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Corticosteroid Dependence of Continuous Hemopoiesis In Vitro with Murine or Human Bone Marrow

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A. Introduction

Continuous mouse bone marrow cultures (Dexter and Testa 1977) have been used to study in vitro normal marrow hemopoiesis and viral or chemical leukemogenesis (Dexter et al. 1977; Greenberger 1978; Greenberger 1979b; Greenberger et al., to be published a; Greenberger et al. 1980; Greenberger et al., to be published b; Greenberger et al., to be published c). Modifications in culture technique, including elimination of a second marrow inoculum ("recharging") and weekly addition of fresh 17-hydroxycorticosteroid (hydrocortisone), have lengthened the period of production of pluripotent hemopoietic stem cells (CFUs) and committed granulocyte-macrophage progenitor cells (GM-CFUc) in vitro (Greenberger et al. 1979a, to be published a). Mouse strain genotype influences the longevity of hemopoiesis in corticosteroid-supplemented cultures (Greenberger et al. 1979c) and is emphasized by the comparison of marrow cultures from AKr/J and NZB mice (Table 1).

We now report a system for continuous hemopoiesis using human marrow. Weekly addition of $10^{-7}M$ hydrocortisone was required; however, other conditions were found necessary for human marrow culture.

Table 1. Influence of mouse strain genotype on the duration of hemopoiesis in corticosteroid-supplemented long-term bone marrow cultures

Mouse strain[a]	Added hydrocortisone concentration *(M)*	Duration of hemopoiesis in 25% fetal calf serum		
		Longest duration production of:[b]		
		GM-CFUc (wks)	Immature granulocytes (wks)	Mature granulocytes wks
AKr/J	10^{-7}	61	61	63
AKr/J	None	3	4	6
NZB	10^{-7}	14[c]	14	16
NZB	None	2	3	3

[a] Contents of one tibia and one femur from 6–8-week-old female mice were inoculated into each 10.0 ml flask and medium changed weekly by removal of all nonadherent cells and medium. Cultures were not recharged with additional marrow

[b] Results are the mean of at least 16 flasks for each strain. GM-CFUc were scored at 7 days in 0.3% agar with 10% L929 cell CSF (Greenberger et al. 1979b). There were <5% toluidine blue positive mast cells detected in AKr/J cultures at 63 weeks or NZB cultures at 16 weeks

[c] Difference from AKr/J highly significant $P < 1001$

B. Materials and Methods

I. Tissue Culture

McCoy's 5A, Dulbecco's modified Eagle's, RPMI 1640, and Fisher's medium were obtained from Gibco. Horse serum and fetal calf serum were obtained from Flow Laboratories, Rockville, Maryland; human serum was obtained from the Blood Products aboratory Sidney Farber Cancer Institute. All media were supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin (Gibco). Marrow from AKr/J or NZB mice was prepared as described (Greenberger et al. 1979b). Human marrow biopsy specimens were obtained intraoperatively from femur or rib, and fragments dissected in McCoy's 5A medium. A single cell suspension was prepared by crushing marrow fragments, filtering through gauze to remove bone spicules, drawing cells through successively smaller gauge needles to a 26-gauge needle, washing several times in McCoy's 5A medium, and transferring at $2.0–4.0 \times 10^7$ nucleated cells in 10.0 ml to 40 cm^2 Corning plastic flasks. Cultures were compared by depopulation using several feeding schedules. The methods for detection of mouse and human GM-CFUc (Greenberger et al. 1979b) and detection of myeloid-esterase (ASD chloroacetate substrate-specific) and nonspecific esterase (alpha-naphthol-buterate substrate) have been described (Greenberger et al. 1979c).

C. Results

I. Method for Growth of Human Long-Term Bone Marrow Cultures

The methods for mouse marrow culture applied to human bone marrow result in poor longevity (Greenberger 1979a; Greenberger, et al., 1979l). Growth of 2.0×10^7 nucleated bone marrow cells in hydrocortisone-supplemented Fisher's medium or Dulbecco's modified Eagle's medium with 25% horse serum or 25% fetal calf serum produced no detectable GM-CFUc after 4 weeks (Table 2). Cultures grown in these media supplemented with hydrocortisone and either 12.5% horse serum plus 12.5% human serum or 12.5% fetal calf plus 12.5% human serum were also ineffective. In marked contrast cultures grown in McCoy's 5A medium supplemented with 12.5% horse serum, 12.5% fetal calf serum, and $10^{-7}M$ hydrocortisone generated GM-CFUc and $>10^5$ granulocytes weekly for over 10 weeks. The ultrastructure of adherent cell areas detected at 14 days after initiation (Fig. 1) was similar to those in mouse cultures (Fig. 2). Twice weekly or once weekly feeding with removal of all nonadherent cells was

Table 2. Results at 10 weeks for hemopoiesis in unrecharged human long-term marrow cultures in McCoy's 5A medium with $10^{-7}M$ hydrocortisone using a single donor femur marrow (amputation specimen) and varying serum combination

Serum combination tested[a]	No. cells per culture ($\times 10^5$)	Mean results at week 10 Percentage immature granulocytes	GM-CFUc per 10^5 cells
1. Horse serum (25%)	0.4	2	0
2. Fetal calf serum (25%) FCS	0.5	6	0
3. Horse serum (25%) switch to FCS (25%) at wk 4	0.2	3	0
4. Fetal calf serum (25%) switched to horse serum (25%) at week 4	0.3	2	0
5. Human serum (pooled) 25%	0.6	2	0
6. Human serum (25%) to wk 4, then horse serum 25%	0.2	3	0
7. Human serum (25%) to wk 4, then FCS 25%	0.7	2	0
8. Horse serum 12.5% + fetal calf serum 12.5%	*6.3*	*15*	*17*
9. Horse serum 12.5% + human serum 12.5%	0.2	2	0
10. Human serum 12.5% + FCS 12.5%	0.3	1	0

[a] 2.4×10^7 nucleated marrow cells were seeded to each of triplicate 10.0 ml flasks and kept at 33°C, 3% CO_2 pH 7–7.5. All media and nonadherent cells were removed each 7th day and fesh medium added. Weekly total cell counts, differential cell counts, and assay for GM-CFUc were performed. Results are the mean of at least three culture flasks for each point. McCoy's 5A medium was supplemented with the additives described previously (Greenberger et al. 1980)

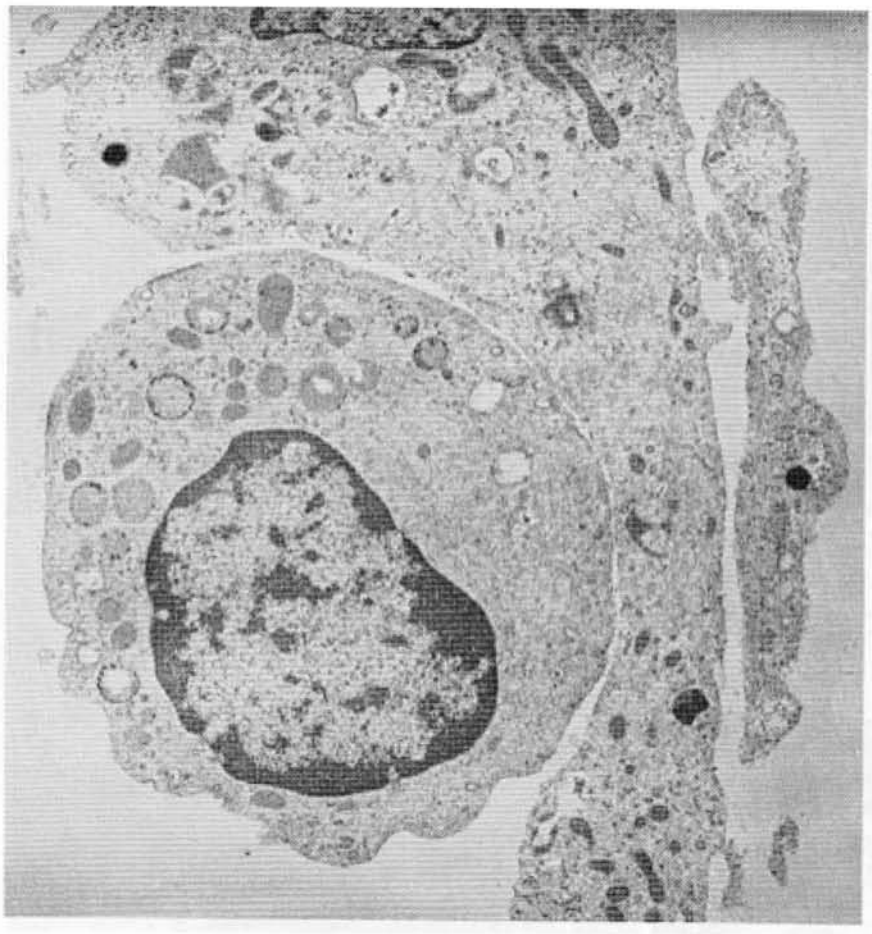

Fig. 1. Ultrastructural appearance of adherent cell microenvironment of human continuous bone marrow culture at 12 weeks prepared as described in the Fig. 1 legend. Note close apposition of early granulocyte and fibroblastic cells (×27,000)

associated with loss of human GM-CFUc by 10–12 weeks. In contrast, cultures depopulated twice weekly of all nonadherent cells with replacement of fresh medium and 50% of the washed nonadherent cells generated GM-CFUc past 15 weeks. Delayed removal of red blood cells 3–5 days after culture initiation (Gartner and Kaplan, see this vol p 276) resulted in a further improvement with GM-CFUc produced for over 18 weeks and in three samples for over 20 weeks. Using the optimal conditions, 3 of 50 amphoteracin-B (fungazone; 0.25 μg/ml) treated cultures established a functional "cobblestone" monolayer that produced GM-CFUc for at least 6 weeks and in 15 beyond 10 weeks. Thus, amphoteracin-B was toxic to human marrow cultures. Granulocytes generated after 10 weeks from each of 42 human donor marrow cultures were positive for myeloperoxidase and ASD-chloroacetate-specific esterase. There were on average 1% erythroid, 10% early granulocyte, 70% late granulocyte, 2%–13% monocytes, 2%–5% lymphocyte, and <1% eosionophils, mast cells, or megakaryocytes detected in nonadherent cells removed weekly between weeks 10–20.

II. Fluctuation in Generation of GM-CFUc in Human Long-Term Marrow Cultures

Weekly assay of GM-CFUc in nonadherent cells harvested over 10–20 weeks revealed differences in the numbers of colony-forming cells within each specimen as well as between individuals. Results with a representative specimen are shown in Fig. 3. Fluctuation in GM-CFUc has been observed in mouse long term bone marrow cultures (Dexter et al. 1977; Greenberger et al. 1980) and fluctuation in CFUs has been shown to occur through a process of clonal succession (Mauch et al. 1980).

D. Discussion

An in vitro culture technique is described for generation of human granulocyte-macrophage progenitor cells and mature granulocytes in excess of 20 weeks in continuous marrow culture. The requirement of freshly added hydrocortisone for continuous human marrow cultures was similar to that for murine cultures; however, major differences included de-

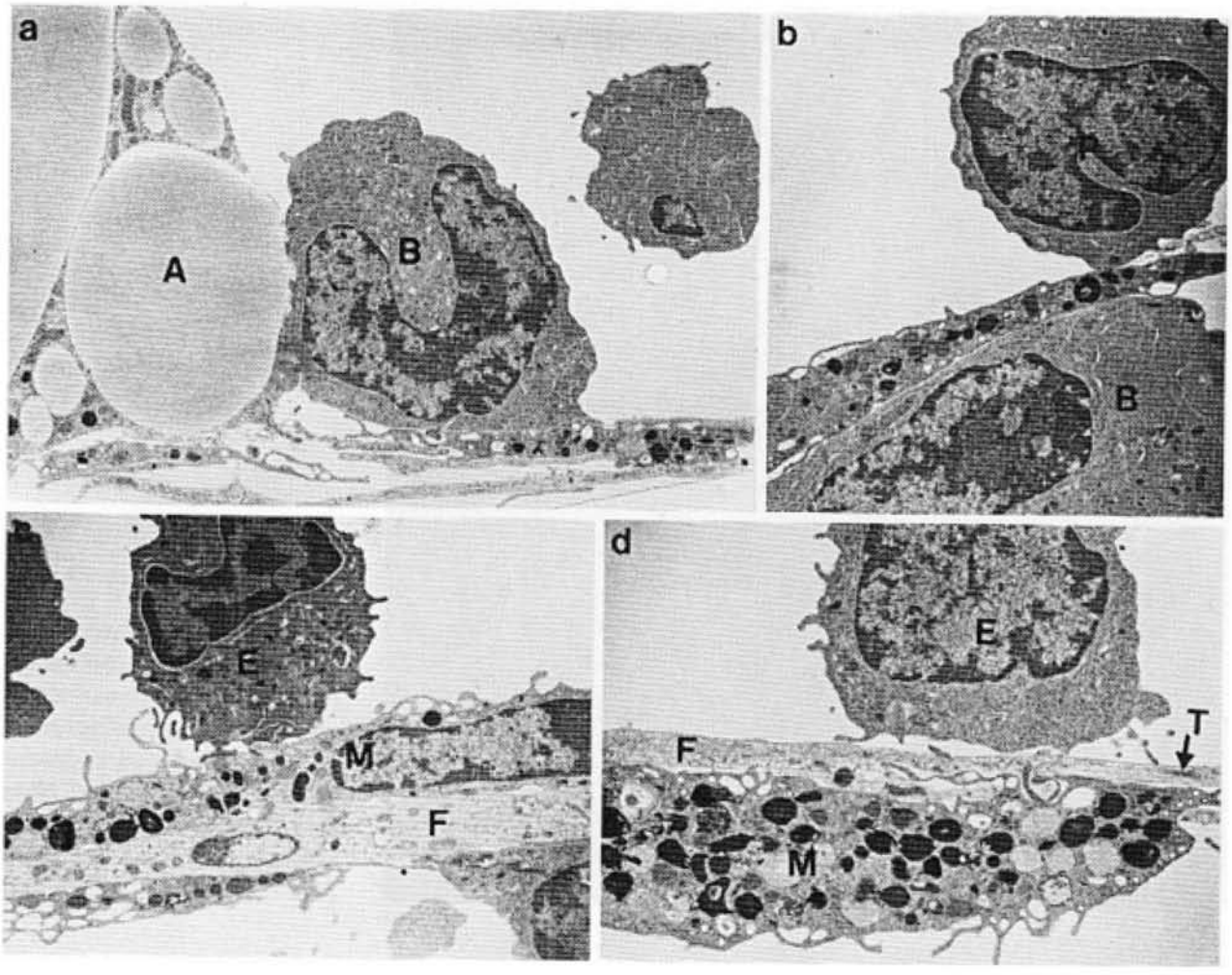

Fig. 2. Ultrastructural appearance of 8 week culture from C3H/HeJ marrow grown in 25% horse serum with $10^{-7}M$ hydrocortisone fixed in glutaraldehyde, postfixed in osmium, and poststained with uranyl acetate and lead citrate. **a–d** interaction between myeloid cells and the cells of the adherent layer. *A*, adipocyte, *B*, band neutrophil, *E*, early myelocyte, *F*, fibroblast, *M*, macrophage, *P*, promonocytic cell, and *T*, tight junction. (×26,400)

layed red blood cell removal and pH 7.0–7.5 with PCO_2 at between 3%–5%. The morphology of the adherent microenvironment in human cultures showed more fibroblastic proliferation, but groups of flat, tightly packed cells termed "cobblestone areas" for mouse cultures were enmeshed within fibroblasts and macrophages in the stroma. Granulocytes produced in human continuous marrow cultures were normal in morphology and synthesized esterase and myeloperoxidase. Individually removed GM-CFUc contained both granulocytes and macrophages in >80% of the colonies tested. Furthermore, granulocytes generated in these cultures have normal physiologic functions including ingestion, respiratory burst activity, degranulation, and bacterial killing (Greenberg et al., unpublished work). Application of human long-term marrow cultures is now feasible for study of the cell biology of hematological diseases.

Acknowledgments

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References

Dexter TM, Testa NG (1976) Differentiation and proliferation of hemopoietic cells in culture. Methods Cell Biol 14:387–405 – Dexter TM, Scott D, Teich NM (1977) Infection of bone marrow cells in vitro with FLV: Effects on stem cell proliferation, differentiation, and leukemogenic capacity. Cell: 12:355–364 – Greenberger JS (1978) Sensitivity of

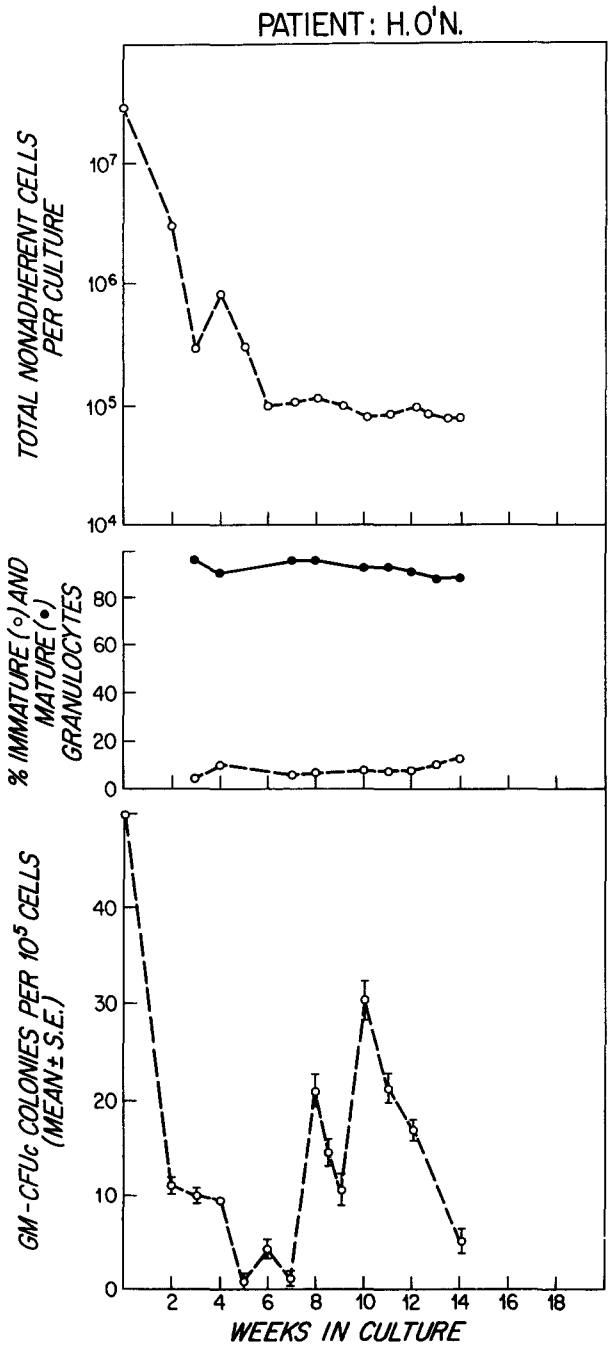

Fig. 3. Kinetics of generation of granulocytes and GM-CFUc in vitro over 14 weeks in a representative human continuous marrow culture. Results are the mean of at leat four cultures at each time point. Cultures were established in McCoy's 5A medium supplemented with $10^{-7}M$ hydrocortisone, 12.5% FCS, and 12.5% horse serum, with delayed removal of red blood cells after 5 days

corticosteroid-dependent, insulin-resistant lipogenesis in marrow preadipocytes of mutation diabetic-obese mice. Nature 275:752–754 – Greenberger JS (1979a) Corticosteroid-dependent differentiation of human bone marrow preadipocytes. In vitro 15:823–828 – Greenberger JS (1979b) Phenotypically-distinct target cells for murine sarcoma virus and murine leukemia virus marrow transformation in Vitro. J Nat Cancer Inst 62:337–348 – Greenberger JS, Davisson PB, Gans PJ (1979a) Murine sarcoma viruses block corticosteroid-dependent differentiation of bone marrow preadipocytes associated with long-term in vitro hemopoiesis. Virology 95:317–333 – Greenberger JS, Donahue D, Sakakeeny MA (1979c) Induction of ecotropic endogenous murine leukemia virus in long-term bone marrow cultures from mouse strains of varying natural leukemia incidence. J. Reticuloendothel Soc 26:839–853 – Greenberger JS, Gans PJ, Davisson PB, Moloney WC (1979c) In vitro induction of continuous acute promyelocytic cell lines in long-term bone marrow cultures by Friend or Abelson leukemia virus. Blood 53:987–1001 – Greenberger JS, Sakakeeny MA, Parker LM (1979l) In vitro proliferation of hematopoietic stem cells in long-term marrow cultures: Principals in mouse applied to man. Exp Hematol (Suppl 5) 7:135–148 – Greenberger JS, Newburger PE, Lipton JM, Moloney WC, Sakakeeny MA, Jackson PL (1980) Virus and cell requirements for Friend virus granulocytic leukemogenesis in long-term bone marrow cultures of NIH swiss mice. J Natl Canc Inst 64:867–878 – Greenberger JS, Eckner RJ, Ostertag W, Colletta G, Boshetti S, Nagasawa H, Karpas A, Weichselbaum R, Moloney WC (to be published a) Release of spleen focus-forming virus (SFFV) from differentiation inducible promyelocytic leukemia cell lines transformed in vitro by Friend leukemia virus. Virology – Greenberger JS, Newburger P, Sakakeeny MA (to be published b) Phorbol myristate acetate stimulates macrophage proliferation and differentiation and alters granulopoiesis and leukemogenesis in long-term bone marrow cultures. Blood – Greenberger J, Wroble LM, Sakakeeny MA (to be published c) Murine leukemia viruses induce macrophage production of granulocyte-macrophage colony stimulating factor in vitro. J Natl Canc Inst – Mauch P, Greenberger JS, Botnick LE, Hannon EC, Hellman S (1980) Evidence for structured variation in self-renewal capacity within long-term bone marrow cultures. Proc Natl Acad Sci USA 77:2927–2930.