PROLIFERATION AND DIFFERENTIATION OF
HEMATOPOIETIC STEM CELLS IN LONG-TERM CULTURES OF
ADULT HAMSTER SPLEEN*

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Studies on the regulatory mechanisms for stem cell differentiation in hematopoietic
and lymphopoietic tissues indicate that an adequate hematopoietic microenvironment
(HM) is an essential requirement both in vivo and in vitro (1–11). Whereas there is
uncertainty about the mechanisms for cellular relationships and function during early
differentiation of stem cells (1–14), the general concept implies special interactions
between stromal cells comprising the matrix of specialized regions of hematopoietic
organs, and stem cell populations that "reside" in or "home" to these regions. The
induction of proliferation may result from direct cell-cell interaction, whereas differ-
entiation may be induced either by cellular interaction, humoral factors produced by
stromal elements, or both.

In the adult animal the spleen is usually considered to have an insignificant role in
hematopoiesis. However, the spleen is the primary hematopoietic organ at certain
stages of fetal development and in pathological conditions of bone marrow failure
such as myelofibrosis. Experimental studies in vivo and in vitro (15, 16) have
demonstrated that adult spleen contains pluripotent stem cells (CFU-S) and commit-
ted progenitor cells (CFU-C). The adequacy of the splenic HM for the in vivo
maintenance of hematopoiesis has been shown by the spleen colony assay of Till and
McCulloch (17) and by implantation of mouse spleen stroma into omenta of irradiated
or normal hosts (18). The findings presented in the cited papers indicate that the
spleen differs from bone marrow in three ways: (a) the CFU-S and CFU-C content in
spleen is lower than in bone marrow, (b) the precursor populations are in the resting
state in the spleen, and (c) the pattern of differentiation imposed on pluripotent stem
cells by the splenic HM is different from that due to the medullary stroma.

From the work cited it appears that the functional specializations of hematopoietic
organs in the adult are a consequence of differences in their HM. As part of an
ongoing program of studies of hematopoiesis in the Syrian hamster, we have under-
taken a series of in vitro experiments to determine if the HM from different
hematopoietic organs impose restraints on the differentiation of stem cells derived
from bone marrow. During the initial experiments in this series we found that

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Abbreviations used in this paper: HM, hematopoietic microenvironment; HSCM, hamster spleen-condi-
tioned medium; CFU-C, committed progenitor cells; CFU-S, pluripotent stem cells.
adherent layers of spleen stromal cells were able to support the proliferation and differentiation of myeloid elements before seeding with bone marrow cells. This result has led to the establishment of independent cultures of adult hamster spleen in which proliferation and differentiation of monocytic, myeloid, and megakaryocytic cells have been maintained for >4 mo. Whereas previous studies on long-term liquid cultures of hematopoietic tissues have been restricted to bone marrow (6–8, 19), this is the first report of the long-term culture of adult spleen and of the maintenance of both proliferation and differentiation of cultured hematopoietic elements without reseeding with fresh stem cells or the addition of exogenous growth factors to the basic culture medium.

Materials and Methods

Cell Cultures. Male and female Syrian hamsters of the F1D (Trenton Labs, Bar Harbor, ME) strain were used in these experiments. All animals were 8–10 wk of age. Spleens were removed aseptically and prepared for culture by either mincing with scissors with direct plating or flushing the spleen with medium using a 25-gauge needle. The nutrient medium was RPMI 1640 with 20% unheated horse serum (Flow Laboratories Inc., Rockville, MD) and gentamycin at a concentration of 10.0 mg/liter of medium. Cells were plated in T-25 flasks (Falcon Labware, Oxnard, CA) in 5 ml of medium at various cell densities. Single-cell suspensions of nucleated cells at levels of 2.6 × 10^6 to 1.3 × 10^7/flask were most successful in providing long-term maintenance of precursor cell populations. Cultures were incubated in an atmosphere of fully humidified 5% CO₂ in air.

Feeding was accomplished by removing one-half of the medium and supernatant cells and replacing the 2.5 ml removed with 2.5 ml of fresh medium. After the initial plating, the cultures were fed at day 3 and 7 with subsequent feedings at weekly intervals. The cells removed at the 3rd- and 7th-d feeding were used to establish subcultures by adding 2.5 ml of fresh medium and replating. In most instances the 7th-d subcultures were more successful in maintaining long-term hematopoiesis than the primary cultures from which they were derived. It is possible that this improved maintenance is due to removal of inhibitory stromal cell elements, which remained with the adherent component of the original cell population (20).

Colony Assays in Semi-Solid Medium. Clonal growth of nonadherent precursor cell populations was assayed weekly in 0.3% agar using the methods described by Johnson and Metcalf (21, 22). Stimulation for colony growth was provided by pokeweed mitogen-stimulated spleen cell-conditioned medium obtained after a 1-wk incubation of hamster spleen cells in the plasma-free medium described by Guilbert and Iscove (23), as modified by Burstein et al. (24). Control experiments on colony formation by fresh bone marrow samples showed linear growth both for number of cells plated and amount of hamster spleen-conditioned medium (HSCM) added. Spleen culture supernatant cells were plated at densities ranging from 1.5 × 10^3 to 2.6 × 10^5 cells in 35-mm petri dishes, the density depending on the number of blast cells present as measured on Wright-stained cytospin preparations. Plates were incubated for 1 wk in 10% CO₂ and then scored for colony number. Colonies were sequentially picked, smeared on slides, and stained with a Papanicolaou method modified for hematopoietic colonies in agar (25). In several experiments the agar disks were fixed in situ with 3% glutaraldehyde, then removed and mounted on slides according to the method of Salmon and Buick (26). These slides were then stained for acetylcholinesterase (27) to confirm the presence of megakaryocytes.

Spleen Colony Assays. To determine if CFU-S were present in the supernatant cell population, the spleen colony assay of Till and McCulloch (17) was used on cultures at 4 wk of incubation. 14 hamsters were exposed to lethal total-body irradiation of 925 rad from a 137Cs gamma-emitting source at a rate of 137 rad/min. After 18 h, eight animals were injected intravenously with supernatant culture cells, four with a suspension of normal spleen cells, and two with medium only. All animals were anesthetized with pentobarbital. Cell suspensions were injected via the lingual vein. The spleens were examined for the presence of surface colonies 10 d after injection. The cellular composition of spleen colonies was determined on paraffin sections stained with hematoxylin and eosin or on frozen sections stained for acetylcholinesterase.
**Cell Morphology.** The morphology of the cells in liquid cultures was monitored and photographed using a Wilde inverted phase contrast microscope. Preparations of supernatant cells were obtained at each weekly feeding by cytocentrifugation (Cytospin, Shandon Southern Instruments, Inc., Sewickley, PA) and stained with Wright's stain. Sudan black and acetylcholinesterase staining were used to determine the presence of lipid-containing cells and megakaryocytes. Cell counts were performed using a TOA automatic cell counter (Microcellcounter CC-108, TOA Medical Electronics Manufacturers, Ltd., San Francisco, California.)

**Results**

In the course of pilot studies for this work it became evident that there was a critical distinction to be made between the cells maintained in culture. These were the categories of adherent cells, cells attached to or associated with adherent cells, and cells floating in the liquid phase (suspension cells). The complex of firmly adherent cells with the cells attached to them appeared to be essential for the maintenance of hematopoietic proliferation and differentiation of adult hamster spleen cultures. The definitive studies presented here confirm and quantify these initial pilot observations.

**Characteristics of the Adherent Layer.** Adherence of stromal cells was complete within 2–2.5 wk. This adherent layer was comprised of two main types of cells: (a) small stellate cells with several short dendritic cytoplasmic processes (Fig. 1), and (b) giant multiprocessed flattened cells (Figs. 2, 3). The surface area of the flasks occupied by adherent cells varied from culture to culture with a pattern of islet or aggregate formation. No culture displayed confluent growth.

Clumps and small chains of blasts, myeloid, megakaryocytic, and monocytic cells were seen on or near both types of adherent cells, but their more intimate topographic connections were with the giant flattened cells (Figs. 2–5). Supernatant cells in actively proliferating cultures were of the same type seen in association with the adherent aggregates and appeared to arise from these islets. This association of adherent and supernatant cells is analogous to the cell-producing islets described by Dexter and his co-workers (6–8) in mouse bone marrow cultures, although the spleen cultures did show differences in stromal cell type as noted below.

The adherent cells, which comprised the base for the islets producing hematopoietic cells, were of reticular or fibroblastic morphology, and typically were flattened giant multiprocessed cells with a small nucleus containing two to six prominent nucleoli. Whereas these cells were usually single, several displayed syncytial connections (Fig. 3). With phase-contrast microscopy the cytoplasm of the giant cells contained small fine dark granules concentrated in the perinuclear zone, a description corresponding to the large flattened cells described by Allen and Dexter (7, 8) as cells of “epithelial” type.

On the surface of the giant cells there were small cells of varying shape (irregular, polygonal, oval, and round) with a small nucleus and short cytoplasmic processes at one of the poles (Figs. 3, 4). The striking peculiarity of these cells was their motility. In the course of a 10–15-min inspection there was a change in location of these cells along the surface of giant cells and a detachment of cell clumps forming near adherent cells (Figs. 4, 5). These small cells were not seen in cultures producing only macrophages or with low levels of hematopoietic cell production and may represent the proliferative cells of the hematopoietic compartment of the cultures.

Later, cells of a third type appeared on the surface of giant flattened cells. These were smaller than the flattened cells, oval or polygonal in shape, with 1–3 visible
Fig. 1. Phase-contrast photomicrograph of stellate cells (arrows) with short dendritic processes adherent to the bottom of the flask. Hamster spleen culture, 10 wk old. × 640.

Fig. 2. Phase-contrast photomicrograph of adherent giant elongated cell (large arrow) with aggregates of hematopoietic cells (small arrows) and individual macrophages (small arrow heads) attached to its surface. Hamster spleen culture, 14.5 wk old. × 430.

Fig. 3. Phase contrast photomicrograph of adherent multiprocessed cells displaying syncytial connection. Small dark cells (arrows) and granulovacuolated cells (small arrow heads) are attached to the surface of adherent cells. The latter demonstrates prominent cytoplasmic granularity. Refractile clusters of hematopoietic cells and macrophages are seen near the surface of adherent cells. Hamster spleen culture, 14.5 wk old. × 430.
Fig. 4. Phase-contrast photomicrograph of part of a cytoplasmic process of a giant adherent cell (large arrow) with attached small dark cells (small arrows) and a clump of blast or myeloid cells (large arrow head). Note that small dark cells, which are beginning to detach, have refractile halos not seen on attached cells. Individual macrophages (small arrow head) may also be seen on the surface of the giant adherent cell. Hamster spleen culture, 15.5 wk old. × 860.

Fig. 5. Phase-contrast photomicrograph of adherent cell with suspended megakaryocytes (large arrow) and macrophages (small arrow). Megakaryocytes display a homogeneous cytoplasm with refractile surfaces in contrast to the granular refractile cytoplasm of macrophages. Other hematopoietic cells are detached from the surface of the adherent cell. Hamster spleen culture, 14.5 wk old. × 860.
cytoplasmic processes, and large black granules alternating with light vacuoles in the cytoplasm (Fig. 3). These cells correspond morphologically to the phagocytic mononuclear cells described in bone marrow cultures which produce myeloid cells for extended periods of time (7-8). Cells of this type were found as a component of the majority of the adherent islets. In some of the cultures with macrophage production only, or in poorly replicating cultures with low cell densities in the supernatant, these cells contained enlarged, irregular cytoplasmic vacuoles (Fig. 6), rather than the more uniform rounded vacuoles that were present in this cell type in cultures supporting active hematopoiesis.

We have been unable to identify any “giant fat cells” in these cultures. Their presence in the adherent layer of mouse bone marrow cultures is believed to be crucial for successful proliferation and differentiation of stem cells and their progeny (6-8). In our hands staining of cells of the adherent layer with Sudan black did not demonstrate the presence of lipid material in the cytoplasm of any cells in several hamster cultures examined.

In all cultures actively producing hematopoietic cells in the supernatant, most of the adherent islands were associated with multiple supernatant cell types, such as both myeloid and megakaryocytic cells (Fig. 5). On occasion, however, small islets associated with a single cell type such as megakaryocytes were seen (Fig. 7). This suggests a limited potential of some adherent cells to direct the differentiation of precursor cells.

**Characteristics of Supernatant Cells.** The average content of nucleated cells in freshly prepared suspensions of adult hamster spleen was: lymphomonocytoid cells, 91%; myeloid cells, 6%; and undifferentiated cells, 3%. There were also a few immature

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**Fig. 6.** Phase-contrast photomicrograph of a culture supporting only macrophage differentiation. Cells with granulo-vacuolated cytoplasms are firmly attached to adherent cells. Hamster spleen culture, 8 wk old. X 860.

**Fig. 7.** Phase-contrast photomicrograph of adherent cells with megakaryocytes (arrows) only attached to the cytoplasmic processes. Hamster spleen culture, 8 wk old. X 450.
erythroid cells. Lymphomonocytoid cells consisted of lymphocytes, monocytes, and plasma cells at all stages of differentiation. Undifferentiated cell counts included infrequent reticular cells ("Ferrata" cells) and rare erythroblasts. Ferrata cells were included because some workers consider them to be stem cells (28, 29).

Differential counts on the supernatant cells showed that during the first 2–3 wk of culture the number of lymphocytoid cells rapidly decreased, while the myeloid series (especially mature granulocytes) increased gradually (Fig. 8) and detectable megakaryocytes appeared (Table I). At the 4th wk, early myelocytic precursors became predominant over mature granulocytes and maintained that dominance for the remaining period of culture. The number of megakaryocytes varied from 2 to 4% as evaluated by characteristic morphology on Wright-stained cytospin preparations. All stages of differentiation from the basophilic to granular azurophilic cells were present in these routine preparations (Fig. 9). Use of acetylcholinesterase staining, however, revealed much larger numbers of the megakaryocytic series (Fig. 10). Some of the cells that were positive for acetylcholinesterase activity were small cells not recognizable as members of the megakaryocytic series by standard morphology with Wright stain, and therefore probably representative of early stages of megakaryocytic differentiation. Some megakaryocytes displayed emperipolesis (Fig. 11), whereas others manifested mitotic activity (Fig. 12).

Undifferentiated cells appeared as aggregates or clumps, which corresponded to the appearance of the cells in the cultures as seen with phase-contrast microscopy. The number of member cells in these groups varied from 10 to 60, with only an occasional cell in the aggregate displaying features useful in determining the type of differentiation. In contrast, other clusters were seen that consisted of cells with identifiable differentiation. These clusters were predominantly myelocytic (Fig. 13) or megakaryocytic.

The supernatant blast cell clusters from spleen cultures showed a heterogeneous morphology, with at least three different types of aggregates identifiable cytologically. The most frequently occurring aggregates were composed of cells that were lymphomonocytoid cells in size and appearance (Fig. 14). They have a high nuclear/cytoplasmic ratio, and in some cells the cytoplasm is virtually undetectable. Chromatin was coarsely clumped in the small cells in these clusters, whereas the monocytoid type had evenly distributed chromatin. The peripheral cytoplasm was deep blue with a prominent light cytoplasmic zone near the nuclear invagination of the larger cells.

Fig. 8. Cytospin preparation of supernatant cells showing clumps of blast cells (small arrow), myeloid cells at different stages of maturation (small arrow head), and megakaryocyte (large arrow head) surrounded by myeloid cells. Hamster spleen culture, 4 wk old. Wright stain × 130.

Fig. 9. Cytospin preparation showing megakaryocytes (arrows) at different stages of differentiation. Myeloid precursors and mature granulocytes (arrow head) are also present. Hamster spleen culture, 5 wk old. Wright stain × 150.

Fig. 10. Acetylcholinesterase-positive cells (arrows) in a cytospin preparation from supernatant cells. Hamster spleen culture, 4 wk old. × 400.

Fig. 11. Phenomenon of emperipolesis displayed in megakaryocytes. Note intact granular leukocytes within the cytoplasm of the megakaryocyte. Hamster spleen culture, 4 wk old. Wright stain × 650.

Fig. 12. Mitotic activity in a megakaryocyte. Hamster spleen culture, 10 wk old. Wright stain × 900.

Fig. 13. An aggregate of myelocytes displaying prominent azurophilic granularity in the cytoplasm. Hamster spleen culture, 13 wk old. Wright stain × 1,000.
HEMATOPOIESIS IN LONG-TERM SPLEEN CULTURES

Table I

| Weeks in culture | Cells/ml (X 10^-6) | Type of cell* |
|------------------|------------------|--------------|
|                  | Myel  | Gran  | Lymph  | Mono  | Mega  | Blast | Ery  |
| 0                | 1.20 ± 0.3† | 6 ± 2 | 0 | 91 ± 8 | § | 0 | 3 ± 1 | § |
| 1                | 0.61 ± 0.02 | 1 ± 1 | 0 | 96 ± 6 | § | § | 3 ± 1 | § |
| 2                | 1.01 ± 0.2 | 1 ± 0.5 | 0 | 94 ± 5 | § | § | 5 ± 2 | § |
| 3                | 1.21 ± 0.4 | 2 ± 1 | 0 | 80 ± 7 | 18 ± 3 | § | 0 | 0 |
| 4                | 1.12 ± 0.2 | 36 ± 4 | 18 ± 2 | 11 ± 2 | 29 ± 4 | 1 ± 0.5 | 3 ± 2 | 0 |
| 5                | 0.83 ± 0.1 | 80 ± 8 | 9 ± 1 | § | 1 ± 0.5 | 1 ± 1 | 9 ± 2 | 0 |
| 6                | 0.41 ± 0.03 | 86 ± 5 | 3 ± 1 | § | 3 ± 1 | 1 ± 0.8 | 8 ± 1 | 0 |
| 7                | 0.48 ± 0.03 | 81 ± 9 | 9 ± 2 | 0 | 1 ± 0.5 | 2 ± 1 | 8 ± 1 | 0 |
| 8                | 0.42 ± 0.01 | 68 ± 6 | 7 ± 2 | 0 | 12 ± 1 | 4 ± 1 | 9 ± 1 | 0 |
| 9                | 0.42 ± 0.02 | 57 ± 4 | 7 ± 1 | 0 | 13 ± 3 | 4 ± 1 | 19 ± 3 | 0 |
| 10               | 0.38 ± 0.01 | 67 ± 5 | 11 ± 2 | 0 | 3 ± 1 | 4 ± 2 | 15 ± 3 | 0 |
| 11               | 0.36 ± 0.03 | 68 ± 6 | 3 ± 2 | 0 | 6 ± 1 | 4 ± 1 | 19 ± 4 | 0 |
| 12               | 0.40 ± 0.04 | 61 ± 6 | 4 ± 1 | 0 | 22 ± 4 | 3 ± 1 | 10 ± 2 | 0 |
| 14               | 0.46 ± 0.03 | 61 ± 5 | 3 ± 2 | 0 | 21 ± 3 | 4 ± 1 | 11 ± 3 | 0 |
| 16               | 0.51 ± 0.02 | 62 ± 6 | 2 ± 2 | 0 | 25 ± 2 | 2 ± 1 | 9 ± 1 | 0 |

Percentages derived from count of 400 cells from three separate cultures.

* Myel, myeloid; Gran, granuloma; Lymph, lymphocyte; Mono, monocyte; Mega, megakaryocyte; Ery, erythrocyte.
† Mean ± SD.
§ Presence of very small number of cells.

Nucleoli were difficult to find in this type of cell and they had the highest incidence of mitotic figures.

The second type was comprised of relatively large cells with abundant pale blue cytoplasm containing a few fine azurophilic granules (Fig. 15). The nuclei had a finely reticulated to moderately coarse chromatin pattern with one to three distinct pale nucleoli. Other undifferentiated cells were of histiocytic type with light blue cytoplasm showing pseudopodial processes, fine evenly distributed chromatin, and one to two paler nucleoli (Fig. 16).

Macrophages were present in all cultures at all periods of observation, although in highly variable numbers. These cells showed basophilic inclusions, vacuoles, or both within their cytoplasm (Fig. 17).

Assays for Precursor Cells. Supernatant cells from cultures at various periods of incubation were plated in soft agar and exposed to HSCM to evaluate the number of

Fig. 14. An aggregate of undifferentiated cells of lymphomonocytoid type with a mitotic figure in one cell. Hamster spleen culture, 13 wk old. Wright stain × 900.
Fig. 15. Undifferentiated cells of the second type with abundant pale blue cytoplasm, reticular chromatin, and several prominent nucleoli. Hamster spleen culture, 11 wk old. Wright stain × 900.
Fig. 16. Undifferentiated cells with light blue irregular cytoplasm, short pseudopodia, and pale nucleoli. Hamster spleen culture, 10 wk old. Wright stain × 1,000.
Fig. 17. Two macrophages (arrows) with basophilic granules and numerous vacuoles in their cytoplasms. Hamster spleen culture, 13 wk old. Wright stain × 1,000.
Fig. 18. Cellular composition of "mixed" colony: granulocytes (small arrow), monocyte (small arrow head), normoblast (large arrow head), and megakaryocyte (large arrow). × 600. Modified Papanicolaou staining.
Fig. 19. A part of another "mixed" colony where granulocytes at different stages of differentiation are seen together with two normoblasts (arrows). × 1,200. Modified Papanicolaou staining.
HEMATOPOIESIS IN LONG-TERM SPLEEN CULTURES

Table II
Colony Formation in Soft Agar Cultures by Supernatant Cells from Hamster Spleen Liquid Cultures

| Weeks in culture | Colonies/10⁴ cells | Blast content % |
|------------------|--------------------|-----------------|
| 0                | 2                  | 4               |
| 1                | 2                  | 5               |
| 2                | 2                  | 5               |
| 3                | 4                  | 5               |
| 4                | ND*                |                 |
| 5                | 22                 | 9               |
| 6                | 20                 | 5               |
| 7                | 25                 | 8               |
| 8                | 28                 | 9               |
| 9                | 48                 | 20              |
| 10               | 48                 | 16              |
| 11               | 113                | 18              |
| 12               | 41                 | 10              |

* Not done.

Table III
Incidence of Mixed Colonies in Randomly Picked Colonies

| Weeks in culture | 2 | 4 | 6 | 8 | 10 | 12 | 14 |
|------------------|---|---|---|---|----|----|----|
| Mixed/total      | 3:3| 2:10| 0:6| 5:12| 3:6| 1:10| 11:13 |

Table IV
Spleen Colony Assay of Supernatant Phase Cells from Spleen Cultures

| Source of cells          | Number of animals | Number of cells injected | Number of surface colonies ± SD |
|--------------------------|-------------------|--------------------------|--------------------------------|
| Normal hamster spleen    | 4                 | 10⁶                      | 10.2 ± 2                       |
| Hamster spleen cultures  | 8                 | 10⁶                      | 28.6 ± 4                       |
| Media only               | 2                 | 0                        | 0                              |

 precursor cells present (21, 22). Colony-forming efficiency was variable (Table II), but it was found that a correlation existed between the number of blast cells present in the supernatant phase and the number of colonies found. The finding that blast cell content is important to colony formation was reinforced by observations on cultures where the only identifiable cells were macrophages and blast cells. In these cultures with no other probable candidate for a precursor cell population, eight colonies were found per 10⁵ cells, and three of these eight were mixed colonies.

The incidence of mixed colonies was also variable but was maintained throughout the period of culture reported here. Sequentially picked colonies were evaluated for mixed colony incidence and later culture periods showed a high percentage (Table III). The cellular content of mixed colonies was confirmed by the staining of whole fixed agar disks for acetylcholinesterase. Observed mixed colonies consisted of two to four different lineages (Figs. 18, 19). The majority of pure colonies were of the granulomonocytic or megakaryocytic series.
The spleen colony assay demonstrated that CFU-S were present in both the normal spleen cell suspension and in the supernatant phase of the spleen cultures (Table IV). The number of CFU-S in the culture was enriched by a factor of 2-3 when compared with normal spleen. A preliminary examination of hematoxylin-eosin-stained spleen sections from the injected animals revealed that mixed, erythrocytic, myeloid, and megakaryocytic colonies were present (data not shown). Evaluation of the relative number of colonies of each type is currently in progress.

Discussion

The results presented here indicate that long-term cultures of adult hamster spleen provide a simple in vitro model of myeloid, monocytic, and megakaryocytic hematopoiesis. Production of differentiated cells and their precursors is maintained without secondary repopulation or the addition of exogenous growth factors. Prolonged maintenance of hematopoietic cells in the supernatant phase provides evidence that an adequate HM is present and that endogenous pluripotent stem cells are able to replace cells depleted by removal at feeding. The presence of pluripotent stem cells is confirmed by the mixed-colony assay in agar cultures stimulated by HSCM (20, 21). The spleen colony assay results indicate that there is an enrichment of the fraction of progenitor cells capable of forming spleen colonies during the establishment of the cultures.

Three points of particular interest arise from these results. First, adult spleen, which does not normally participate in production or maintenance of myeloid or megakaryocytic cells in vivo, is able to produce these cells in vitro. In this regard, the functional capacity of the spleen reverts to functions present in embryonic life or in the clinical state of extramedullary hematopoiesis. This suggests that regulatory suppressors not present in vitro are present in the in vivo environment. Second, the combination of in vitro splenic hematopoiesis and the hematopoietic function of the spleen in periods of fetal life before bone marrow establishment or during extramedullary hematopoiesis suggests that normally functioning bone marrow might produce factors that regulate splenic hematopoietic function in the intact animal. Third, the stromal cellular component of the HM found in spleen cultures is similar to that found in bone marrow cultures, and the differentiated cell populations that arise in these two environments are comparable. This indicates that some regulatory properties of the two HM are similar and that differences in their function in the animal probably result from different interactions with regulatory factors, or that regulatory factors are present in the intact animal which are not produced by any of the cellular components in an in vitro system. Because erythropoiesis and lymphopoiesis are not seen in long-term in vitro systems of either spleen or bone marrow, but occur normally in vivo, these factors could be stimulatory in function.

The cytology of the adherent islet in the spleen culture is quite similar to that seen in bone marrow culture. The flattened giant cell is probably an important participant since its persistence is associated with the maintenance of long-term growth. In our system, these cells always displayed an intimate topographical relation with small cells, which may represent the earliest hematopoietic cell component of the cultures. We have not seen fat cells (7, 8) in these cultures, however. The maintenance of long term hematopoiesis in cultures devoid of the giant fat cell suggests that it is not an essential stromal component. If this type of cell does have an essential function in
murine bone marrow cultures, then that function in the spleen cultures is dependent upon a cell type that has no morphological similarity.

Another cellular feature of the spleen culture is the stellate cell (Fig. 1), which is morphologically consistent with the dendritic cell described by Steinman et al. (30-33). The ability of these cells to stimulate the mixed leukocyte reaction suggests that this function may be maintained in these cultures. Experiments are in progress to determine if these cells also have a function in the maintenance of in vitro hematopoiesis in the spleen system.

The data of Golde et al. (16) indicate that the spleen contains CFU-C, which increase in number under the influence of colony stimulating factors derived from pregnant mouse-uterine extracts. Their experiments were on responses of mouse spleen maintained in Marbrook diffusion chambers for 14 d, and showed a peak CFU-C content at 7-10 d of culture. Adherent cell populations were not assayed. The long-term maintenance of CFU-C in the hamster spleen cultures and the maturation of these cells to mature granulocytes and megakaryocytes indicates that the cell populations that are present are capable of continuing synthesis of regulatory factors which bring about differentiation as well as proliferation of the committed precursor population. It is of interest that the maintenance of the progenitor cells and their differentiated progeny continues without the addition of supplemental hormones or growth factors to the basic culture medium. In a preliminary report on murine spleen in culture, it was found that even with the addition of hydrocortisone to the medium, the cultures survived only 6 wk and converted to macrophage morphology within this period (34). The hamster spleen culture is different from mouse bone marrow culture in this regard also. Successful establishment of mouse marrow cultures is dependent on a secondary plating of 3-wk cultures with fresh marrow or the addition of hydrocortisone to the culture medium (19). The cultures described here do not require these manipulations. Because there is some variance of the adherent cell populations between the hamster system and the mouse system, comparative studies of the two species may be instructive in determining the regulatory role of the various adherent cells.

Summary

We have found that in liquid cultures of spleen cells of adult Syrian hamsters of the F1D strain, the hematopoietic microenvironment is adequate to sustain proliferation of splenic stem cells for periods of >4 mo, and permits granulocytic, monocytic, and megakaryocytic differentiation without secondary repopulation or addition of exogenous growth factors to the basic medium of RPMI 1640 plus 20% horse serum. Intimate topographical relations are established between spleen stromal cells and hematopoietic cell components of the culture in adherent "cell-producing" islets. Some of these islets are associated with multiple hematopoietic cell types such as myeloid, monocytic, and megakaryocytic cells. Other islets are associated with a single cell type such as megakaryocytes, which suggests a limited potential of some adherent stromal cells to direct the differentiation of precursor cells. Cultures of this type provide a simple and convenient model for investigation of the mechanisms controlling differentiation of hematopoietic stem cells, not only for granulocytic and monocytic cells, but for megakaryocytic cells as well.
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