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Recloned Colonies Positive for T-Cell-Associated Antigens Derived from Mixed Hemopoietic Colonies (CFU-GEMM)[1] (41422)

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Abstract. Human pluripotent hemopoietic progenitors (CFU-GEMM) can be identified by their ability to form colonies in culture that contain granulocytes, erythroblasts, macrophages, and megakaryocytes. Forty-six individual mixed colonies from four individuals were prepared by cytocentrifugation and examined for cells positive for human T-cell markers using the monoclonal antibodies E 2-22 and OKT 3 and fluorescently labeled goat $F(ab')_2$ anti-mouse IgG antibody. Sixteen primary mixed colonies were found to be positive for the monoclonal antibodies E 2-22 and OKT 3. Seventy-nine primary mixed colonies derived from these four individuals were aspirated, dispersed, and then recloned. Twenty-three primary mixed colonies formed 97 secondary hemopoietic colonies. All cells in 50 recloned hemopoietic colonies were positive for the antibodies directed against T cells.

A culture assay for human pluripotent hematopoietic progenitors (CFU-GEMM) was described (1). These primitive precursors form mixed hemopoietic colonies containing neutrophilic and eosinophilic granulocytes, erythroblasts, macrophages, and megakaryocytes (2). The addition of a medium conditioned by leucocytes in the presence of phytohemagglutinin (PHA-LCM) and erythropoietin (EPO) is required for such colony formation.

In this study we provide experimental evidence that some mixed colonies may contain other lineages, i.e., cells positive for antigens usually associated with T lymphocytes. Recloning experiments indicated that some primary mixed colonies formed secondary hemopoietic colonies that are again reactive with monoclonal antibodies directed against T cells.

Material and Methods. *Bone marrow samples.* Bone marrow from four individuals was collected (after informed consent) in syringes containing preservative-free heparin. These individuals were assessed as putative bone marrow transplant donors.

Preparation for leucocyte conditioned medium. A conditioned medium was prepared from peripheral leucocytes of normal individuals as previously described (3). To summarize this process 10^6 leucocytes were incubated with 1% human serum albumin (HSA) (v/v, Sigma), IMDM, (Iscove's modified Dulbecco's medium, Gibco Laboratories, Grand Island, N.Y.) and 1% PHA (Burroughs Wellcome, Research Triangle Park, N.C.). This material, PHA-LCM, was harvested after 4 days of incubation at 37° in a humidified atmosphere with 5% CO_2.

Preparation of cell suspensions. Mononuclear cells of density less than 1.077 g/ml were obtained after centrifugation in Ficoll–Paque (Pharmacia Fine Chemicals). Antibody-coated plastic dishes were prepared by allowing purified monoclonal antibodies to bind to the plastic surface. These dishes were washed twice, 2×10^6 mononucleated cells were added to the coated dishes and incubated for 1 hr. The final concentration of the antibody E 2-22[3] (4) (cellular distribution 100% of peripheral T lymphocytes, IgG 2a) was 1:2000. This antibody was used for three experiments.

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[3] The E 2-22 monoclonal antibody was obtained from Cedarlane Laboratories, Milton, Ontario, Canada, and donated by Dr. Richard L. Krogsrud. The specificity of the antibody is for E-positive cell and B-cell CLL. This specificity is similar if not identical, to that described by Royston *et al.* (4).

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In one panning experiment OKT 3 (5) was used. After 1 hr of incubation the dishes were gently shaken and the supernatant containing nonadherent and T-cell-depleted cells was plated as outlined. The supernatants were analyzed for their ability to form E-rosettes (6). No detectable E-rosette formation could be observed. The same supernatants were analyzed for T cells by immunofluorescence using the OKT 3 antibody. The examined aliquots contained less than 3–4% cells which stained positive for OKT 3. The remaining petri dishes were air dried and cells adhering to the plastic surface were examined for their content of T cells by immunofluorescence using FITC-labeled goat $F(ab')_2$ anti-mouse IgG antibody (Cappel, Cochranville, Pa.).

Approximately 60–70% of cells adhering to the plastic surface stained for the OKT 3 or E 2-22 antibodies. These cells appeared to be lymphocytes in stained smears.

Culture conditions. Three-tenths milliliter of nonadherent (7) and T-cell-depleted supernatant was mixed with 30% fetal calf serum (FCS), 5% PHA-LCM, IMDM, and methylcellulose as viscous support to yield a final concentration of 0.9% (w/v). In order to obtain an adequate number of mixed hemopoietic colonies, 10–12 petri dishes (35 mm, Lux) were routinely plated. The cultures were incubated at 37° in humidified atmosphere at 5% CO_2. Four days later 1.0 unit of erythropoietin (Connaught, Step III) was added to the culture dish. After an additional 8–10 days of incubation the cultures were examined under an inverted microscope. Erythroid bursts (BFU-E) (8), granulocytic colonies (CFU-C) (9), and mixed hemopoietic colonies (CFU-GEMM) (2) were identified by their characteristic morphological appearance. The latter type of colonies contained cells with the red color typical of hemoglobin admixed with colorless and translucent cells of various sizes. The different types of colonies were removed from the cultures for further analysis by means of micropipet.

Recloning of hemopoietic colonies. Individual erythroid bursts (BFU-E), granulocytic colonies (CFU-C), and mixed hemopoietic colonies (CFU-GEMM) derived from these individuals were aspirated, dispersed, and placed in Linbro microtiter wells (10). Secondary plates contained 2.5% PHA-LCM and 0.5% PHA (Burroughs Wellcome). Erythropoietin was not added to the cultures. The microtiter plates were incubated at 37° humidified atmosphere with 5% CO_2. The Linbro plates were examined for secondary hemopoietic colonies 8 days later. They were counted and individual colonies were aspirated (10).

Immunofluorescence staining of primary and secondary hemopoietic colonies. Individual primary hemopoietic colonies were removed from the cultures on Day 12. Secondary colonies were aspirated 6–8 days after replating.

For immunofluorescence the specimens were incubated with 10 μl of the monoclonal antibody E 2-22 or OKT 3 (Ortho Pharmaceutical Company, Raritan N.Y.) for 1 hr and washed twice. Then they were exposed for 1 hr to 15 μl of FITC-conjugated goat $F(ab')_2$ anti-mouse IgG antibody (4). Cells derived from these colonies were placed on glass slides and evaluated under a fluorescence microscope with a specific filter combination for FITC-labeled specimens. Twenty-five individual colonies were found to be negative when stained with anti-mouse IgG alone.

Characterization of the monoclonal antibody. Erythroid bursts (BFU-E) and granulocytic colonies (CFU-C) were plucked by micropipette on Day 14 and were subjected to immunofluorescence staining.

T-Cell colonies were grown from peripheral blood of five volunteers (11, 12). Colony growth was stimulated by the addition of 0.5% PHA (Wellcome HA 15) with 5×10^{-5} M mercaptoethanol. They were harvested on Day 7 at random from the cultures and examined for their ability to form E-rosettes (5).

Furthermore, individual colonies were assessed using the monoclonal antibody E 2-22. In addition, in one experiment a random sample of 40 individual colonies was subdivided in two groups. Twenty colonies were assessed using the OKT 3 antibody (4). The second group was analyzed using the E 2-22 antibody. In each group all cells

of these individual colonies stained positive for OKT 3 or E 2-22 antibody. These cells appeared to be lymphocytes in stained smears.

Results. *Analysis of primary mixed colonies by immunofluorescence.* Forty-six hemopoietic mixed colonies (CFU-GEMM) were examined for the coexistence of lymphopoietic lineages (Table I). Thirteen mixed hemopoietic colonies were found to be positive for antibody E 2-22. Three mixed hemopoietic colonies grown from the individual M.P. were positive for the OKT 3 antibody (Table I). The number of positive cells varied and ranged between 8 and 42 in each clone. In contrast to CFU-GEMM, cells derived from individual BFU-E and CFU-C colonies did not react with E 2-22 or OKT 3.

Recloning of primary mixed colonies. Seventy-nine individual mixed colonies with the described typical appearance were plucked and recloned in Linbro wells (10). Twenty-three primary mixed colonies formed two different types of colonies. The first type was compatible with CFU-C. The second type was a very compact colony consisting of approximately 250–300 cells. The density of the colonies was higher in the peripheral areas of the wells, a typical feature for T-cell colonies. The number of secondary colonies per well varied greatly.

Examination of recloned hemopoietic colonies derived from primary mixed colonies by immunofluorescence using OKT 3 and E 2-22. We examined 97 secondary colonies for their expression of T-cell antigens by immunofluorescence using E 2-22 and OKT 3 (Table II). Fifty analyzed secondary clones reacted with the E 2-22 or OKT 3 antibody. In contrast to the primary mixed colonies, basically all cells were positive in the 50 secondary colonies. Cells derived from primary and secondary colonies which stained positive for the antibodies were lymphocytes in stained smears. In 140 examined BFU-E and CFU-C colonies no immunofluorescence staining could be observed. BFU-E and CFU-C colonies did not form secondary clones after replating.

Discussion. Culture assays for pluripotent murine and human progenitors that promote the growth of mixed colonies containing different hemopoietic lineages have been described (13–17). Recloning experiments of mixed hemopoietic colonies indicated that some primary mixed colonies formed secondary colonies, thus fulfilling criteria of stem cells (18). When primary mixed colonies were replated in the presence of 5% PHA-LCM and 1 unit of EPO approximately 25–30% of these colonies formed secondary CFU-C, BFU-E, and mixed colonies (19).

In our present study single cell suspensions of primary mixed colonies were exposed to 2.5% PHA-LCM and 0.5% PHA. There was no exogenous erythropoietin added. After 6–8 days of culture we found secondary colonies positive for monoclonal T-cell antibodies by immunofluorescence. This observation suggests that those recloned colonies are lymphoid cells. It was found that approximately 50% of the recloned colonies did not react with the

TABLE I. Identification of Colonies Positive for the Monoclonal Antibody by Immunofluorescence

Patient	No. of BFU-E		No. of CFU-C		No. of CFU-GEMM		
	Analyzed	Positive	Analyzed	Positive	Analyzed	Positive	
						E2-22	OKT 3
E.D.	50	0	50	0	12	4	n.d.
F.S.	35	0	40	0	14	6	n.d.
G.B.	55	0	50	0	11	3	n.d.
M.P.	31	0	69	0	9	n.d.	3

Note. Number of positive cells per colony ranged from 27 to 75 cells (46.9 ± 13.9, mean ± SD).

TABLE II. IMMUNOFLUORESCENCE ANALYSIS OF SECONDARY HEMOPOIETIC COLONIES AFTER REPLATING OF REDISPERSED MIXED HEMOPOIETIC COLONIES (CFU-GEMM)

Patient	No. of CFU-GEMM		Total No. of secondary colonies	No. of secondary colonies positive for	
	Replated	Secondary colonies		E2-22	OKT3
E.D.	19	7	28	17	n.d.
F.S.	21	5	19	11	n.d.
G.B.	23	6	33	14	n.d.
M.P.	16	5	17	n.d.	8

Note. Number of positive cells per colony ranged from 90 to 300 cells (234 ± 47, mean ± SD).

antibodies. These clones were found to be granulocytic colonies according to morphological criteria.

It is very unlikely that cells positive for the monoclonal T-cell antibodies within primary mixed hemopoietic colonies are a contaminant of T cells because of unselective and incomplete depletion procedure. The supernatants containing nonadherent and T-cell-depleted cells which were analyzed for their ability to form E-rosettes revealed no detectable E-rosette formation. In addition, the examined supernatants contained less than 3–4% cells that stained positive for OKT 3 antibody. In contrast to primary mixed colonies, BFU-E and CFU-C colonies were negative by immunofluorescence using the same antibodies.

When single cell suspensions derived from BFU-E and CFU-C colonies were replated no secondary colony formation could be observed in each individual experiment. Only single cell suspensions of primary mixed hemopoietic colonies formed secondary colonies. Secondary hemopoietic colonies derived from CFU-GEMM revealed two types of colonies. The first type of clones did not stain by immunofluorescence. These were compatible with CFU-C. The second type of colonies stained positive for OKT 3 or E 2-22. These cells, however, were lymphocytes. Secondary T-cell colonies derived from multilineage hemopoietic progenitors suggest that the renewed clones are part of the differentiation program of CFU-GEMM (1, 2, 10, 19, 20), as T cells or T-cell colonies do not renew. Further evidence for the existence of a common stem cell for myeloid and lymphoid cells is provided by two observations. Fialkow *et al.* (21) studied patients with chronic myelocytic leukemia who were heterozygous at the X-linked glucose-6-phosphate dehydrogenase locus to determine if lymphocytes had the same stem cell origin as the leukemic myeloid cells. They found a population of lymphocytes which has a single enzyme phenotype and T-cell characteristics, suggesting that T lymphocytes may arise from CML stem cells (22). Abramson *et al.* (23) used the technique of Wu *et al.* (24) to generate stem cells bearing unique, radiation-induced chromosome aberrations. By the use of these markers it was possible to identify cells as members of the same clone in the recipient animal. Their data provided cytogenetic evidence for the existence of a pluripotent stem cell capable of differentiating into myeloid and lymphoid progeny including both B and T lymphocytes in the mouse.

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