CONCISE REPORT

Identification of T Lymphocytes in Human Mixed Hemopoietic Colonies

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The addition of a T-cell growth-promoting medium (PHA-TCM) to culture conditions that support growth of multilineage hemopoietic colonies enhances the proliferation of cells with lymphoid morphology within these colonies. These cells were identified as T lymphocytes by their ability to form rosettes with SRBC and by their reaction with monoclonal antibodies (OKT3, OKT4) directed against T-cell-specific surface components. They continue to proliferate extensively under the influence of PHA-TCM after transfer of mixed colonies into liquid suspension culture. Supportive evidence for a common progenitor of myeloid and lymphoid cells within single mixed colonies is provided by Y-chromatin body analysis of E-rosette positive and negative cells in colonies grown in cocultures of male and female bone marrow cells.

HUMAN PLURIPOTENT hemopoietic progenitors (CFU-GEMM) in bone marrow and peripheral blood form colonies that contain cells of different hemopoietic lineages when grown under appropriate culture conditions. The assay is quantitative with a plating efficiency of approximately 10% normal mononuclear bone marrow cells. Analysis of their cellular composition revealed that neutrophilic granulocytes and erythroblasts are predominant in all colonies, while megakaryocytes, macrophages, and eosinophils are only seen in some. In addition to these myeloid cells, mononuclear cells of lymphoid morphology may be observed occasionally.

We are now reporting culture conditions that consistently support the development of T lymphocytes in some mixed colonies. Evidence is provided suggesting a common progenitor for T lymphocytes and myeloid cells within individual mixed colonies.

MATERIALS AND METHODS

Culture Conditions for Mixed Hemopoietic Colonies

Bone marrow samples from consenting normal bone marrow transplant donors were aspirated into heparinized syringes. Mononuclear cells of density less than 1.077 g/ml were prepared by density centrifugation in 60% Percoll. T lymphocytes were removed as previously described by incubation with 2.5-aminomethylisothiouronium (AET; Sigma, St. Louis, Mo.) treated sheep erythrocytes (SRBC abbreviated as E; Woodlyn Laboratories, Guelph, Ontario) and subsequent centrifugation in a second Percoll gradient.

These T-cell-depleted populations were admixed with Iscove’s modified Dulbecco’s MEM (IDMEM, Gibco, Grand Island, N.Y.), 5 x 10^5 M 2-mercaptoethanol, 30% fetal calf serum (FCS, Flow Laboratories), and 1 U of sheep erythropoietin (EPO; Step III, Connaught Laboratories, Willowdale Ontario). Methylenediamine (0.9%) and two sources of stimulators: 2.5% of medium conditioned by normal, E-rosette-forming T cells in the presence of 1% phytohemagglutinin (PHA; HA 15 Wellcome) (PHA-TCM) and 2.5% of regularly used PHA-induced leukocyte conditioned medium (PHA-LCM). PHA-TCM was replaceable by 0.5% PHA, freshly prepared in IDMEM. Aliquots of 1 ml containing 5 x 10^6 or 10^6 cells were placed in 35-mm Petri dishes (Lux) and incubated for 11-13 days at 37°C in humidified atmosphere supplemented with 5% CO2. The cultures were examined for the presence of mixed colonies, erythroid bursts, and granulocytic colonies using an inverted microscope. Colonies of all types were removed by micropette for further analysis to be performed either on individual or subsequently pooled colonies.

Assessment of Colonies

E-Rosette Formation

Individual or pooled colonies were placed into round-bottom flexible microtiteration plates (Microtiter, Cooke), resuspended in 0.5 ml IDMEM supplemented with 10% FCS containing 5 x 10^-6 AET-treated SRBC, and incubated for 1 hr at 4°C. The proportion

| Table 1. Frequency of E-Rosette-Forming Cells and Cells That Stain Positive With OKT-Monoclonal Antibodies in Pooled Mixed Colonies, Erythroid Bursts, and Granulocytic Colonies |
|---|---|---|---|---|---|
| Experiment | No. of Colonies | Analysis | Mixed Colonies | Pooled Erythroid Bursts | Granulocytic Colonies |
| 1 | 100 | E-rosetting | 30/300 | 0/300 | 0/268 |
| 2 | 20 | E-rosetting | 60/1,000 | 0/1,000 | 0/1,000 |
| 3 | 100 | E-rosetting | 106/1,000 | 0/1,000 | 10/1,000 |
| 4 | 40 | E-rosetting | 115/1,000 | 1/1,000 | 2/1,000 |
| 5 | 30 | E-rosetting | 39/1,092 | 4/1,275 | 14/874 |
| OKT 3 | 26/1,092 | 3/1,275 | 14/874 |
| OKT 4 | 24/533 | — | — |
| OKT 8 | 0/579 | — | — |

*No. of positive cells/total no. of examined cells.*
of E-rosette-forming cells was determined by phase-contrast microscopy.

**T-Cell Surface Markers on E-Rosette-Forming Cells**

Pooled colonies were thoroughly washed in IMDMEM supplemented with 5% FCS and 0.2% sodium azide. Twenty microliters of normal rabbit IgG were added to avoid nonspecific binding of IgG. Aliquots were incubated for 30 min at 4°C with one of three monoclonal antibodies directed against various T-cell subpopulations (OKT3 PAN, OKT4 IND, OKT8 SUP: generously provided by Ortho Pharmaceutical Corp., Raritan, N.J.), washed with IMDMEM and stained for a further 30 min at 4°C with FITC-labeled goat anti-mouse affinity-purified IgG preparation (Tago, Inc., Burlingame, Calif.). After careful washing, cells were incubated with SRBC as described above. Following this procedure, slides were prepared by cytocentrifugation. The slides were fixed with 95% ethanol, rinsed in PBS, mounted with PBS-glycerol, and sealed with clear nail lacquer. The preparations were examined under phase contrast for E-rosettes and under fluorescence for cells that stained positive with the monoclonal antibodies.

**Liquid Suspension Culture of Individual Colonies**

Single colonies were transferred into round-bottom microtitration plates containing 0.1 ml of IMDMEM, with 20% FCS and 20% PHA-TCM. The cultures were maintained at 37°C in humidified atmosphere containing 5% CO₂ with medium changes every 3–5 days.

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Fig. 1. (A) Wright stain of individual mixed colony showing cells of myeloid and lymphoid morphology. (B) E-rosette-forming cells in mixed colonies. (C and D) Demonstration of a single E-rosette-forming, OKT3-positive T lymphocyte observed in a mixed colony. (E) A tightly cohesive sphere of T lymphocytes grown from a single mixed colony 14 days after transfer into liquid suspension culture.
days. Colonies that demonstrated a substantial increase in cell number were examined after 14 days of liquid suspension culture for E-rosette-forming cells and stained with OKT3.

**Y-Chromatin Analysis of E-Rosette Positive and Negative Cells in Individual Mixed Colonies**

Individual colonies grown in cultures that contained bone marrow cells of male and female origin were incubated with AET-treated SRBC. Slides prepared by cytocentrifugation were fixed in 95% ethanol and stained with 0.5% quinacrine-HCl. E-rosette positive and negative cells in each colony were identified by phase-contrast microscopy and subsequently assessed by fluorescence for the presence of a Y-chromatin body.

**RESULTS**

**T Lymphocytes in Mixed Colonies**

Pooled and individual mixed colonies, erythroid bursts, and granulocytic colonies were examined for the presence of T lymphocytes by testing for two different properties: E-rosette formation and identification of surface markers using the monoclonal antibodies (OKT3, OKT4, OKT8).

E-rosette-forming cells were consistently identified in cell suspensions of pooled mixed colonies (Table 1). Their frequency varied in 5 experiments from 4% to 12%. The morphological appearance of some E-rosette-forming cells resembled that of mature lymphocytes, while others displayed a more primitive phenotype (Fig. 1B). Simultaneous examination of E-rosette-forming cells with monoclonal antibodies was performed in one experiment (Fig. 1C and D).

The majority, but not all E-rosette-forming cells stained positive with OKT3 and OKT4. No reaction with OKT8 was observed.

Rosettes are only rarely observed in suspensions of pooled erythroid bursts. The frequency remained within the background level of each experiment. Similar observations for pooled granulocytic colonies were made in 3 of 5 experiments. The background level for E-rosette-forming cells was exceeded in two studies (1%–2%).

Analysis of individual colonies yielded similar results (Fig. 2). In all 5 experiments, some mixed colonies were identified that contained from 1% to 60% of E-rosette-forming cells. The frequency of positive colonies varied between 30% and 100%. Erythroid bursts did not contain E-rosette-forming cells. With one exception, granulocytic colonies were also negative. One colony of morphological appearance typical for granulocytic colonies was found to contain 50% E-rosette-forming cells among other mononuclear, nongranulocytic cells.

**Proliferation of T Cells Derived From Mixed Hemopoietic Colonies in Liquid Suspension Culture**

Cells in some mixed colonies continued to proliferate extensively under the influence of PHA-TCM (Table 2) after transfer into liquid suspension culture. The size of some colonies increased within 14 days up to 20,000 cells that typically formed a tightly cohesive sphere (Fig. 1E). Almost all of these cells formed...
rosettes with SRBC and stained positive with OKT3. This phenomenon of continuing T-cell proliferation was not observed when erythroid bursts and granulocytic colonies were placed into liquid suspension culture.

**Y-Chromatin Analysis of Mixed Colonies**

A cocultivation experiment between cells of male and female origin was performed to examine the origin of lymphoid and myeloid components within individual mixed colonies. Eleven mixed colonies were initially subjected to rosette formation, and E-rosette positive and negative cells subsequently analyzed for the presence of a Y-chromatin body. In five colonies, E-rosette positive and negative cells did not contain the Y-chromatin body; in five, the Y-chromatin body was present in approximately 50% of all examined E-rosette positive and negative cells. In one mixed colony, the Y-chromatin body was observed in 12% of all cells.

**DISCUSSION**

Culture conditions are described that support the growth of T lymphocytes in mixed hemopoietic colonies. Their identification is based on their ability to form rosettes with SRBC and on their reaction with monoclonal antibodies directed against surface components of T lymphocytes. The extensive proliferation of T lymphocytes within mixed colonies observed under the influence of PHA-TCM in liquid suspension culture suggests a similar proliferative potential as that of normal T cells exposed to T-cell growth factor.1,12 The common origin of lymphoid and myeloid cells within individual mixed colonies is strongly supported by results of the Y-chromatin body analysis performed on E-rosette positive and negative cells in individual mixed colonies that were grown in a coculture experiment of male and female bone marrow cells. The data are consistent with observations reported for the different myeloid cell lineages found in mixed colonies.1,4

The finding of T cells in pooled granulocytic colonies is not completely understood but is likely related to a problem discriminating granulocytic colonies from a new type of T-cell colony recently observed under these culture conditions. These T-cell colonies persist for as long as 14 days and can only be discriminated from granulocytic colonies by detailed cellular analysis. This possibility is supported by data in Fig. 2. Cytologic assessment of what appeared to be a typical granulocytic colony demonstrated a large component of E-rosette-forming cells among mononuclear, non-granulocytic cells.

The reproducible coexistence of myeloid and lymphoid components within individual mixed colonies may permit monitoring of events that regulate the early stages of myeloid and lymphoid differentiation. This might be further facilitated by the use of appropriate cell surface markers. In addition, it may now be feasible to determine whether lymphoid cells originating in pluripotent progenitors are phenotypically homogeneous or diverse.

**REFERENCES**

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