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Influence of a Fibroblastoid Cell Line Derived from Murine Bone Marrow (H-1 Cells) on Stem Cell Proliferation[1] (41768)

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Abstract. A murine fibroblastoid cell line (H-1) with properties similar to those of adventitial reticular cells can support granulopoiesis and the development of mononuclear phagocytes *in vitro*. In the current study the effect of these cells on stem cell maintenance *in vitro* was assessed. The H-1 cells were unable to support CFU_s replication in liquid culture, while treatment of some stem cells with H-1 conditioned medium appeared to inhibit their proliferation.

Several studies have indicated that the proliferation of murine hemopoietic stem cells (CFU_s) is influenced by the bone marrow stroma (1–3) and indeed marrow adherent cells grown *in vitro* have been shown to support hemopoiesis *in vivo* (4, 5) and *in vitro* (6, 7). These adherent cells were described as phagocytic mononuclear cells, epithelial cells, and giant fat cells (7, 8) although more recent studies have suggested that many of the adherent cells are of a fibroblastoid nature (9, 10). Recently a cell line with fibroblastoid morphology was established from the adherent layer of a liquid murine bone marrow culture (11, 12). These cells influence both granulopoiesis and erythropoiesis *in vitro* (13). The current study was undertaken to ascertain the influence of these cells on CFU_s survival and proliferation *in vitro*.

Materials and Methods. *Cell culture.* The H-1 cells were routinely cultured in Fischer's medium (GIBCO) supplemented with 10% horse serum (Flow Laboratories) and penicillin and streptomycin (GIBCO, 100X). The cultures were incubated at 33°C in an atmosphere of 5% carbon dioxide in air and subcultured weekly, at a split ratio of 1:10 using a trypsin–versene dispersant (GIBCO, IX).

For experimental purposes 25-cm^2 Corning flasks were seeded with 0.6, 1.2, and 1.8×10^4 H-1 cells in Fischer's medium supplemented with antibiotics with or without 10^{-7} *M* hydrocortisone sodium succinate. In addition, 25% horse serum was added, this being the serum concentration routinely used in the Dexter liquid culture system. The cultures were incubated at 33°C, fed twice weekly and used after 1 or 2 weeks. At this time, they were washed with Fischer's and recharged either with 10^7 fresh C57Bl/6J bone marrow cells, or 1.5×10^6 cells taken from liquid bone marrow cultures, suspended in 10 ml supplemented Fischer's medium. They were fed weekly by demidepopulation.

Liquid bone marrow cultures (DLTLC) were derived from the bone marrow of 8-week-old C57Bl/6 mice according to the method of Dexter *et al.* (14) with the addition of 10^{-7} *M* hydrocortisone sodium succinate (15). The cultures were recharged with 10^7 freshly isolated syngeneic cells after 3 weeks and fed weekly by demidepopulation.

When necessary the bone marrow adherent layers or the H-1 derived adherent layers were harvested by treatment with trypsin-EDTA (GIBCO, IX).

Conditioned medium. The medium was harvested from 7-day-old H-1 cultures grown in Fischer's supplemented with 2% horse serum and antibiotics. It was clarified by centrifugation at 400*g* for 10 min, filtered through a 0.22-μm Millipore filter and stored at −20°C until further use.

Incubation of hemopoietic cells in conditioned medium. Some 1.5×10^6 bone marrow cells (1.5×10^6/ml) were suspended in 100,

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75, 50, 25, and 0% conditioned medium, the dilution being made in Fischer's medium supplemented with 2% horse serum and incubated at 33°C in 5% CO_2 for 5 hr. The cells were taken from normal C57Bl/6 mice or mice injected with phenylhydrazine (50 mg/kg body weight) to provide a source of rapidly proliferating CFU_s (16, 17) [^{3}H]TdR (25 μCi/ml) or equivalent amounts of cold thymidine were added to duplicate cell suspensions for the last 30 min of incubation. The suspensions were then placed on ice, washed with cold medium, and adjusted to a cell concentration of 4×10^5/ml.

CFU_s assays. CFU_s were detected by injecting cells (40,000–100,000) in 0.1 ml of Fischer's medium intravenously into each of 15 mice previously irradiated with 730 rad of X-rays. The number of colonies per spleen (CFU_s) was counted after 10 days and the mean and standard error of each group calculated.

Results. *Inability of H-1 cells to support CFU_s proliferation.* H-1 adherent layers, established from varying cell numbers, were recharged with 10^7 bone marrow cells and fed weekly by demidepopulation. Cultures were maintained in the presence and absence of hydrocortisone. The CFU_s present in the nonadherent fractions were assessed by the inoculation of harvested cells (5×10^4) into irradiated mice at 2, 4, and 6 weeks of culture. After 6 weeks, the adherent layers were harvested and 10^5 cells inoculated into irradiated recipients. Regardless of the presence or absence of hydrocortisone in the medium, the nonadherent CFU_s concentrations detected per flask fell from 3×10^3 to 100 after 2 weeks. By 6 weeks, no CFU_s could be found either in the nonadherent or adherent fractions of the cultures, suggesting that the H-1 cells were not capable of supporting CFU_s proliferation in liquid culture.

However, it has been shown that the nonadherent proliferating cells in a liquid culture system are derived in the first weeks after recharging from the adherent cell layer and only subsequently do the recharging cells contribute to the proliferating CFU_s (18). Hence ability of H-1 adherent layers to support CFU_s adapted to culture was subsequently determined.

One-week-old H-1 cell layers derived from 1×10^5 cells were inoculated with either 1.5×10^6 nonadherent cells, 1.5×10^6 adherent cells, or a mixture of 0.75×10^6 adherent and 0.75×10^6 nonadherent cells taken from a DLTLC recharged 7 weeks earlier. The number of CFU_s present in these inocula were determined. After incubation for 4 weeks, the number of CFU_s present in both the adherent layers and the nonadherent fractions was assessed. As can be seen in Table I the H-1 adherent layers were incapable of supporting CFU_s proliferation.

Effect of H-1 cell conditioned medium on survival of CFU_s. When normal or rapidly proliferating stem cells were incubated in various concentrations of H-1 conditioned medium for 5 hr, no detrimental effect on their survival could be detected. However, the presence of 25% H-1 conditioned medium appeared to reduce the proportion of CFU_s, from phenylhydrazine treated mice, in DNA synthesis (Table II). Increasing amounts of H-1 medium had no further effect.

Discussion. It has been suggested that stem cell proliferation is under the control of various

TABLE I. CFU_s REMAINING IN CULTURES AFTER 4 WEEKS WHEN H-1 CELLS WERE INOCULATED WITH NONADHERENT AND/OR ADHERENT CELLS FROM ESTABLISHED DEXTER LIQUID CULTURES (1.5×10^6 CELLS INOCULATED)

Nature of cells used for inoculum	CFU_s inoculated per flask	CFU_s per flask at 4 weeks	
		Adherent cells	Nonadherent cells
1.5×10^6 Nonadherent cells	75 ± 5	15 ± 3	2 ± 0.6
1.5×10^6 adherent cells	31 ± 4	0	3 ± 1
0.75×10^6 adherent cells + 0.75×10^6 nonadherent cells	53 ± 5	14 ± 3	0

TABLE II. EFFECT OF H-1 CONDITIONED MEDIUM ON SURVIVAL OF CFU_s *in Vitro*

Source of marrow	Treatment[a]	CFU_s detected in 4×14^4 cells after incubation in H-1 conditioned medium for 5 hr				
		0%	25%	50%	75%	100%
Normal mice	—	12.2 ± 0.8	12.3 ± 0.9	11.9 ± 0.7	13.3 ± 0.8	11.2 ± 0.7
	[^{3}H]TdR	8.1 ± 0.6	8.7 ± 0.9	9.4 ± 0.6	8.6 ± 0.9	8.6 ± 0.5
PHz mice	—	9.1 ± 1.0	9.1 ± 1.0	9.6 ± 0.6	9.5 ± 0.9	9.7 ± 0.5
	[^{3}H]TdR	4.5 ± 0.5	7.1 ± 0.6	6.9 ± 0.5	6.7 ± 0.8	6.8 ± 0.9

[a] Cultures were treated in duplicate and 15 mice injected per sample.

regulatory substances produced by bone marrow cells (17–22). In the liquid bone marrow culture many of the CFU_s are found to be in the adherent layer (23) while even those in the nonadherent fractions are thought to be closely associated with the adherent cells (24). This has led to the suggestion that the adherent stromal cells produce important factors for stem cell proliferation and indeed Blackburn and Patt (25, 26) showed that marrow stromal factors increased the survival of CFU_s *in vitro*. The adherent layer is composed of various cell types and the question as to which cell type influences stem cell maintenance and proliferation is unanswered.

In the current study some culture conditioned CFU_s were retained in the adherent layers of H-1 cultures reinoculated with nonadherent DLTLC cells (Table I). However, the total number of stem cells in the culture rapidly decreased. This result suggested that these marrow derived fibroblastoid cells were unable to support the replication of CFU_s. Indeed long-term survival may require factors from many cell types, while short-term effects may be modulated by specific factors acting on particular populations.

No evidence was obtained from the production of a toxic factor by the H-1 cell, although it is possible that a noncytotoxic substance inhibiting the entry of some susceptible CFU_s into DNA synthesis, is produced by the H-1 cells. This notion is supported by the fact that exposure of phenylhydrazine-treated marrow to 25% or greater concentrations of H-1 conditioned medium reduced the [^{3}H]TdR induced suicide (Table II), although no effect was seen on cells from normal marrow. Different populations of stem cells have been reported and it is possible that those responding to phenylhydrazine exposure are of a particular population, susceptible to the H-1 factor. Indeed it has been reported that a CFU_s proliferation inhibitor is produced under steady state conditions in long term bone marrow cultures (27, 17) and that cycling stem cells can be blocked at various stages by inhibitory substances (17, 29). Wright *et al.* (29, 30), suggested that one cell producing this inhibitory factor was a bone marrow macrophage, although peritoneal macrophages and various cell lines derived from the mononuclear phagocytic series failed to elicit the inhibitory substance. The results reported in the current study suggest that at least one type of fibroblastoid cell found in the bone marrow may also produce a factor which can interfere with the replication of some CFU_s.

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