In Vitro Commitment by Hemopoietins of Murine Marrow Exocolony Forming Units (CFU)? Preliminary Report (36680)

L. DELMONTE AND D. M. MUMFORD
(Introduced by W. T. Butler)

Departments of Anatomy, Microbiology and Obstetrics and Gynecology, Baylor College of Medicine, Houston, Texas 77025; and Department of Pediatrics, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

The effects of specific hemopoietins and of nonspecific exogenous stimuli, such as endotoxins and foreign proteins, on the total and differential spleen colony forming unit (CFU) potential of hemopoietic tissues have been studied in detail by many investigators using the Till–McCulloch (1) endogenous and exogenous spleen colony systems.

Curry, Trentin and Wolf (2) and Wolf and Trentin (3) have demonstrated that the E/G (erythrocytic:granulocytic) ratio of colonies observed in the spleen and marrow of hosts grafted intravenously with hemopoietic cells from untreated donors is host rather than donor tissue-dependent. This ratio cannot be altered by post-treatment of the hosts with hemopoietins.

We have shown (4, 5) that donor treatment with hemopoietins and nonspecific agents alters the total number and E/G ratio of host spleen exocolonies in such a way as to allow us to differentiate between the effects of erythrocytic and granulocytic cell line-specific hemopoietins as well as between hemopoietins and nonspecific stimuli such as endotoxin and foreign proteins.

In vivo studies are limited by the interdigitating action of stromal and humoral factors on the stem cells. The currently available *in vitro* cloning systems—the soft agar (6) and plasma clot (7) culture systems—appear to support growth of only hemopoietin-dependent, finitely mitotable, committed hemopoietic stem cells. Suspension cultures of bone marrow cells, with (8) and without (9) soft agar feeder underlay have been shown to permit survival of exocolonizing precursor cells. However, no reports are available to date as to the differential patterns of *in vitro* cultured marrow-derived spleen colonies.

In an attempt to study the direct effects of hemopoietins on the transplantable marrow CFU compartment(s), we used the combination of an *in vitro* suspension culture system and the *in vivo* spleen exocolonizing system.

Materials and Methods. A total of forty-five 8- to 10-week-old A/J male mice were used as femoral bone marrow donors.

For each experimental run, marrow cells from 9 donors were suspended in MEM (culture medium) and pooled. The viable nucleated cell concentration of each pool was adjusted to 5×10^5/ml and was dispensed in 10-ml volumes into culture tubes. Immediately prior to incubation the tubes received either 0.5 ml MEM, 0.5 ml renal granulopoietic factor (GPF) (10), or 0.5 erythrocyte stimulating factor (ESF) in the form of dialyzed heparinized anemic horse plasma.[1] The cell suspensions were incubated in a humidified atmosphere at 37°, 20% CO_2 in air.

The culture medium consisted of Eagle's minimal essential medium supplemented with 20% fetal calf serum, 1% nonessential amino acids, 1% *l*-glutamine, 1% sodium pyruvate and 4.2% $NaHCO_3$, plus 5 units penicillin and 5 μg streptomycin/ml.

Cultured cells were harvested after 0, 1, 3, 24, 48 and (MEM and GPF groups only) 72 hr incubation. The cells were concentrated in conical bottom tubes into an underlay of 50% fetal calf serum and washed once with MEM. Their final resuspension in MEM was adjusted to yield approximately 6 spleen surface colonies in supralethally irradiated hosts: 25×10^3, 50×10^3, 75×10^3, $125 \times$

[1] Kindly supplied by Dr. G. V. VanHoosier.

10^3, 250 $\times$ 10^3, and 125 $\times$ 10^4 viable nucleated cells harvested at 0, 1, 3, 24, 48 and 72 hr, respectively. Prior to and following centrifugation, total and viable (trypan blue dye-excluding) nucleated cell numbers were counted.

Eight- to ten-week-old male A/J hosts were given 750 R ($LD_{99/30}$) whole body irradiation delivered at a rate of 130 R/min in air from a G-E Maxitron 250 X-ray source (250 kVp, 30 mA, Th III filter with 1 mm Al and 0.5 mm Cu, HVL 3.0 mm Cu in air). Three to 5 hr later, the hosts were injected intravenously with 0.5 ml of the appropriately adjusted cultured cell suspension. Host spleens were harvested 9 days later and fixed in Bouin's solution. All surface colonies, regardless of their size, were counted at 9$\times$ magnification with a dissecting microscope.

A total of 1951 colonies were then classified differentially from hematoxylin- and eosin-stained, 5 μ-thick, longitudinal, subserial sections taken at 200-μ intervals throughout the length of 213 spleens.

Five replicate runs were performed for the GPF and MEM groups; three replicate runs for the ESF group.

Results. No significant intergroup differences were observed in the total nucleated cell and CFU survival rates (Fig. 1); CFU numbers decreased exponentially as a function of time, only 20% of the inoculated CFU surviving by 24 hr and less than 10% by 48 hr. Viable nucleated cell numbers, on the other hand, appeared to decrease exponentially only for 24 hr, and then plateaued at about 40–55% of initial values.

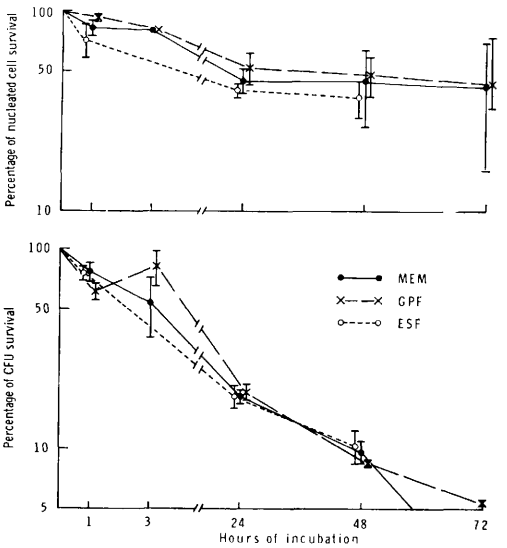

FIG. 1. *In vitro* survival of nucleated cells and CFU from femoral marrow cultured in the presence of GPF and ESF. (mean $\pm$ standard error).

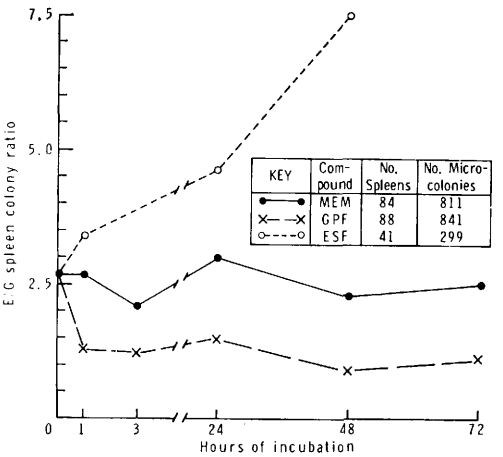

FIG. 2. *In vitro* shift of E/G ratio of transplantable femoral marrow CFU cultured in the presence of GPF and ESF.

Most striking was the marked divergence in the differential retransplantation potential of the *in vitro* cultured CFU over the entire 72-hr experimental period (Fig. 2): within as little as 1 hr one could detect a significant hemopoietin-specific shift of the E/G colony ratios from the normal 2.1–3.0 to 1.3 in the GPF- and 3.4 in the ESF-treated marrow-derived host spleens. By 48 hr the hemopoietin-treated CFU showed maximal response, yielding E/G colony ratios of 0.9 in the GPF- and 7.5 in the ESF-treated marrow-derived host spleens. No changes were observed during the 72-hr incubation period in the control cultures' differential exocolonizing potential, host spleen colony E/G ratios remaining within normal limits (2.1–3.0).

Discussion. The above *in vitro* data appear to indicate that hemopoietins may have other functions besides those of differentiating and maturing agents.

One possibility is that hemopoietins permit preferential survival of the appropriately committed hemopoietin-sensitive colony

formers and/or selective destruction of the inappropriately committed cells in suspension culture. However, this alternative is unlikely in view of the identical nucleated cell and transplantable CFU survival curves of the 3 experimental groups. Our data differ slightly from those of Silver and Erslev (9), who, in an *in vitro* suspension culture system containing both fetal calf and mouse serum, observed that ESF had a slightly protective effect on the absolute survival potential of exocolonizing marrow CFU. To date no differential counts on cultured marrow-derived spleen colonies have been reported.

A second possibility is that hemopoietins selectively trigger proliferation of the appropriately committed CFU. This has been demonstrated *in vitro* for ESF (11) and appears to apply also to CSF (granulocytic colony stimulating factor) (12). But it may not be true for GPF since in preliminary studies we (G. Stockman, L. Delmonte and D. M. Mumford) have been unable to demonstrate that GPF increases *in vitro* ^{3}H-thymidine incorporation by marrow cells and since GPF does not support colony formation in soft agar (L. Delmonte, unpublished data).

Another possibility is that, among other functions, hemopoietins selectively commit undifferentiated CFU to the hemopoietin-specific cell line, as has been proposed by Kretchmar (13). Previous *in vivo* findings have indicated that GPF and ESF evoke a time- and dose-dependent increase in a pool of committed CFU which (a) are retransplantable from a primary into a secondary host without losing their cell line-specific commitment (L. Delmonte, unpublished data) and (b) appear to compete for a common stem cell (5). Yet the findings of Curry, Trentin and Wolf (2) and Wolf and Trentin (3) have suggested that most, although not all, transplantable CFU are uncommitted and become committed upon contact with the cellular hemopoietic inductive microenvironment (HIM).

As a hypothetical working model, we propose a coexistence theory of CFU commitment which could account for our own and various other laboratories apparently conflicting *in vivo* and *in vitro* findings:

1. Pluripotential CFU are permissively but reversibly committed by the cellular microenvironment (HIM) they seed on and, unless rendered irreversibly committed by a hit by the appropriate hemopoietin, revert to their uncommitted state upon removal from their cellular microenvironment.

2. Pluripotential CFU are irreversibly committed by the humoral environment (hemopoietins), retain their commitment upon removal from this environment, and respond to the appropriate hemopoietin regardless of the cellular microenvironment on which they seed.

Further studies are necessary to test the validity of the various tentative explanations discussed for the present *in vitro* findings.

Summary. The spleen colony technique was used to test the *in vitro* effects of GPF (calf renal granulopoietic factor) and ESF (erythrocyte stimulating factor-rich anemic horse plasma) on the survival and differential colonizing potential of A/J mouse marrow cells in suspension culture. The quantitative survival of nucleated cells and transplantable colony forming units remained unaffected by the addition of either hemopoietin. Beginning at 1 hr and increasing progressively, the erythrocytic:granulocytic ratios of cultured marrow-derived spleen colonies shifted markedly in the direction of the hemopoietin-dependent cell line. Alternate theoretical explanations for this phenomenon are discussed.

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