marrow-derived adherent cells from a particular mouse strain can stimulate the growth of hemopoietic cells from syngeneic and genetically incompatible mice. Furthermore, the maintenance of stem cells and the production of granulocyte precursor cells is not compromised in semiallogeneic or allogeneic bone marrow combinations. These results contrast with the in vivo situation in which heavily irradiated F_1 hybrid mice are capable of rejecting hemopoietic grafts of certain parental strains (10, 11). This phenomenon has been called hybrid resistance, but since bone marrow graft rejection has been demonstrated also in irradiated allogeneic and xenogeneic hosts, it appears to be a wider manifestation of a hemopoietic histocompatibility (Hh) or genetic resistance mechanism (12). The expression of resistance is determined by noncodominant Hh genes controlling the expression of Hh alloantigens on the surface of hemopoietic cells and by immune response genes which

govern reactivity to Hh alloantigens (9). Recently, in vitro models of F_1 hybrid anti-parent responses have been reported, in which cytotoxic F_1 effector cells can be generated against normal or neoplastic parental hemopoietic target cells (13, 14).[1] The effector cells in the in vitro systems are Thy-1 positive and are generated by a nylon wool nonadherent and Thy-1-positive population of spleen cells, which appears to contrast with the T-cell independence of in vivo Hh reactions (14).[1] The absence of Thy-1 cells in continuous marrow cultures could provide an explanation for our failure to detect Hh resistance in this system. However, the present system for maintaining continuous stem cell proliferation and interaction between these cells and adherent marrow "microenvironmental" cells (5, 6) is more analogous to the in vivo situation than any previously reported in vitro systems. The observation that athymic nude mice are more efficient than normal animals in rejecting bone marrow allografts (15) and that the resistance is abrogated by agents toxic to macrophages (16) suggests that in vivo resistance is effected by an as yet poorly understood cell-mediated mechanism which may involve macrophages or a subpopulation thereof. Since macrophages are an integral (and possibly essential) component of the bone marrow-derived adherent population of this culture system, it remains possible that our failure to detect allogeneic or hybrid resistance was due to the inoculation of too many allogeneic or semiallogeneic cells (since it is known that in vivo resistance can be overcome if sufficient numbers of cells are injected (9, 11, 12). Alternatively, it is possible that the cells responsible in vivo for the transplantation resistance phenomenon are lost during the 3-wk culture period, in which the adherent layer becomes established. Experiments examining these possibilities are in progress.

The culture system described offers a means of analyzing in vitro the cellular mechanisms involved in marrow transplantation resistance, and the data obtained further suggest that the culture system can be exploited for the growth of bone marrow cells from noninbred species.

Summary

A culture system is described in which bone marrow-derived adherent cells can support prolonged proliferation and differentiation of genetically incompatible stem cells and precursor cells. The results suggest that the reactive cells responsible in vivo for host transplantation resistance and for graft-versus-host disease are selectively lost or inhibited in such cultures, which may provide a vehicle for studying some of the cellular mechanisms involved in transplantation resistance.

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