Spleen Colonies Produced by Cells Obtained from Colonies Grown *In Vitro*[1] (36028)

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Bone marrow cells from animals and man are capable of giving rise to hematopoietic colonies *in vivo* (1, 2) and/or *in vitro* (3, 4). In the *in vivo* system cells are transplanted into lethally irradiated mice and colonies appear on the spleen between 5–10 days after irradiation. These colonies differentiate into erythrocytic, granulocytic, megakaryocytic, or mixed cell types. In the *in vitro* system, colonies develop from marrow and spleen of animals (3), as well as from human blood and marrow (4, 5) when grown in a soft gel medium. These colonies consist of granulocytic or large mononuclear cells and require a stimulatory substance for growth to occur.

The cells giving rise to the *in vivo* and *in vitro* colonies most likely represent two distinct cell types which are closely related (6–9). The cell giving rise to *in vivo* colonies is a pluripotent stem cell, and the cell giving rise to *in vitro* colonies an early differentiated cell committed to granulopoiesis. The *in vitro* colonies could therefore result from self replication of the committed cell, "feed-in" from the pluripotent stem cell, or both. If "feed-in" from the pluripotent pool is occurring, pluripotent stem cells persist in developing *in vitro* colonies; and Dicke (10), using the solid agar method, has suggested that pluripotent stem cells persist in developing *in vitro* colonies. This possibility was investigated by injecting cells from colonies grown in methylcellulose into irradiated hosts and observing the development of spleen colonies.

Materials and Methods. Donor and recipient mice were (C57 Bl ♀ × DBA ♂) F_1 hybrid males raised in our laboratory from stock obtained from Jackson Laboratories. Animals ranged between 8 and 12 weeks of age for all experiments.

Cells for injection were obtained from colonies grown *in vitro* by an adaptation of the soft agar method described by Bradley and Metcalf (3). Methylcellulose (Dow Chemical) as described by Ichikawa *et al.* (11) and modified by Wortin *et al.* (12) was used in place of agar. Bone marrow cells were removed from the femurs of normal mice and dispersed in CMRL-1066 tissue culture medium. Cells were then suspended in 1.4% methylcellulose containing CMRL-1066, 10% horse serum, and "conditioned medium" prepared from incubated peripheral leukocytes (13) as the stimulatory substance and incubated at 37° in 7.5% Co_2. After various periods of growth, individual colonies were removed with a Pasteur pipette, pooled, and washed twice in CMRL-1066, resuspended, and counted in preparation for injection. A fraction of cells for injection were also exposed to 900 R of irradiation prior to injection.

Recipient mice for *in vivo* colony studies were exposed to irradiation (900 R) delivered by a cesium 137 source, 35 cm from target with an exposure rate 60 R/min.

The assay of spleen colony formation was performed as described by Till and McCulloch (1). Within 4 hr after irradiation mice were injected intravenously with a known number of cells obtained from colonies grown in tissue culture as described above. Ten days later mice were killed and the spleens were removed and fixed in Bouin's solution for counting of colonies. Sixteen hours before killing, mice were injected subcutaneously with 0.1 μCu of ^{59}Fe; and the uptake of radioactive iron into the spleens was measured.

Histologic observation of *in vivo* colonies was made from 5 serial sections made at 5

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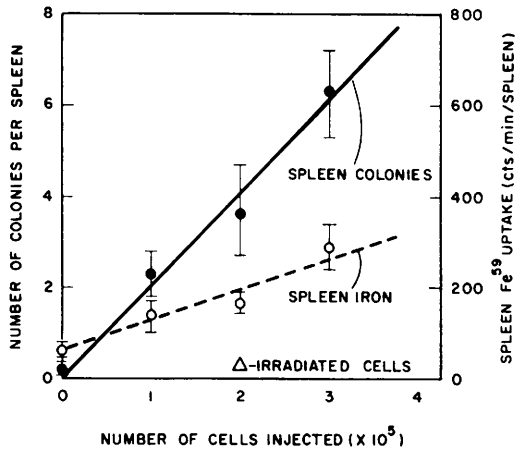

FIG. 1. Relationship between number of spleen colonies, spleen iron, and number of cells injected. Values represent the mean and standard error of 10 mice/group. Cells for injection were obtained after 4 days growth *in vitro*. (●——) Spleen colonies; (○——) spleen iron; (Δ) number of colonies from 2×10^5 irradiated cells.

μ intervals through the center of the spleen sectioned along its longitudinal axis and stained with hematoxylin and eosin.

Results. The relationship between the number of 4 day *in vitro* colony cells injected and the number of colonies formed on the spleens of irradiated recipients is shown in Fig. 1. A progressive increase in number of spleen colonies was observed as the number of cells injected was increased. Although the number of spleen colonies was small, these differed significantly ($p<.05$) from that of control animals. An increase in ^{59}Fe incorporation by the spleen paralleled the increase in number of colonies. Cells which were exposed to irradiation (900 R) prior to injection did not result in colony formation which differed significantly from control animals. In two duplicate experiments, the results followed a similar pattern to that described above.

The relationship between duration of cell growth *in vitro* and their ability to form spleen colonies *in vivo* is seen in Table I. The injection of cells obtained after 3, 4, or 5 days of growth resulted in spleen colony formation which differed significantly ($p<0.05$) from control animals, with cells derived after 3 days of growth containing the greatest concentration of spleen colony forming cells. Cells obtained after 6–10 days of *in vitro* growth produced only a few colonies which did not differ significantly from control ($p<0.1$). The cells obtained for injection after 3–4 days of *in vitro* growth were predominantly granulocytic in appearance and those obtained between days 6–10 were almost entirely large mononuclear (macrophages) cells.

To determine if spleen colony forming cells were perhaps all from the media, the concentration of spleen colony forming cells removed by aspirating *in vitro* colonies was compared to the same volume of media aspirated from the plates after colonies had been removed. These results revealed nearly a 3-fold greater concentration of spleen colony forming cells in the media containing the *in vitro* cells, suggesting that spleen colony forming cells were found in the *in vitro* colonies.

Histologically spleen colonies were either erythrocytic, granulocytic, megakaryocytic, or mixed cell type and were similar to those observed after transplantation of normal marrow or spleen cells.

Discussion. The results of this study suggest that pluripotent stem cells persist in or in close proximity to developing *in vitro* colonies arising from mouse marrow when grown in a soft gel medium. These cells retain their ability to form spleen colonies *in vivo* when transplanted into lethally irradiated recipients. That these are pluripotent stem cells is sug-

TABLE I. Relationship Between Duration of *in Vitro* Colony Cell Growth and Ability to Form Spleen Colonies.

Duration of *in vitro* cell growth (days)	No. of colonies[a]	^{59}Fe uptake spleen (cpm)
Control	0.1 ± 0.1	43 ± 3
3	4.6 ± 0.6	220 ± 60
4	1.3 ± 0.4	120 ± 32
5	2.0 ± 0.8	100 ± 28
6	0.4 ± 0.2	48 ± 7
7	0.3 ± 0.2	43 ± 6
10	0.4 ± 0.2	47 ± 7

[a] Colonies/spleen per 10^5 injected cells expressed as the mean and standard error of 6–10 mice/group. Control animals did not receive cells.

gested by the finding of erythrocytic, granulocytic, and megakaryocytic colonies in the spleen which are similar to those observed after the transplantation of normal marrow or spleen cells (14).

It seems unlikely that the increase in number of spleen colonies represented stimulation of endogenous spleen colony formation (2) rather than being produced from the transplanted cells. The good correlation between the number of cells injected and the number of spleen colonies produced, as well as the failure of irradiated cells to produce colonies, favor growth from the transplanted cells.

Cells removed after 3, 4, or 5 days of *in vitro* growth resulted in significant colony formation with cells removed after 3 days of growth containing the greatest concentration of colony forming cells. Cells removed after 6 days of growth did not result in significant colony formation.

It cannot be determined if the spleen colony forming cells found in the *in vitro* colonies had merely persisted in the *in vitro* cultures or had actually replicated in culture. Since the concentration of spleen colony forming cells in these cultures was slightly less than that of the original marrow innoculum, if replication occurred it was not extensive. The decreasing concentration of spleen colony forming cells in maturing *in vitro* colonies suggests that replication of the former either ceases or at least fails to keep pace with replication of more mature cells.

Assuming that spleen colony forming cells are found in the *in vitro* colonies, the following possible hypotheses concerning their relationship to *in vitro* colonies may be considered, but cannot be distinguished: (i) Spleen colony forming cells are closely related to their presumed progeny, the *in vitro* colony forming cell, and may replicate independently within the colony for a period of time and give rise to a certain number of *in vitro* colonies; (ii) *in vitro* colony forming cells are able to "de-differentiate" to spleen colony forming cells during *in vitro* colony growth; or (iii) *in vitro* and *in vivo* colony forming cells are in the same compartment.

The finding of pluripotent stem cells persisting in developing *in vitro* colonies is not entirely unexpected. In the *in vivo* system, colonies which have differentiated into erythrocytic, granulocytic, or megakaryocytic cell lines contain pluripotent stem cells capable of repopulating the spleen of irradiated recipients (15). The finding of such cells in the *in vitro* colonies suggests that these more primitive cells perhaps "feed" into the committed granulocytic stem cell pool and that both cell types may contribute to colony formation *in vitro*.

Summary. Cells grown *in vitro* from mouse bone marrow are capable of forming hematopoietic spleen colonies when injected into lethally irradiated recipient mice. Cells derived after 3–5 days of *in vitro* growth resulted in significant colony formation with the 3 day colonies containing the greatest concentration of spleen colony forming cells. Histologically, spleen colonies were erythrocytic, granulocytic, megakaryocytic or mixed cell types. These results suggest that pluripotent stem cells are contained within or in close proximity to developing *in vitro* colonies and may contribute along with the committed stem cell to the formation of granulocytic and mononuclear cell colonies *in vitro*.

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