Proc. Natl. Acad. Sci. USA
Vol. 77, No. 8, pp. 4756–4759, August 1980
Cell Biology

Long-term culture of human bone marrow cells

(hematopoiesis/adipocytes)

SUZANNE GARTNER AND HENRY S. KAPLAN

Cancer Biology Research Laboratory, Stanford University School of Medicine, Stanford, California 94305

Contributed by Henry S. Kaplan, May 5, 1980

ABSTRACT **A method has been described for the long-term culture of human bone marrow cells in liquid medium. Hematopoiesis, as measured by the production of granulocytic-macrophage progenitor cells (CFU_c), continued for at least 20 weeks and was dependent upon the presence of a marrow-derived adherent layer of cells. As in the case of murine marrow liquid cultures, the adherent layer consisted of mononuclear phagocytic cells, endothelial cells, and lipid-laden adipocytes, the latter being essential for long-term hematopoiesis. Optimal growth conditions included McCoy's medium supplemented with fetal bovine serum, horse serum, and hydrocortisone and incubation at 33°C. Horse serum in conjunction with hydrocortisone appeared essential for the growth of adipocytes.**

Hematopoiesis depends upon the complex interaction of growth and regulatory factors, most of which are poorly understood. In recent years the development of clonal culture systems in semisolid media for the detection of granulocyte–macrophage (1–3), erythroid (4), lymphoid (5–7), and megakaryocytic (8) progenitors has contributed greatly to investigations of hematopoiesis. Unfortunately, such systems possess two major limitations: (*i*) observations are restricted to relatively short time intervals, and (*ii*) the interaction between different kinds of cells cannot be easily studied. Short-term liquid culture methods have also been investigated. Golde and Cline (9) described the culture of human marrow cells in liquid medium, using an *in vitro* diffusion chamber in which cells grew both in suspension and on a dialysis membrane. Proliferation and maturation of granulocytes and macrophages in these chambers persisted for 4 weeks.

Dexter *et al.* (10) reported the development of a liquid system for the cocultivation of mouse thymus and bone marrow in which granulocyte–macrophage progenitor cells (CFU_c; colony-forming unit—culture) were generated for at least 10 weeks and pluripotent stem cells (CFU_s) were present for 14 days. The same group (11–13) later developed a method for the long-term culture of mouse bone marrow cells alone in liquid medium. Such cultures produce both CFU_c and CFU_s for several months. Hematopoiesis in this system is dependent upon the presence of a marrow-derived adherent population consisting of three cell types: phagocytic mononuclear cells, endothelial cells, and giant lipid-laden adipocytes. Initially, only certain lots of horse serum had the ability to stimulate the growth of these essential adipocytes. Greenberger (14) reported that "deficient" lots of horse serum could be reconstituted with corticosteroids.

The importance of the development of a long-term liquid culture system for human bone marrow is obvious. Moore and Sheridan (15) reported the establishment of human marrow cultures, using conditions similar to those described by Dexter *et al.*, but CFU_c production was limited to 6–8 weeks. More recently, Moore *et al.* (16) reported sustained long-term hematopoiesis in liquid cultures of marrow from a subhuman primate, the tree shrew (*Tupaia glis*). We now describe a method for the long-term culture of human marrow cells, based on modifications of the murine system.

MATERIALS AND METHODS

Media. Fischer's complete growth medium consisted of Fischer's medium (GIBCO no. 320-1735) supplemented with 0.1 μM hydrocortisone sodium succinate (Upjohn) and 25% horse serum (HoS; Flow Laboratories, McLean, VA), 25% fetal bovine serum (FBS; Microbiological Associates, Bethesda, MD), or 12.5% each of HoS and FBS. McCoy's complete growth medium consisted of the following: McCoy's 5a medium, modified (GIBCO no. 430-1500), 0.1 μM hydrocortisone, 1% sodium bicarbonate solution (GIBCO no. 670-5080), 1% minimal essential medium sodium pyruvate solution (GIBCO no. 320-1360), 1% minimal essential medium vitamin solution (GIBCO no. 320-1120), 0.8% minimal essential medium amino acids solution (GIBCO no. 320-1135), 0.4% minimal essential medium nonessential amino acids solution (GIBCO no. 320-1140), 200 mM 1% L-glutamine (GIBCO no. 320-5030), 1% penicillin/streptomycin solution [10,000 units of penicillin per ml, 10,000 μg of streptomycin per ml] (GIBCO no. 600-5140), and 25% HoS, 25% FBS, or 12.5% of each. Only freshly prepared medium was used for the initiation and maintenance of cultures. All serum was heat-inactivated at 56°C for 1 hr and stored frozen prior to use. In the presence of 0.1 μM hydrocortisone, all lots of HoS tested were able to support the development of a good stromal layer. Only those lots of FBS that sustained the growth of our fastidious human lymphoma cells (17, 18) were used for these cultures.

Initiation and Maintenance of Cultures. Normal marrow specimens were obtained from resected ribs of patients undergoing thoracotomy. Immediately after removal from the patient, the rib was cut into segments 2–3 cm in length and immersed in cold, Ca^{2+}-free Dulbecco's phosphate-buffered saline (GIBCO) supplemented with 1% penicillin/streptomycin solution and beef lung sodium heparin (Upjohn), 10 units/ml final concentration. All extraneous connective tissue was cut away from the segments and they were gently rinsed with fresh Ca^{2+}-free Dulbecco's phosphate-buffered saline. Care was taken not to flush the marrow from the segments at this step. By using two 6-inch blunt-ended forceps, the rib segments were pried apart longitudinally. With the tip of a forcep, the marrow

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: FBS, fetal bovine serum; HoS, horse serum; CFU_c, colony-forming unit—culture (granulocyte–macrophage progenitor cells); T cell, thymus-derived lymphocyte; B cell, bone marrow-derived lymphocyte.

was scraped into a 100-mm glass or plastic petri dish containing 20 ml of cold complete growth medium (two dishes were used for the marrow from each resected rib). The medium containing the marrow clumps was then transferred to a 50-ml centrifuge tube (Corning) and pipetted moderately vigorously to break apart the cell clumps and create a single-cell suspension. (In our hands, cultures initiated from single-cell suspensions have consistently proven superior to those in which cell clumps were seeded.) Viable cells were counted, using 0.04% trypan blue; viability was consistently 98–100%. Wright's/Giemsa-stained slides of the fresh specimens were also prepared to ensure that the marrow was normal. About 1–2 × 10^9 nucleated cells were usually recovered from a rib. From such suspensions 2 × 10^7 viable nucleated cells in 10 ml of complete growth medium were seeded into plastic T-25 flasks (Corning) or 1 × 10^7 nucleated cells were seeded into Cluster[6] 35-mm wells (Costar, Vineland, NJ) containing glass coverslips (Corning). Cultures were incubated at 33°C in 5% CO_2 in air. [Our early experiments demonstrated the superiority of 33°C over 37°C in terms of supporting long-term hematopoiesis, although 37°C accelerated the development of the stromal layer. In contrast, Moore and Sheridan (15) reported that 37°C was superior for their human marrow cultures.] Weekly feedings consisted of removal of 5 ml of spent medium containing nonadherent cells and addition of 5 ml of fresh medium. The nonadherent cells were counted, used for morphologic and cytochemical studies, and assayed for CFU_c.

CFU_c Assay. Human peripheral blood mononuclear cells (1 × 10^6) were suspended in 1-ml volumes of 0.5% Noble agar (Difco) in McCoy's 5a medium supplemented with 15% FBS as described by Pike and Robinson (3) and seeded into 35-mm wells of Cluster[6] plates. Viable bone marrow cells (1 × 10^5) in 1-ml volumes of 0.3% Noble agar in McCoy's 15% FBS medium were overlayed on the 0.5% agar layer. Only 0- to 1-day-old underlayers were used. Clusters and colonies were scored at day 12–14. Clusters contained 20–50 cells and colonies contained more than 50 cells. CFU_c values represent the average of at least three cultures, and three wells per culture were assayed.

Cytochemical Stains. For morphologic characterization, nonadherent cells and coverslip cultures were stained with Wright's/Giemsa; cytochemical tests were also performed for nonspecific esterase and myeloperoxidase activity (19).

RESULTS

Adipocyte-containing confluent stromal layers could be initiated and maintained in Fischer's growth medium supplemented with 25% HoS and 0.1 μM hydrocortisone (see Table 1). Greenberger (20) also reported the induction of lipogenesis in adipocytes in human marrow cultures by corticosteroids. Although the mixed adherent cell population in such cultures

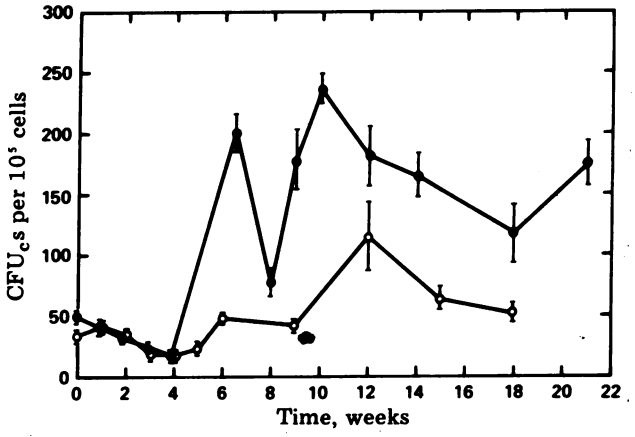

FIG. 1. Serial CFU_c production in long-term human marrow liquid cultures. The results represent the means ± SEM of three wells for each of three cultures. ●, Hyperproliferative pattern; ○, homeostatic pattern.

can be maintained for periods of at least 1 year, active hematopoiesis, as measured by the numbers, morphology, and CFU_c-forming capacity of viable nonadherent cells recovered, is more limited. Fischer's growth medium supplemented with 0.1 μM hydrocortisone and 25% HoS or 12.5% HoS/12.5% FBS was able to support the generation of CFU_c-forming cells for up to 12 weeks in some cases. However, the number of such colony-forming cells was usually less than 50 per 10^5 cells assayed. Fischer's medium supplemented with 0.1 μM hydrocortisone and 25% FBS did not sustain either the development of adipocytes or hematopoiesis.

Table 1 also suggests that FBS alone, although able to support the growth of other adherent cells, failed to provide for the initial growth, differentiation, or both of adipocytes. In contrast, McCoy's complete growth medium supplemented with both FBS and HoS not only provided adequate nutrition for the early development and maintenance of stromal layers containing abundant adipocytes but also contributed either directly or indirectly (through the stromal microenvironment) to long-term hematopoiesis. Cultures grown in McCoy's complete growth medium supplemented with 25% HoS required longer for growth to confluence than those grown in the presence of both HoS and FBS. Hematopoiesis in such cultures did not approach the levels observed in cultures supplemented with both sera.

The generation of cells with colony-forming ability in these long-term cultures appeared to follow two different patterns, designated the "hyperproliferative" and "homeostatic" patterns. The hyperproliferative pattern describes cultures in which the number of CFU_cs generated exceeds 100 per 10^5 cells in serial assays over several weeks. In contrast, the homeostatic

Table 1. Characteristics of human bone marrow cultures in liquid medium

Culture medium*	% serum		Adherent population			Nonadherent population > 8 weeks				
						Monocytoid‡		Myeloid‡		CFU_c-forming
	FBS	HoS	Time to confluence, days	Longevity, months	Presence of adipocytes†	Immature	Mature	Immature	Mature	capacity
Fischer's	—	25	14–21	>12	3+ to 4+	1+	4+	1+	1+ to 2+	1+
Fischer's	25	—	12–14	>6	0 to 1+	0 to ±	4+	0 to ±	0 to ±	0 to ±
Fischer's	12.5	12.5	12–14	>6	3+ to 4+	1+	4+	1+	1+ to 2+	0 to 1+
McCoy's	—	25	14–21	>6	3+ to 4+	1+	3+	1+	1+ to 2+	1+
McCoy's	25	—	7–12	>6	0 to ±	0 to ±	2+	0 to ±	0 to ±	NT§
McCoy's	12.5	12.5	7–10	>6	3+ to 4+	1+ to 2+	3+	2+ to 4+	2+ to 4+	2+ to 4+

* All cultures were supplemented with 0.1 μM hydrocortisone; see *Materials and Methods* for additional additives.
† Stromal layers of very old cultures were composed almost exclusively of adipocytes.
‡ Based on Wright's/Giemsa, nonspecific esterase, and myeloperoxidase staining characteristics.
§ NT, not tested; too few nonadherent cells were recovered after 8 weeks.

pattern describes a steady-state situation in which a lower level of CFU_cs, usually between 25 and 75 per 10^5 cells, is continuously present in the cultures. An example of each pattern is shown in Fig. 1. Of nine different rib specimens incubated in McCoy's complete growth medium supplemented with both FBS and HoS, four showed the hyperproliferative pattern and five the homeostatic pattern. With the other growth media only the homeostatic pattern was observed, and only in rare instances were CFU_cs detected beyond 12 weeks. With all growth media and at all time intervals most colonies were large, containing more than 500 cells. An example is shown in Fig. 2. Adipocyte colonies were also occasionally seen in the agar cultures. After approximately 8 weeks the numbers of nonadherent cells recovered weekly usually varied between 2×10^5 and 2×10^6 per culture. Before 7–8 weeks, between 1×10^6 and 1.5×10^7 cells were routinely recovered. Such recovery frequencies were common to both the hyperproliferative and homeostatic patterns.

Fig. 1 shows an important facet of the kinetics of the generation of CFU_cs in these cultures. The numbers of CFU_cs do not increase during the first 4 weeks of culture. In the murine system, Dexter *et al.* (13) described the "re-charging" of cultures at 4 weeks by the addition of fresh marrow. Addition of fresh cells was found to be unnecessary for prolonged hematopoiesis in *Tupaia glis* marrow cultures (15, 16). We have found that recharging did not enhance the production of CFU_cs in our cultures and in some cases proved deleterious either by destroying the stromal microenvironment or by decreasing the number of CFU_cs recovered. In all cases, the number of CFU_cs began to increase between 4 and 6 weeks. In both the proliferative and homeostatic patterns, CFU_c production continued for at least 20 weeks.

Fig. 3 illustrates the appearance of a typical long-term culture. A most important feature is the presence of "cobblestone"-like areas, presumably regions of hematopoiesis from which the nonadherent cells appear to arise. Similar aggregations of cells have been described in the murine system (21). Lipid-containing adipocytes and a diverse stromal cell population are also apparent in this culture.

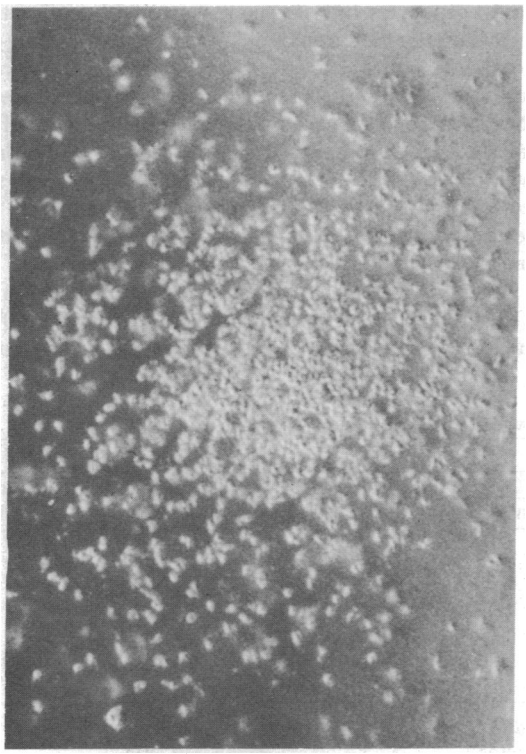

FIG. 2. Agar culture colony arising from a CFU_c harvested from a 14-week-old human marrow culture in liquid medium. (×25.)

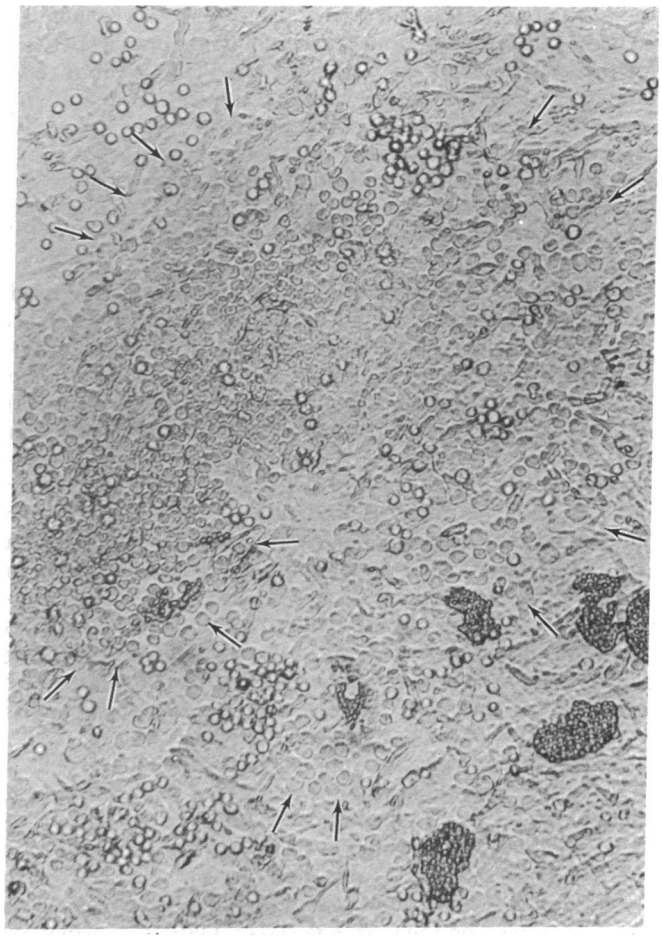

FIG. 3. A human cell culture at 6 weeks, illustrating adipocytes and cobblestone areas of active hematopoiesis (circumscribed by arrows). (×100.)

Coverslip cultures gently washed extensively to remove nonadherent cells and stained for myeloperoxidase activity demonstrated that cobblestone areas did indeed contain both myeloid and monocytoid cells. Coverslip cultures stained for nonspecific esterase activity revealed monocytoid cells scattered throughout the stromal layer, in islands of hematopoiesis, and sometimes in large tight clusters within the stromal layer. In general, nonspecific esterase-positive cells were much more intensively stained outside of the cobblestone areas.

Table 1 indicates the relative abundance of different morphologic cell types in the nonadherent cell population. In older (greater than 8 weeks) cultures grown in Fischer's or McCoy's medium with FBS only, the vast majority of nonadherent cells were mature macrophages. This finding is in agreement with that of Moore and Sheridan (15) in long-term human cultures grown in Fischer's medium with HoS. With Fischer's medium plus 25% HoS or 12.5% FBS/12.5% HoS, immature monocytoid cells and both immature and mature myeloid cells were also present but in smaller numbers. In contrast, cultures grown in McCoy's FBS/HoS growth medium contained many more immature monocytoid and myeloid cells, as well as mature myeloid cells. The numbers of myeloid cells often exceeded the numbers of mature macrophages in the McCoy's medium cultures, unlike the situation in Fischer's medium. In younger cultures (less than 8 weeks), monocytoid and myeloid cells at

various stages of differentiation were generally observed, and fewer differences were detected between the different growth media.

DISCUSSION

It has been demonstrated that hematopoiesis in long-term liquid cultures of murine (21) and *Tupaia glis* (16) marrow is dependent upon the presence of lipid-laden adipocytes. Our results suggest that this is also the case for human marrow. HoS appears to be essential for the initial growth, differentiation, or both of the adipocytes. In limited studies, cultures grown in the presence of freshly collected pooled human serum and 0.1 μM hydrocortisone with or without the addition of FBS failed to develop mature adipocytes.

It seems apparent that both the hyperproliferative and homeostatic patterns of CFU_c production reflect the actual *de novo* generation of progenitor cells in culture. The numbers of CFU_cs detected weekly in such cultures exceed the number of stem cells initially seeded. Moreover, serial CFU_c assays would have been expected to reveal a rapid depletion of CFU_c as weekly samples were withdrawn if CFU_cs were merely persisting rather than proliferating in the cultures. This interpretation is further supported by the fact that production of CFU_cs did not begin to increase until about 4 weeks. The differences between the two patterns may be attributable to age and other constitutional factors affecting individual hematopoietic activity; they may have been more readily apparent because most of our rib specimens came from older patients. Greenberger *et al.* (22) has demonstrated that the capacity for long-term hematopoiesis in murine marrow cultures differs considerably from strain to strain.

We believe that the numbers of nonadherent cells harvested in the samples of spent medium do not accurately reflect the numbers of nonadherent cells present in the cultures. The nonadherent cells tend to "hover" closely over the stromal layers. Phase-contrast microscope observations immediately after feeding of the cultures reveal that, even with gentle agitation of the culture vessel, large numbers of distinctly nonadherent cells remain close to the stromal layer. Possibly the viscosity of the growth medium enhances this effect. More vigorous agitation results in some destruction of the delicate stromal microenvironment and cessation of hematopoiesis.

Erythroid (BFU-E) (23) and megakaryocytic (CFU-M) (24) progenitors are also produced in long-term murine cultures. Jones-Villeneuve *et al.* (25) recently reported the persistence for at least 16 weeks of pre-T cells in long-term mouse marrow cultures. It is of great interest that we have established three permanent polyclonal cell lines of Epstein–Barr virus-transformed B-lymphoblastoid cells from marrow cultures of different patients. All three arose from what appeared to be hematopoietically exhausted stromal layers, one developing at 3 months, one at 4 months, and one at 6 months after initiation of the cultures. All were derived from cultures with Fischer's medium and 25% HoS. In the case of the 6-month-old stromal layer, no evidence of cobblestoning remained, and no nonadherent cells had been recovered for 2 months prior to the appearance of the B cells. Moore and Sheridan (15) reported the conversion of 10% of their human cultures to a lymphoblastoid morphology by the sixth week of culture. We are at present uncertain as to whether our observations represent the generation of B cells from immature precursors in these old cultures or the delayed outgrowth of long-lived Epstein–Barr virus-transformed mature B cells. Given the complexity of the stromal microenvironment, it is quite possible that small numbers of mature B cells could have survived undetected.

Although there are still many improvements to be made in the application of this culture system to human marrow, we believe the modifications we have described allow the routine establishment of such cultures in a reproducibly successful way. We have recently applied this methodology to normal sternal and leukemic iliac crest marrow aspirates. The establishment of long-term cultures (now at 4 months) from such specimens has proven surprisingly successful, considering the limited numbers of cells obtained from some of the leukemic specimens in particular. Hence we believe that this culture system provides an approach for the study of normal human hematopoiesis and may permit investigators to address questions regarding the nature of the transformed cell population in neoplastic diseases of the hematopoietic system.

We thank Dr. James B. D. Mark for providing the rib specimens, Glenda Garrelts for the nonspecific esterase tests, Dr. Werner Henle for the Epstein–Barr nuclear antigen determinations, and Dr. Joel Greenberger for helpful discussions. This work was supported by research contract NO1-CP-91044 from the National Cancer Institute. S.G. is the recipient of a predoctoral traineeship in a Cancer Biology Training Program supported by training grant CA-09302 awarded by the National Cancer Institute.

1. Pluznik, D. H. & Sachs, L. (1966) *Exp. Cell Res.* **43**, 553–563.
2. Bradley, T. R. & Metcalf, D. (1966) *Aust. J. Exp. Biol. Med. Sci.* **44**, 287–299.
3. Pike, B. & Robinson, W. (1970) *J. Cell. Physiol.* **76**, 77–84.
4. Stephenson, J. R., Axelrad, A. A., McLeod, D. L. & Shreeve, M. M. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1542–1546.
5. Metcalf, D., Warner, N. L., Nossal, G. J. V., Miller, J. F. A. P., Shortman, K. & Rabellino, F. (1975) *Nature (London)* **255**, 630–631.
6. Fibach, E., Gerassi, E. & Sachs, L. (1976) *Nature (London)* **259**, 127–128.
7. Sredni, B., Kalechman, Y., Michlin, H. & Rozenszajn, L. A. (1976) *Nature (London)* **259**, 130–132.
8. Metcalf, D., MacDonald, H. P., Odartchenko, N. & Sordat, L. B. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1744–1748.
9. Golde, D. & Cline, M. (1973) *Blood* **41**, 45–57.
10. Dexter, T. M., Allen, T. D., Lajtha, L. G., Schofield, R. & Lord, B. I. (1973) *J. Cell. Physiol.* **82**, 461–470.
11. Dexter, T. M. & Lajtha, L. G. (1974) *Br. J. Haematol.* **28**, 525–530.
12. Dexter, T. M. & Testa, N. G. (1976) in *Methods in Cell Biology*, ed. Prescott, D. M. (Academic, New York), Vol. 14, 387–405.
13. Dexter, T. M., Allen, T. D. & Lajtha, L. G. (1977) *J. Cell. Physiol.* **91**, 335–344.
14. Greenberger, J. S. (1978) *Nature (London)* **275**, 752–754.
15. Moore, M. A. S. & Sheridan, A. P. C. (1979) *Blood Cells* **5**, 297–311.
16. Moore, M. A. S., Sheridan, A. P. C., Allen, T. D. & Dexter, T. M. (1979) *Blood* **54**, 775–793.
17. Epstein, A. L. & Kaplan, H. S. (1974) *Cancer* **34**, 1851–1872.
18. Kaplan, H. S., Goodenow, R. S., Gartner, S. & Bieber, M. M. (1979) *Cancer* **43**, 1–24.
19. Yam, L. T., Li, C. Y. & Crosby, W. H. (1971) *Am. J. Clin. Pathol.* **55**, 283–290.
20. Greenberger, J. S. (1979) *In Vitro* **15**, 823–828.
21. Allen, T. D. & Dexter, T. M. (1976) *Differentiation* **6**, 191–194.
22. Greenberger, J., Donahue, D. & Sakakeeny, M. (1979) *J. Reticuloendoth. Soc.* **26**, 839–853.
23. Testa, N. G. & Dexter, T. M. (1977) *Differentiation* **9**, 193–195.
24. Williams, N., Jackson, H., Sheridan, A. P. C., Murphy, M. J., Elste, A. & Moore, M. A. S. (1978) *Blood* **51**, 245–255.
25. Jones-Villeneuve, E. V., Rusthoven, J. J., Miller, R. G. & Phillips, R. A. (1980) *J. Immunol.* **124**, 597–601.