Cytotoxic T-Cell Clones Derived From Pluripotent Stem Cells (CFU-GEMM) of Patients With Hodgkin's Lymphoma

By A. A. Fauser, H. A. Neumann, K. G. Bross, L. Kanz, and G. W. Lühr

Pluripotent stem cells (CFU-GEMM) give rise to multi-lineage hemopoietic colonies in culture. The cellular composition revealed that mixed colonies contain cells of different myeloid lineages and mononuclear cells with T-cell surface antigens. T lymphocytes of primary colonies and replated secondary colonies from 5 patients with Hodgkin's lymphoma (stage I-II) were identified by their reaction with the monoclonal antibody OKT-8. Replated secondary clones do act functionally as cytotoxic cells using K562 as target cells. Evidence for a common progenitor of myeloid and lymphoid cells is provided by analysis of individual secondary colonies with the use of OKT-3, OKT-4, OKT-8, VIM-D5, and IgM + D antibodies for each individual clone. Primary mixed and replated secondary colonies revealed OKT-8-positive cells. No reaction with OKT-3, OKT-4, VIM-D 5, or IgM + D was observed. In mixed colonies grown from putative bone marrow transplant donors, only OKT-3-positive cells could be observed. Secondary replated colonies did not stain for OKT-8 and failed to lyse 51Cr-labeled K562 cells.

Pluripotent Stem Cells (CFU-GEMM)

From human bone marrow can be identified by their ability to give rise to mixed hemopoietic colonies in culture. Mixed colonies may contain cells of different hemopoietic lineages. In addition, compelling evidence is available that mixed colonies contain mononuclear cells with T-cell surface antigens. This observation supports the view that a common progenitor exists for lymphocytes and myeloid cells in bone marrow.

In this article we examined primary mixed hemopoietic colonies and replated secondary colonies from 5 patients with Hodgkin's lymphoma (stage I-II) for lymphoid markers. We found a consistent pattern of cytotoxic T lymphocytes within individual primary mixed colonies and replated secondary hemopoietic colonies. Secondary colonies act functionally as cytotoxic cells using chromium-labeled K562 target cells.

Materials and Methods

Patients

Bone marrow samples were obtained from the iliac crest of 5 consenting patients with Hodgkin’s lymphoma (stage I-II) undergoing clinical investigation and from 2 putative bone marrow transplant donors (after informed consent).

Preparation of Leukocyte Conditioned Medium

Conditioned medium was prepared from peripheral leukocytes from a patient with hemochromatosis (HLA-A5, B7). Briefly, 10^6 leukocytes were incubated with 1% human serum albumin (HSA) (v/v, Sigma, St. Louis, Mo.) IMDM (Iscove’s modified Dulbecco’s medium, GIBCO Laboratories, Grand Island, N.Y.), and 1% PHA (Borroughs Wellcome, Research Triangle Park, N.C.). This material (PHA-LCM) was harvested after 4 days of incubation.

Preparation of Cell Suspensions

The specimens were aspirated into heparinized syringes. Mononuclear cells of density less than 1.077 g/ml were obtained after centrifugation in Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden). Antibody-coated plastic dishes were prepared by allowing purified mononuclear antibody (OKT-3) to bind to the plastic surface. Usually, 2 x 10^6 mononuclear cells of density less than 1.077 g/ml were added to the thoroughly washed coated dishes and incubated for 1 hr. After 1 hr of incubation, the dishes were gently shaken, and the supernatant containing nonadherent T-cell-depleted cells was removed. The supernatants were analyzed for their ability to form E-rosettes. No detectable E-rosette formation could be observed. 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 OKT-8 antibodies. These cells appeared to be lymphocytes in stained smears.

Culture Conditions for Mixed Hemopoietic Colonies

Supernatants containing 1.5 x 10^6 cells were admixed with 30% human fresh plasma, 5% PHA-LCM, IMDM, 2 x 10^{-3} M mercaptoethanol, and methylcellulose at a final concentration of 0.9% (w/v). A total of 1.8 x 10^6 mononuclear cells were cultured in 12 replicate dishes containing 1.5 x 10^6 cells. The incubation was performed at 37°C in a humidified atmosphere at 5% CO2. After 4 days of incubation, 1 U of erythropoietin (Connaught, Step III) was added to the culture. Mixed hemopoietic colonies were identified at day 12 by their characteristic morphological appearance. These colonies contained cells with the red color typical for hemoglobin, admixed with colorless and translucent cells of various size. For further analysis, mixed hemopoietic colonies were removed by micropipette from the cultures.

Recloning of Hemopoietic Colonies

Individual mixed hemopoietic colonies (CFU-GEMM) were replated as single cell suspensions in Linbro microtitir wells. In order to stimulate lymphopoietic proliferation, secondary plates contained 2.5% PHA-LCM and 0.5% PHA (Wellcome) and no erythropoietin was added. After 7 days of culture, the Linbro plates were scored for secondary hemopoietic colonies. They were

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counted and individual colonies were picked at random by micropette as described for primary colonies and were examined using the peroxidase antiperoxidase staining procedure (PAP).10,11

**Cell-Mediated Lympholysis Assay**

Secondary colonies were assayed for cell-mediated cytotoxicity against targets by using a 51Cr-release assay. The target used was K562, a human leukemic cell line. Target cells were labeled with 51Cr as described elsewhere.12

Individual secondary colonies were placed separately in microtiter well (Linbro 76-022-05) and 51Cr-labeled target cells (2 x 10⁶) were then added. The supernatant was sampled and counted after 4 hr of incubation of 37°C. A well containing colony cells was considered to exhibit cytotoxic activity if its 51Cr-release value was greater than the mean plus 2 standard deviations for the group of wells from which colony cells were omitted. Fractional specific 51Cr-release was calculated as 

\[ \text{Fractional specific } \frac{\text{counts}}{\text{counts}} \]

Step 2: Rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen).

Step 3: Swine anti-rabbit immunoglobulin (Dakopatts).

Step 4: Peroxidase-anti-peroxidase complex from rabbit (Dakopatts).

Step 5: Diamonobenzidine–H₂O₂, followed by postfixation with OsO₄. The slides are covered with glycerin and coverglass.

Controls were performed with β₂-microglobulin and the different sandwich antiser. The number of cells in each reaction area (i.e., each antibody) ranged from 2000 to 5000 cells for primary colonies and 60–250 cells for secondary colonies. Cells were scored positive for a particular antibody if more than 10 cells were positive, i.e., revealing dark brown stained cell membranes as indicated in Fig. 1.

**RESULTS**

**OKT-8-Positive Lymphocytes in Mixed Colonies**

Mixed hemopoietic colonies were grown from 5 patients with Hodgkin's disease and 2 putative bone marrow transplant donors. The frequency of mixed hemopoietic colonies observed is shown in Table 1. Individual mixed colonies were examined for the presence of T lymphocytes. Lymphocytes were identified by surface markers using the monoclonal antibodies OKT-3, OKT-4, OKT-8,13 and allantiserum to β₂-microglobulin. OKT-8-positive cells were found in approximately 30–40% of individual mixed colonies of patients with Hodgkin's disease, as indicated in Table 2. The number of positive cells ranged from 0 to 68 cells per colony. In a total of 53 analyzed mixed colonies from 5 individual experiments, 17 clones were identified that stained for OKT-8 (Table 2).

Individual erythroid bursts (BFU-E)14 and granulocytic colonies (CFU-C)15 were found to be negative for OKT-3, OKT-4, OKT-8, or IgM + D antibodies. Twenty-four individual mixed hemopoietic colonies grown under identical conditions from putative marrow transplant donors failed to reveal any OKT-8-positive cells. As indicated in Table 3, cells within mixed colonies reacted with OKT-3.

![Fig. 1. A 120-fold magnification of OKT-8-positive cells (PAP-method) derived from a mixed hemopoietic colony.](http://www.ashpublications.org/blood/article-pdf/60/6/1317/588378/1317.pdf)

**Table 1. Number of Mixed Colonies (CFU-GEMM), Erythroid Bursts (BFU-E), and Granulocytic Colonies (CFU-C)**

<table>
<thead>
<tr>
<th>Patient</th>
<th>CFU-GEMM*</th>
<th>BFU-E†</th>
<th>CFU-C†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.M.</td>
<td>47</td>
<td>74</td>
<td>51</td>
</tr>
<tr>
<td>D.S.</td>
<td>40</td>
<td>83</td>
<td>35</td>
</tr>
<tr>
<td>S.P.</td>
<td>57</td>
<td>51</td>
<td>44</td>
</tr>
<tr>
<td>D.I.</td>
<td>61</td>
<td>69</td>
<td>70</td>
</tr>
<tr>
<td>E.E.</td>
<td>39</td>
<td>70</td>
<td>45</td>
</tr>
<tr>
<td>Control</td>
<td>A.D.</td>
<td>79</td>
<td>97</td>
</tr>
<tr>
<td>S.C.</td>
<td>44</td>
<td>56</td>
<td>63</td>
</tr>
</tbody>
</table>

*1.8 x 10⁶ total.
†1.5 x 10⁶.
Table 2. Identification of Colonies Positive for the Monoclonal Antibody OKT-8 by PAP

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of BFU-E (Pos/Total)</th>
<th>No. of CFU-E (Pos/Total)</th>
<th>No. of CFU-GEMM (Pos/Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.M.</td>
<td>0/10</td>
<td>0/10</td>
<td>3/10</td>
</tr>
<tr>
<td>D.S.</td>
<td>0/10</td>
<td>0/10</td>
<td>3/12</td>
</tr>
<tr>
<td>S.F.</td>
<td>0/10</td>
<td>0/15</td>
<td>4/8</td>
</tr>
<tr>
<td>D.I.</td>
<td>ND</td>
<td>ND</td>
<td>5/14</td>
</tr>
<tr>
<td>E.E.</td>
<td>0/10</td>
<td>0/10</td>
<td>2/9</td>
</tr>
<tr>
<td>Control</td>
<td>A.D.</td>
<td>0/12</td>
<td>0/15</td>
</tr>
<tr>
<td></td>
<td>S.C.</td>
<td>0/15</td>
<td>0/14</td>
</tr>
</tbody>
</table>

Number of positive cells per colony ranged from 0 to 68 cells.

Analysis of Secondary T-Cell Colonies Derived From Mixed Hemopoietic Colonies

A number of primary mixed colonies formed secondary colonies upon replating in the presence of 2.5% PHA-LCM and 0.5% PHA. After 7 days of culture, individual secondary colonies were picked at random by micropipette and analyzed for 6 different antibodies (Table 3). A total of 50 individual secondary colonies derived from different microtiter wells were examined for β2-microglobulin, IgM + D, OKT-3, OKT-4, OKT-8, and VIM-D5. All examined cells within each individual clone were OKT-8 and β2-microglobulin positive (Fig. 1). No reaction with OKT-3, OKT-4, VIM-D5, or IgM + D was observed (Table 3). However, secondary colonies derived from transplant donors stained positive for OKT-3; no reaction could be observed with the use of OKT-8. Erythroid bursts (BFU-E) and granulocytic colonies (CFU-C) did not form secondary colonies.

Direct Cytotoxicity of Recloned T-Cell Colonies Derived From Primary Mixed Hemopoietic Colonies Against K562 Cells

Individual secondary T-cell colonies derived from mixed hemopoietic colonies (CFU-GEMM) of patients with Hodgkin’s disease are capable of lysing K562 cells. It is of note that colony cells producing the cytotoxicity have not apparently been previously exposed to K562, thus, they are exhibiting spontaneous cytotoxicity toward these targets. The majority of cells within secondary T-cell colonies are OKT-8 positive, thus we conclude that these cells act functionally as cytotoxic cells. Results of two independent experiments are shown in Fig. 2. Colony cells were tested for cytotoxicity against 2 × 10⁶ ⁵¹Cr-labeled K562 target cells in a 4-hr ⁵¹Cr-release assay. Replated secondary colonies of marrow transplant donors were not capable of lysing ⁵¹Cr-labeled K562 cells.

DISCUSSION

Experimental evidence was provided that appropriate culture conditions support the growth of lympho-

Table 3. Peroxidase-Antiperoxidase Analysis of Individual Secondary Hemopoietic Colonies

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of Replated CFU-GEMM</th>
<th>Secondary Colony Formation</th>
<th>OKT-3</th>
<th>OKT-4</th>
<th>OKT-8</th>
<th>VIM-D5</th>
<th>IgM + D</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.M.</td>
<td>24</td>
<td>7</td>
<td>0/10</td>
<td>0/10</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>D.S.</td>
<td>22</td>
<td>4</td>
<td>0/12</td>
<td>0/12</td>
<td>12/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>S.F.</td>
<td>30</td>
<td>8</td>
<td>0/10</td>
<td>0/10</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>D.I.</td>
<td>19</td>
<td>6</td>
<td>0/10</td>
<td>0/10</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>E.E.</td>
<td>12</td>
<td>5</td>
<td>0/8</td>
<td>0/8</td>
<td>8/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>Control</td>
<td>A.D.</td>
<td>20</td>
<td>5</td>
<td>7/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>S.C.</td>
<td>13</td>
<td>4</td>
<td>5/14</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
</tr>
</tbody>
</table>

Number of positive cells per colony ranged from 95 to 350 cells (205 ± 53, mean ± SD).
The assay was found to be linear using nonadherent T-cell-depleted bone marrow cells. Mixed colonies were found to originate from one single cell when plated at a density of 1.5 x 10^3/plate, as assessed by G6PD analysis. OKT-3 and OKT-4 positive cells were described in mixed hemopoietic colonies of bone marrow transplant donors. No reaction was observed with OKT-8 antibodies.

We observed T cells in primary mixed colonies derived from putative bone marrow donors that stained positive with OKT-3. The same staining pattern could be observed in recombined colonies derived from primary mixed colonies. The examination of primary mixed hemopoietic colonies and recombined colonies derived from 5 patients with Hodgkin's disease, all having stage I-II at the time of diagnosis, revealed that the majority of the analyzed primary and secondary clones stained positive for OKT-8. However, no reaction with OKT-3 or OKT-4 could be observed. In addition, the secondary colonies from these patients act functionally as cytotoxic cells using K562 as targets. Mixed hemopoietic colonies and secondary T-cell colonies grown under identical conditions, derived from 2 putative bone marrow donors who served as controls, did not contain or consist of OKT-8-positive cells. This observation may be explained by the sensitivity of detection of a small subpopulation of T cells in the primary colonies. The examined colonies did react with OKT-3. In addition, the OKT-3-positive colonies were not capable of lysing ^51Cr-labeled K562. Other forms of lymphoma or cancer have not yet been examined to determine the generality or specificity of our observations with mixed hemopoietic colonies derived from marrow of patients with Hodgkin's disease. Additional studies during and after appropriate therapy to patients with Hodgkin's disease may serve to provide indications of changes in hematoipoiesis induced by malignant transformation and the subsequent effects of therapy.

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REFERENCES