where F_I is the fractional influx oxygen concentration.
7. M. Denny, *Science* **208**, 1288 (1980); T. F. Jensen and I. Holm-Jensen, *J. Comp. Physiol.* **137**, 151 (1980).
8. G. A. Bartholomew and T. M Casey, *Science* **195**, 882 (1977).
9. A. F. Bennett, *Annu. Rev. Physiol.* **400**, 447 (1978); ——— and J. A. Ruben, *Science* **206**, 649 (1979).
10. J. Piiper and P. Spiller, *J. Appl. Physiol.* **28**, 657 (1970).
11. P. Pasquis, A. Lacaisse, P. Dejours, *Respir. Physiol.* **9**, 298 (1970).
12. D. F. Hoyt and C. R. Taylor, *Am. Zool.* **19**, 899 (1979).
13. Supported in part by grant PCM 79-02890 from the National Science Foundation.

4 December 1980

Continuous Lines of Basophil/Mast Cells Derived from Normal Mouse Bone Marrow

Abstract. *Nonadherent tissue culture cell lines were established from normal bone marrow of a variety of mouse strains. The lines possessed morphological and histochemical markers of the basophil/mast cell and contained committed stem cells for metachromatic cells. Their derivation from normal marrow and their lack of tumorigenicity despite long-term culture makes these cell lines potentially important for studies of the mechanisms of allergic reactions and inflammation as well as the differentiation pathways involving this subset of hematopoietic cells.*

Techniques for growth of normal hematopoietic cells in culture have recently been developed (*1*, *2*). With the use of these procedures, multipotential stem cells (CFU-s), mixed colony-forming cells, and committed stem cells (CFU-c), including granulocyte/macrophage, erythroid, megakaryocytic, and lymphoid progenitor cells, can be generated in primary culture for up to several months (*2–6*). However, the establishment of continuous cell lines of normal hematopoietic cells has so far been limited to the lymphocyte series, in which a specific growth factor for T lymphocytes has been isolated and shown to stimulate T cell proliferation in vitro (*7*, *8*). The loss of self-renewal capacity of nonlymphocyte stem cells in culture may be attributable to irreversible, spontaneous differentiation, but the mechanism is unknown.

Certain cells are reported to release growth factors for the proliferation in culture of specific subpopulations of normal hematopoietic cells (*9–13*). A growth-stimulating factor (or factors), released by the BALB/c mouse myelomonocytic leukemia cell line WEHI-3 (*14*) has been reported to support continuous growth of mouse bone marrow cells in suspension culture (*15*). In the present report, we demonstrate the establishment, in WEHI-3–conditioned medium (W-CM) (*16*), of bone marrow cell lines that have characteristics of basophilic granulocytes or tissue mast cells and contain committed stem cells for metachromatic cells (CFU-META).

Cultures were initiated with bone marrow cells of NIH Swiss and 129/J mice with the use of Greenberger's modification (*3*) of Dexter's system (*2*). After 4 to 6 weeks in primary culture, nonadherent cells in the population were transferred into Dulbecco-Vogt modified minimum essential medium supplemented with 30 percent fetal bovine serum (Colorado Serum Company) and 10 percent W-CM. Transferred cells were maintained at 37°C in 7 percent CO_2 in air. For the first few passages, fibroblast- and macrophage-like cells attached to the surface of the petri dish. However, within five to ten cell transfers at weekly intervals, adherent cells in the culture were eliminated. In the absence of W-CM, nonadherent cells could not be maintained in suspension culture; only fibroblastic adherent cells survived. Optimal concentrations of W-CM for growth of nonadherent cells ranged from 8 to 12 percent.

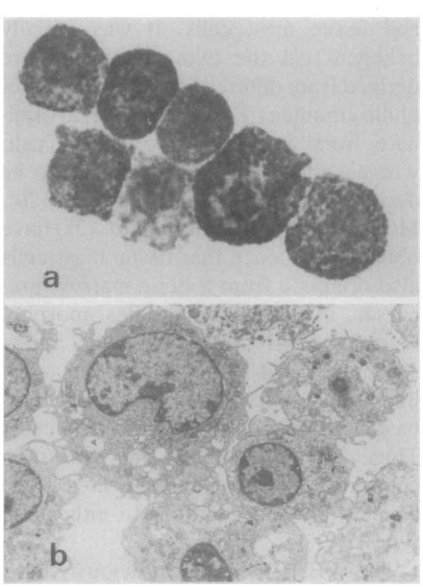

Fig. 1. (a) Photomicrograph of the BM-2 bone marrow cell line stained by Wright-Giemsa (×480). (b) Electron micrograph of the BM-2 cell line (×5300).

Cells were routinely passaged weekly at a concentration of 5×10^4 cells per milliliter; the cell doubling time ranged from 50 to 60 hours. Cell lines derived in this manner have been maintained continuously for more than 20 months.

In order to examine the reproducibility of the system, long-term cultures of fresh bone marrow cells from AKR/J, CBA/N, C57BL/6N, C57BL/KsJ, C57L/J, C58/J, 129/J, and NIH Swiss strains of mice were attempted according to the methods described (*16*). Five to ten individual bone marrow cultures were initiated with each strain. Nonadherent cells were easily passaged and could be maintained indefinitely with NIH Swiss, 129/J, C57BL/6N, and C57BL/KsJ bone marrow cultures. Under the same conditions with the AKR/J, CBA/N, C57L/J, and C58/J strains, it was difficult to maintain nonadherent cells in successive culture for more than 3 months. It has been reported that adipocyte colony formation in primary bone marrow cultures correlates with the ability of such cultures to generate CFU-s and CFU-c of the granulocyte series (*3*). We observed that adipocyte colony formation in the primary culture appeared to correlate with success in establishing nonadherent bone marrow cell lines.

Typical morphological features of cells in the established cell lines included cytoplasmic granules that stained dark violet to red-purple by the Wright-Giemsa method (Fig. 1a). The nuclei of most cells were round to oval, but segmented nuclei were also observed. Examination by electron microscopy (Fig. 1b) showed that cell surfaces were roughened by microvilli and that the cells invariably contained numerous immature granules. These findings suggested that the established lines consisted of cells of the basophil/myeloid series.

The bone marrow cell lines were next analyzed by histochemical techniques for enzymes characteristic of different hematopoietic subpopulations. The results (Table 1) with representative cell lines indicated the lack of detectable myeloperoxidase, which is normally associated with myeloid cells of the neutrophil series (*17*). Lysozyme activity, which is generally associated with cells of the monocyte-macrophage series (*18*), was also absent. In contrast, toluidine blue staining showed that a high percentage of the cells contained metachromatic granules, which are diagnostic for tissue mast cells or basophilic granulocytes (*17*).

Nonspecific esterase is a histochemical marker reported in monocyte-macrophages (*17*). Cell lines examined were

0036-8075/81/0417-0333$00.50/0

Table 1. Biochemical characterization of established nonadherent bone marrow cell lines. Histochemical staining was performed following cytocentrifugation of nonadherent bone marrow cell lines. Staining for naphthol AS-D chloroacetate esterase and α-naphthyl acetate esterase (nonspecific esterase) (Sigma) was performed according to the instructions. Staining for myeloperoxidase and toluidine blue staining for metachromasia were performed according to the methods of Yam *et al.* (*17*). At least 1000 cells were examined in each smear. Lysozyme activity was examined by lysoplate assay (*18*). Myelomonocytic leukemia cells, used as a positive control, contained >15.0 μg of lysozyme per 10^6 cells. Histamine content was examined by the enzymatic isotopic assay (*21*). Histamine content of highly mature mast cells derived from peritoneal exudates of normal mice was 3.0 μg per 10^6 cells.

Cell line	Mouse strain	Percentage of cells positive in histochemical staining for				Lysozyme (micrograms per 10^6 cells)	Histamine (nanograms per 10^6 cells)
		Myeloperoxidase	Metachromasia	Nonspecific esterase	Chloroacetate esterase		
BM-1	NIH Swiss	< 1.0	> 90.0	80.0 to 99.0	75.0 to 90.0	< 0.2	53
BM-2	129/J	< 1.0	> 95.0	80.0 to 95.0	60.0 to 80.0	< 0.2	300

positive for this enzyme (Table 1); however, chloroacetate esterase, an enzyme reported for tissue mast cells and neutrophilic granulocytes (*17*), was also demonstrated in the majority of cells. In fact, double staining revealed that more than 50 percent of the cells contained both activities. Whether nonspecific esterase activity reflects a previously undetected stage in the differentiation of the basophil/mast cell or an aberrant effect of growth in culture remains to be determined. As a further test of the basophil/mast cell origin, histamine content (*19, 20*) of the cell lines was analyzed by the enzymatic isotopic assay (*21*), and histamine was readily detected in all cultures tested. Recently, surface receptors for mouse immunoglobulin E have been detected (*22*), indicating that the cell lines possess additional highly specific and functional markers of basophil/mast cells. Thus, we conclude that the nonadherent bone marrow cell lines established under our culture conditions were composed of cell populations within the basophil/mast cell series.

In order to investigate the stage of differentiation of the cell lines, we performed CFU-s assays according to established methods (*23*). When 5×10^6 cultured cells were inoculated intravenously into lethally irradiated mice, no spleen colonies were observed, which suggested that the cell lines lacked multipotential stem cells. In contrast, when the cells were assayed for CFU-c (*24, 25*) in semisolid methylcellulose-containing medium supplemented with 20 percent horse serum and 20 percent W-CM, we observed 20 to 30 clusters (5 to 50 cells) and one to three colonies (> 50 cells) per 10^5 cells. Histochemical analysis of both clusters and colonies revealed that most of the cells contained metachromatic granules and exhibited a range of differentiation. These results substantiated the presence of CFU-META within the bone marrow lines.

Spontaneous transformation leading to leukemogenic cell lines has been reported in bone marrow cultures (*26*). Tests for tumorigenicity were performed in order to determine whether continuous propagation of the cell lines led to spontaneous transformation. Cells (5×10^6) from established cell lines were inoculated subcutaneously or intraperitoneally into syngeneic newborn mice but no detectable tumors were observed during a period of 1 year. Thus, continuous propagation of the bone marrow lines in culture was not associated with establishment of a transformed malignant cell population.

There has been controversy concerning the differentiation pathways involved in generation of basophilic myeloid cells and tissue mast cells. It was initially believed that the two cell types were derived from different precursors—basophilic granulocytes were thought to originate from a bone marrow stem cell, whereas mast cells were believed to be derived from connective tissue cells (*20*). More recently, Kitamura *et al.* (*27*) have obtained evidence that tissue mast cells also originate from a bone marrow precursor. In the present studies, morphological findings and histochemical analysis showed that within the bone marrow lines some cells could be classified as basophils and others as tissue mast cells. Thus, committed bone marrow stem cells in these lines may be at a stage capable of differentiation to either cell type.

The WEHI-3 line has previously been shown to release factors that can stimulate colony formation of committed stem cells for megakaryocyte, granulocyte, and macrophage series (*4, 28, 29*), as well as long-term growth in normal neutrophilic progenitor cells (*30*). In preliminary experiments, semipurified mature mast cells from mouse peritoneal exudates could not be maintained for more than 2 weeks in the presence of W-CM. Yet metachromatic cells, which initially comprised a distinct minority in primary bone marrow cultures, were selected in medium containing W-CM. These observations suggest that WEHI-3 cells release an additional stimulatory factor (or factors) whose target is at the committed stem cell stage of metachromatic cell differentiation.

The RBL line, which was established from a rat basophilic leukemia (*31*), has been the only available tissue culture line comprised of cells of the basophil/mast cell series. The presently described cell lines were derived from normal bone marrow and have remained nontumorigenic despite long-term culture. Thus, they should be particularly useful for investigation of normal differentiation pathways of this subset of hematopoietic cells, as well as in studies of allergic reactions and inflammation. These cell lines may also be of use in investigations aimed at the development of tissue culture assays for neoplastic transformation of hematopoietic cells by RNA tumor viruses.

KENJI NAGAO
Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205
KENJIRO YOKORO
Department of Pathology, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Hiroshima 730, Japan
STUART A. AARONSON*
Laboratory of Cellular and Molecular Biology, National Cancer Institute

References and Notes

1. T. M. Dexter and L. G. Lajtha, *Br. J. Haematol.* **28**, 525 (1974).
2. T. M. Dexter and N. G. Testa, *Methods Cell Biol.* **14**, 387 (1976).
3. J. S. Greenberger, *Nature (London)* **275**, 752 (1978).
4. N. Williams, H. Jackson, A. P. C. Sheridan, M. J. Murphy, Jr., A. Elste, M. A. S. Moore, *Blood* **51**, 245 (1978).
5. J. F. Eliason, N. G. Testa, T. M. Dexter, *Nature (London)* **281**, 382 (1979).
6. E. V. Jones-Villeneuve, J. J. Rusthoven, R. G. Miller, R. A. Phillips, *J. Immunol.* **124**, 597 (1980).
7. D. A. Morgan, F. W. Ruscetti, R. Gallo, *Science* **193**, 1007 (1976).
8. S. Gillis, M. M. Ferm, W. Ou, K. A. Smith, *J. Immunol.* **120**, 2027 (1978).
9. P. E. Austin, E. A. McCulloch, J. E. Till, *J. Cell. Physiol.* **77**, 121 (1970).
10. J. W. Parker and D. Metcalf, *J. Immunol.* **112**, 502 (1974).
11. D. Metcalf and G. R. Johnson, *J. Cell. Physiol.* **96**, 31 (1978).
12. ———, T. E. Mandel, *ibid.* **98**, 401 (1979).
13. G. Nabel, J. S. Greenberger, M. A. Sakankeeny, H. Cantor, *Proc. Natl. Acad. Sci. U.S.A.*, in press.

14. P. Ralph, M. A. S. Moore, K. Nilsson, *J. Exp. Med.* **143**, 1528 (1976).
15. J. S. Greenberger, P. B. Davisson, P. J. Gans, W. C. Moloney, *Blood* **53**, 987 (1979).
16. Bone marrow cells were removed from the femur and tibia of 8- to 12-week-old male mice, and about 2×10^7 cells were inoculated into plastic petri dishes (Falcon 3003) containing 10 ml of Fischer's medium (Associated Biomedic Systems) supplemented with 25 percent heat-inactivated horse serum (Flow Lab.) and $10^{-7}M$ hydrocortisone-21-sodium succinate (Sigma). Cultures were incubated at 33°C in a fully humidified atmosphere containing 7 percent CO_2 in air. Five milliliters of old medium were removed, and the same volume of fresh medium was added weekly. Cultures were not recharged with additional marrow. For preparation of W-CM, WEHI-3 cells were seeded at a concentration of 1×10^5 cells in 100-mm petri dishes in RPMI 1640 medium supplemented with 10 percent fetal bovine serum at 37°C in 7 percent CO_2 in air. Tissue culture fluids were collected at 3 days, dialyzed against three changes of distilled water (supernatant:distilled water = 1:10) at 4°C at daily intervals, and filtered through a 0.45-μm membrane (Millipore).
17. L. T. Yam, C. Y. Li, W. H. Crosby, *Am. J. Clin. Pathol.* **55**, 283 (1971).
18. E. F. Osserman and D. P. Lawlor, *J. Exp. Med.* **124**, 921 (1966).
19. J. F. Riley, *Ann. N.Y. Acad. Sci.* **130**, 151 (1963).
20. J. Laszlo and R. W. Rundles, *Hematology*, W. J. Williams *et al.*, Eds. (McGraw-Hill, New York, 1977), p. 659.
21. S. H. Snyder, R. J. Baldessarini, J. Axelrod, *J. Pharmacol. Exp. Ther.* **153**, 544 (1966).
22. T. Ishizaka and K. Ishizaka, personal communication.
23. J. E. Till and J. E. McCulloch, *Radiat. Res.* **14**, 213 (1961).
24. T. R. Bradley and D. Metcalf, *Aust. J. Exp. Biol. Med. Sci.* **44**, 287 (1966).
25. D. H. Pluznik and L. Sachs, *J. Cell. Physiol.* **66**, 319 (1965).
26. M. A. S. Moore and T. M. Dexter, *Transplant. Proc.* **10**, 83 (1978).
27. Y. Kitamura *et al.*, *J. Exp. Med.* **150**, 482 (1979).
28. H. E. Broxymeyer and P. Ralph, *Cancer Res.* **37**, 3578 (1977).
29. N. Williams, R. R. Eger, M. A. S. Moore, N. Mendelsohn, *Differentiation* **11**, 59 (1978).
30. J. S. Greenberger, *J. Supramol. Struct.*, in press.
31. A. Kulczycki, Jr., C. Isersky, H. Metzger, *J. Exp. Med.* **139**, 600 (1974).
32. We gratefully acknowledge J. S. Greenberger and Y. Kitamura for their helpful advice and discussions. We thank T. Ishizaka and K. Ishizaka for examining receptors for mouse immunoglobulin E and histamine content, and F. Hirata and J. Axelrod for instruction in the performance of the isotopic histamine assay. WEHI-3 cells were provided by P. Ralph. Supported in part by a grant-in-aid for cancer research from the Japanese Ministry of Education, Science and Culture.
* Address reprint requests to S.A.A.

25 August 1980; revised 23 October 1980

Bovine Babesiosis: Protection of Cattle with Culture-Derived Soluble *Babesia bovis* Antigen

Abstract. *Adult Hereford* (Bos taurus) *cattle were protected from severe reactions and death caused by the tick-borne protozoan hemoparasite* Babesia bovis, *3 months after vaccination with a cell culture–derived immunogen. The immunogen consisted of filtered, freeze-dried supernatant fluid collected from long-term cultures of* Babesia bovis *in vitro. It was reconstituted with saponin adjuvant and injected twice subcutaneously at 2-week intervals. Serum collected from vaccinated cattle caused thickening of the merozoite surface coat, aggregation, and lysis of merozoites in vitro. This reaction was identical to that caused by serum from immune carrier cattle suggesting that the protective antigen present in culture fluids is merozoite surface coat antigen. No mortality occurred among vaccinated cattle, whereas mortality among unvaccinated cattle and* Babesia bigemina*–immune cattle was 62.5 percent indicating that immunity to bovine babesiosis is species-specific.*

Bovine babesiosis, caused by the intraerythrocytic protozoan parasite *Babesia bovis (= argentina)*, is an acute, tick-borne hemolytic disease that affects cattle throughout the world (*1*). The severity of the disease is attributed to erythrocyte destruction and plugging of capillaries with parasitized erythrocytes leading to impaired organ function (*1*). Mortality is high, often exceeding 50 percent of affected cattle (*2*). Chemotherapeutic agents effective against the parasite are commercially available and recovered animals are immune to disease from homologous or heterologous strains of *B. bovis* (*3*). Therefore, immunoprophylaxis is directed toward prevention of the disease rather than infection and has traditionally been achieved through "premunition" (*4*), that is, by infecting cattle with parasites and treating them with drugs as necessary. Immunity to babesiosis persists for at least 6 months after cattle have been freed of infection through drug treatment or their own immune system (*5*). The development of immunity as a result of natural infection provides the rationale for attempting to develop an effective inactivated vaccine for bovine babesiosis. We report here that cattle can be protected against babesiosis for an extended period of time by vaccination with soluble antigen extracted from the medium of long-term cultures of *B. bovis*–infected erythrocytes. We present evidence that merozoite surface coat antigen is present in the culture medium, and that this antigen may be responsible for the induction of the immunity.

Bovine babesiosis is similar in many ways to human malaria caused by *Plasmodium* spp. (*6*). Furthermore, several species of *Babesia* infect man, producing a syndrome that has often been misdiagnosed as malaria (*6, 7*). Recent progress toward the development of an inactivated vaccine for babesiosis has closely paralleled similar studies in malaria research (*8*). Both parasites have been grown in vitro and their respective culture-derived antigens have been partially purified and characterized (*9, 9a*). *Babesia bovis* merozoites are abundant in culture supernatant fluids and possess a loosely adhering surface coat visible under the electron microscope. The infectivity of culture-derived merozoites is destroyed by freeze-thawing. The success of recent immunization and challenge experiments with killed, culture-derived *Plasmodium* (*10*) and *Babesia* (*11*) is attributed to the antigenic composition of the extraerythrocytic invasive stage of these parasites, that is, the merozoite. Specifically, the merozoite surface coat elicits the production of antibodies that prevent erythrocyte invasion by parasites and facilitates their immune destruction by phagocytic cells (*9a, 12*). However, preparations of malaria and babesia merozoites are contaminated with erythrocyte stroma, and this limits their usefulness as a vaccine. Soluble *B. bovis* antigens prepared from medium of primary cultures by salt precipitation or pervaporation induce short-term protection from tick-borne babesiosis when combined with Freund's complete or incomplete adjuvant (*11*).

In the present study the immunogen was extracted from the culture medium of actively growing cultures of *B. bovis*–infected bovine erythrocytes (*13*) that had been maintained in vitro for 196 days (approximately 98 generations) (*14*). Culture medium was collected over a 24-hour growth period during which parasitemia increased from 1.1 to 5.1 percent. After centrifugation at 11,877*g* for 15 minutes at 4°C, the cell-free supernatant fluid was filtered through a membrane with 0.22-μm pore size, freeze-dried in 28-ml portions (0.66 g of total protein), and stored at 4°C. Immediately before being used each portion was solubilized with 5 ml of sterile distilled water, mixed with 1 mg of saponin adjuvant (*15*), and inoculated subcutaneously into each of four Hereford *(Bos taurus)* cattle, each aged 18 months. A booster injection was given 2 weeks later. Four control cattle were each inoculated as above with lyophilized cell-free supernatant fluid from uninfected erythrocyte cultures. The only adverse effects of immunization were mild local swelling and increased body temperature for 48 to 72 hours.

The four immunized cattle and the four controls were challenged by exposing each of them to 1000 *Boophilus microplus* larvae from a *B. bovis*–infected

0036-8075/81/0417-0335$00.50/0