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Induction and Long-Term Maintenance of Thy-1 Positive T Lymphocytes: Derivation From Continuous Bone Marrow Cultures

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In the present study we investigated the presence of T-lymphocyte progenitors in the long-term murine bone marrow culture system described by Dexter: mature Thy-1 antigen-bearing T lymphocytes are lost in these cultures after a few days. By culturing nonadherent cells from such cultures in the presence of a supernatant of concanavalin A-stimulated spleen cells, a source of T-cell growth factor, we found that Thy-1 positive blast cells proliferated together with a second population of Thy-1 negative cells. These two populations of cells have been maintained in long-term in vitro cultures by passaging the cells in fresh conditioned medium at regular intervals. Moreover, we have been able to establish pure cultures of the Thy-1-bearing blast cells after separating them from the non-T cells using their adherence property to plastic surfaces. Long-term cultures of T lymphocytes can thus be established from long-term marrow cultures as well as from the spleen, thymus or fresh bone marrow.

Key words: T lymphocytes, TCGF, continuous marrow cultures

A few years ago, Dexter et al [1–4] described an in vitro system for the long-term culture of murine bone marrow: this system provides adequate microenvironment for the continued production of pluripotent stem cells (CFU-S) and precursors of the granulocyte-macrophage [1–3], erythroid [5], and megakaryocytic [6] series. In contrast, it has been demonstrated that mature Thy-1 positive T lymphocytes and surface immunoglobulins bearing B lymphocytes disappear during the first week of culture. However, long-term cultured bone marrow cells retain the capacity to fully reconstitute the immunohematopoietic system of lethally irradiated mice without occurrence of graft versus host disease [7, 8]. It has not been demonstrated whether T-cell reconstitution was dependent upon the replication of pluripotent stem cells or upon the continuous generation of T-cell progenitors in the continuous bone marrow cultures (CMC).

In 1976, Morgan et al [9] described an in vitro system for the long-term culture of T lymphocytes from normal human bone marrow using a mitogen-stimulated peripheral blood leukocyte-conditioned medium as a source of T-cell growth factor. Thereafter, this

Abbreviations used in this paper: CFU-C, granulocyte-macrophage colony-forming unit; CFU-S, spleen colony-forming unit; CM, a 50:50 mixture of ConA leukocyte-conditioned medium and supplemented RPMI 1640 medium; CMC, continuous marrow cultures; ConA, Concanavalin A; RITC, rhodamine isothiocyanate conjugated.

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system has been applied to the long-term cultivation of mitogen- as well as antigen-stimulated human [10–13] and murine T lymphocytes [14–16].

The present study was undertaken to determine whether T cells that proliferate in response to T-cell growth factor can be induced from long-term cultured mouse bone marrow cells.

MATERIALS AND METHODS

Animals

Mice of either sex were employed in these studies at the age of 6–12 weeks. Inbred DBA/2 mice were obtained from Jackson Laboratory and hybrid (C57B1/6 × DBA/2)F1 (BDF1) mice were obtained from Cumberland View Farms.

Preparation of T-Cell Growth Medium

The procedures were as described by Rosenberg et al [14]. Splenic leukocytes of BDF1 mice were suspended at 10^7/ml in RPMI-1640 medium supplemented with 10% fetal bovine serum (Flow Laboratory) and containing 10 μg concanavalin A (Calbiochem, San Diego) per ml. The cells were aliquoted in 10 ml volumes in 25 cm^2 tissue culture flasks (Corning), which were then incubated upright for 48 hr at 37°C in a humidified incubator containing a gas mixture of 5% CO_2 and 95% air. At the end of the incubation period the cell-free supernate was collected and filtered. A 50:50 mixture of this supernate and supplemented RPMI medium was employed in all T-cell cultures and is hereafter abbreviated as CM.

Establishment of Continuous Bone Marrow Cultures

The establishment of CMC was as previously described [3, 17]. In brief, the contents of single mouse femurs were flushed into 25 cm^2 plastic culture flasks (Corning) containing 10 ml Fischer's medium supplemented with 25% horse serum and antibiotics (all from Grand Island Biological Co.). The flasks were gassed with a 5% CO_2-air mixture for 20 sec and capped tight prior to incubation at 33°C. Over a 3-week period necessary for the establishment of the adherent layer, the cultures were fed weekly by removal of half the growth medium and nonadherent cells and addition of an equal volume of fresh medium. At the end of the 3-week period, 7 to 10 × 10^6 fresh syngeneic bone marrow cells were added to the adherent layer. Thereafter, half the growth medium and suspension cells were removed from the cultures with addition of fresh medium at weekly intervals. The age of the cultures is designated as number of weeks following repopulation with the second bone marrow inoculum. The nonadherent cells removed were pooled, centrifuged, and resuspended in CM at 10^6/ml for establishment of T-cell cultures or in McCoy's medium for assay of granulocyte-macrophage colony-forming cells (CFU-c) in semisolid agar using WEHI-3 conditioned medium as a source of colony-stimulating factor [18], or were Wright-stained for differential counts.

Culture of Cells Recovered From Continuous Marrow Cultures in CM

Cells recovered from CMC were washed once and resuspended in CM at 10^6/ml. The cells were then cultured in 10 ml volumes in Falcon 25 cm^2 tissue culture flasks at 37°C in a humidified incubator containing a gas mixture of 5% CO_2 and 95% air. Viable cells were collected after 1 week of culture and passaged in fresh CM at 5 × 10^4/ml at intervals of 3–7 days.

Assay for Thy-1.2 Antigen-Bearing Cells by Immunofluorescence

Cells cultured in CM were assayed for surface Thy-1.2 antigen by indirect immunofluorescence. Three to 5×10^5 cells were treated for 30 min at 4°C with 50 μl of a hybridoma-derived IgM anti-Thy-1.2 antibody diluted 1:200. Control cells were treated with 50 μl of RPMI 1640 medium alone. The cells were then layered on fetal bovine serum, centrifuged, and exposed for 10 min to 50 μl of a μ-chain specific rhodamine conjugated goat-anti-mouse IgM (α-IgM-RITC) [19]. Treated cells were then washed through fetal bovine serum and then RPMI medium. The number of fluorescent cells was counted using a Zeiss fluorescent microscope equipped with phase-contrast objectives. A minimum of 100 cells were counted.

RESULTS

Generation of T Cells From Cultured Marrow Cells

The ability of CM to induce and sustain the long-term growth of Thy-1 positive cells from cultured marrow cells was examined using a set of BDF1 CMC. Over a period of 7 weeks, the CMC were subjected to weekly depopulation. The recovered cells were pooled, counted, and studied for the ability to generate T cells in CM. During this period, the cultures maintained an average CFU-c incidence of 30–35 colonies per 10^4 cells; total suspension cells per flask decreased from approximately 10^7 to 2×10^6, whereas differential counts revealed a shift from predominantly granulocytic to an increasing proportion of monocytic cells (Table I).

Cell recovery after the first week of culture in CM was consistently around 50% of the input number. At this time, granulocytes, monocytes, and some blast cells, together with some unidentified cells with basophilic granulations, could be detected from a Wright-stained smear. With passaging at weekly intervals, the granulocytes and monocytes were soon lost from the cultures. Within 2–3 weeks, the cultures contained only 2 kinds of cells: a minor population of large cells that bear resemblance to the T cells derived from splenocytes in having foamy cytoplasm and occasionally a few cytoplasmic granules, and a major population of much smaller cells with cytoplasm densely packed with large basophilic granules.

Despite the absence of detectable Thy-1-bearing cells in the input marrow (personal unpublished data), immunofluorescent studies revealed that the former population that represented 4–12% of the total was strongly Thy-1 positive, whereas the latter population of granulated cells was Thy-1 negative (Table I). We had previously observed that T cells derived from cultures of normal spleen, thymus, and bone marrow in the presence of CM became adherent to the bottom of the plastic culture flasks within 2–3 weeks. The Thy-1 positive cells developing from CMC likewise displayed adherence property. Both the adherent T cells and the nonadherent Thy-1 negative cells were dependent upon CM for growth and weekly increase in population size of 4–8-fold was consistently observed, with relatively little change in the proportions of the two cell types.

Establishment of Pure Cultures of Thy-1 Positive Cells From Continuous Marrow Cultures

A set of 3- and 4-week-old CMC of DBA2 mice was used in these studies. Typical blast cells were observed after 1 week of culture in CM. The cells were passaged every 3–4 days instead of at weekly intervals. This procedure resulted in an increase in the proportion of blast cells to over 25% (Table II). In contrast, cells passaged at weekly intervals

TABLE I. Generation of T Cells From Cells Recovered From Continuous Marrow Cultures of BDF1 Mice and Subcultured in CM

Age of CMC (weeks after repopulation)	Culture condition of CMC					No. of passages in CM[b]	No. of days in CM	% Thy-1.2-positive cells by immunofluorescence[c]
	No. of cells/flask $\times 10^6$	No. of CFU-c/10^4 cells	Differential counts (%)					
			Granulocytic	Monocytic	Undifferentiated[a]			
1	ND	ND[d]	–	ND	–	3	28	9.3
						4	35	4.0
2	9.2	30	77	7	10	1	14	12.0
						2	21	12.7
4	6.2	ND	55	32	10	5	42	4.9
5	ND	ND	–	ND	–	1	11	3.4
						2	17	11.7
						3	24	7.8
6	2.0	35	–	ND	–	3	28	11.0
7	1.8	30	40	50	9	2	21	4.4
						4	35	1.7

[a]Undifferentiated cells refer to hemopoietic cells showing no sign of differentiation along any of the hemopoietic differentiation pathways.

[b]Cells were recovered from a set of 20 BDF1 continuous marrow cultures at weekly intervals, washed, and resuspended in CM at a concentration of 10^6/ml. Viable cells were recovered from CM every 6–7 days, tested, and subcultured at 5×10^4/ml.

[c]The percentage of Thy-1.2-positive cells by indirect immunofluorescence. A minimum of 100 cells were counted. Control cells treated with α-IgM-RITC alone were always negative (ie, <1%).

[d]ND, not done.

TABLE II. Establishment of Long-Term Pure Cultures of T Lymphocytes From a 3-Week-Old Continuous Marrow Culture of DBA2 Mice

Age of CMC	Time of fractionation of adherent cells[a] Passage no.	Time of testing Passage no.	Time of testing Days in CM	Percentage of blast cells[b]	Percentage of Thy-1.2-positive cells by immunofluorescence[c]
3 weeks		2	12	25	ND[d]
	3	3	19	>99	>99
		4	22	>99	ND
		6	27	>99	>99

[a]Cells recovered from CMC were suspended in CM at 10^6/ml. After 1 week in culture, the viable cells were recovered and subcultured at 5×10^4/ml every 3–4 days. After 3–5 passages, when the lymphoid cells became adherent, they were separated from the majority of nonadherent granulated cells and further subcultured in CM at 5×10^4 ml.

[b]The percentage of lymphoid cells were enumerated from a Wright-stained smear. A minimum of 100 cells were counted.

[c]The percentage of Thy-1.2-positive cells were enumerated by indirect immunofluorescence. A minium of 100 cells were counted. Control cells treated with α-IgM-RITC alone were always negative (ie, >1%).

[d]ND, not done.

did not show any increase in the proportion of blast cells (data not shown). As the blast cells became adherent to the bottom of the culture vessel, they were separated from the majority of the nonadherent granulated cells by first removing the latter by pipetting, then by washing with fresh medium to remove the adherent blast cells. Wright-stained smears of the adherent fraction revealed 90–100% blasts. Pure cultures of such blast cells were established by further passaging the cells every 3–4 days. Subsequent testing by immunofluorescence showed that more than 99% of the cells were Thy-1 positive. (Table II).

The morphology and growth pattern were identical to those observed for long-term T-cell cultures established from the spleen, thymus, or bone marrow: the doubling time was around 24 hr, the saturation density was around 10^6 per ml, and the cells were totally dependent on continuous presence of TCGF: when passaged in RPMI alone or with ConA, they died within 24 hr. Cytochemical reactions for peroxydase, specific esterase, and alkaline phosphatase performed on these cells were all negative.

DISCUSSION

It has been demonstrated that stimulation of murine or rat spleen cells with the mitogen concanavalin A induces the release of a growth factor for T lymphocytes (TCGF also designated interleukin 2). In the continuous presence of TCGF, long-term cultures of T lymphocytes can be established from spleen cells provided they have been stimulated by a mitogen or an antigen [14–16]. Using a murine ConA splenic leukocytes-conditioned medium we have established long-term cultures of T cells from the spleen, thymus, and bone marrow of mice (personal unpublished data): 100% of the cultured cells expressed Thy-1 antigen by an indirect immunofluorescence assay.

More recently, Gillis et al [20] demonstrated that a conditioned medium of ConA-stimulated rat spleen cells allowed generation of Thy-1-bearing cytotoxic T lymphocytes (CTL) from antigen-activated spleen cells of athymic nude mice.

Our experiments on nude bone marrow cells and T-cell-depleted normal marrow [21] showed that a ConA spleen cells-conditioned medium was capable of inducing expression of Thy-1 antigen on T-cell progenitors. Thus such a conditioned medium not only acts as a proliferating factor for mature T lymphocytes but also as an inducer of T-cell differentiation.

We used this property to investigate the maintenance of T-cell precursors in the long-term marrow culture system described by Dexter et al [3]. Mature and immunocompetent T cells are not maintained in CMC as demonstrated by the observation that cultured marrow cells do not respond to PHA [8], the immunohematopoietic system of lethally irradiated mice could be fully reconstituted with semiallogeneic cultured marrow cells without the complications of graft-versus-host reaction [7], and established marrow adherent layer maintained the continued production and differentiation of hemopoietic stem cells when repopulated with allogeneic and semiallogeneic bone marrow cells [22]. In contrast, after culturing the cells from CMC in the presence of a murine ConA-conditioned medium, we consistently observed the proliferation of Thy-1-bearing T cells together with a second population of Thy-1 negative granulated cells: The nature of the latter population is under current investigation. We used the adherence property of the T cells in culture to separate them from the non-T cell population. Using this procedure, pure populations of T cells have been obtained and further propagated in vitro: Their morphology and growth pattern are exactly similar to the long-term cultured T cells generated from spleen, thymus, and bone marrow. Their growth is totally dependent on the continuous presence of TCGF.

Shrader et al [23] found that a small number of Thy-1 positive cells are maintained in continuous marrow cultures up to the age of 7 weeks. This has not been found by others and, in our experiments, using immunofluorescence, we were unable to detect any surface Thy-1-bearing cells in CMC. However, as we did not treat the cells with anti-Thy-1 antibody and complement before culture in CM, we cannot exclude the possibility that proliferation of a very small number of undetectable Thy-1-positive cells occurred.

Our findings are in agreement with the results of Schrader et al [23], who found that terminal desoxynucleotidyl transferase-positive cells are maintained in CMC: T-cell progenitors are known to be TdT-positive.

In a recent paper, Jones-Villeneuve et al [24] demonstrated that long-term marrow cultures up to the age of 16 weeks contain T-cell precursors (CFU-T), forming colonies when plated in semisolid medium in the presence of a conditioned medium of PHA-stimulated human leukocytes. Part of the cells from these colonies were Thy-1-positive in a cytotoxicity assay, whereas the precursor in CMC was not sensitive to lysis with anti-Thy-1 antibody and complement. In addition, they found that cells from these colonies had helper activity for cytotoxic T lymphocytes. We do not know whether or not the cells we have obtained in suspension culture came from the same T-cell precursor. Experiments are carried out by the investigators whether or not precursors of CTL are maintained in continuous marrow cultures.

The ability to maintain T-cell precursors in vitro for prolonged periods in CMC and to induce their maturation and further growth in long-term cultures opens new approaches to the analysis of T-cell development. In particular, the analysis of the apparent inability of cultured marrow cells to elicit a GvH reaction following transfer to histoincompatible recipients should provide insight into mechanisms of T-cell tolerance and T-cell diversification.

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