Identification of B cells in multilineage hematopoietic colonies derived from cells of patients with lymphocytic lymphoma

-pluripotent stem cells/myelolymphoepoietic progenitors/human stem cells in lymphoma-

A. A. FAUSER*, L. KANZ, AND G. W. LÖHR

Medizinische Klinik, Albert-Ludwigs-Universität, Hugstetterstrasse 55, Freiburg, Federal Republic of Germany

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ABSTRACT

Pluripotent stem cells from human bone marrow can be identified in culture by their ability to form multilineage hematopoietic colonies containing different myeloid lineages and T cells of different phenotypes. The observation of a common progenitor of myeloid and lymphoid cells in normal and disturbed hematopoiesis prompted the question of whether B cells are part of the differentiation program of stem cells. The availability of hybridomas of azaguanine-resistant T-cell lines secreting monoclonal growth factors for B cells and clinical conditions that are considered to originate from malignan-

B cells might facilitate this investigation. We were able to identify surface immunoglobulin and B-cell-associated antigen-positive cells within such colonies, indicating that B cells are generated from a myelolymphopoietic stem cell. This report describes the presence of B cells in these colonies derived from bone marrow cells of patients with non-Hodgkin lymphoma.

In the past, developmental methods for examining hematopoiesis began to influence concepts of studying hematological malignancies. A different approach became possible as cell culture procedures were established for committed hematopoietic progenitors and, more recently, for noncommitted multilineage hematopoietic stem cells (1–5). These primitive progenitors derived from human bone marrow can be identified in culture by their ability to form mixed hematopoietic colonies containing basophilic, eosinophilic, and neutrophilic granulocytes, erythrocytes, erythroblasts, macrophages, megakaryocytes, and T cells of different phenotypes. The observation of a common progenitor of myeloid and lymphoid cells in normal and disturbed hematopoiesis leads to the question of whether B cells are part of the differentiation program of human stem cells in culture. In the present study, we attempted to induce B-cell differentiation of marrow cells from patients with non-Hodgkin lymphoma.

We were able to identify surface immunoglobulin (sgl) and B-cell-associated antigen-positive cells within such hematopoietic colonies containing different myeloid lineages.

MATERIALS AND METHODS

Patients. Bone marrow samples were obtained from eight patients with lymphocytic lymphoma (well-differentiated lymphocytic lymphoma).

Preparation of Leukocyte Conditioned Medium. Conditioned medium was prepared from peripheral leukocytes of normal individuals (6). Briefly, 10^6 leukocytes were incubated with 1% (vol/vol) human serum albumin (Sigma)/Is-

cove's modified Dulbecco's medium (Gibco)/1% phytohe-
magglutinin (Wellcome HA 15, Research Triangle Park, NC). This material (leukocyte conditioned medium) was har-

vested after 3–4 days of incubation at 37°C in humidified 5% CO2.

Preparation of Cell Suspensions. Bone marrow samples were aspirated into heparanized syringes. Mononuclear cells (density, <1.077 g/ml) were obtained after centrifugation in Ficoll-Paque (Pharmacia). Mononuclear cells were incubated sequentially in antibody-coated dishes using B1, B2 (Coulter) (7, 8), and BA1 (Hybritech, San Diego, CA) (9) for 20 min at 4°C. The supernatant containing nonadherent and B-cell-depleted cells was plated as outlined. The supernatants were analyzed for B cells by the peroxidase-antiperoxidase slide technique (10, 11) using the same antibodies as described above. The examined aliquots contained <1% cells that stained positive for B1, B2, or BA1. Normal bone marrow gave similar depletion by the same procedures.

Assessment by Fluorescence-Activated Cell Sorting. Mononuclear bone marrow cells from these patients were examined for cells positive for B-cell-associated antigens using a fluorescence-activated cell-sorting system. Cells were incubated with the monoclonal antibodies in B1 and B2 and subsequently labeled with the F(ab')2 fragment of goat-anti-mouse IgG and IgM. Nonviable cells could be identified by labeling the cell suspension with ethidium bromide. The cell preparations were analyzed for forward light scatter, perpendicular light scatter, fluorescein isothiocyanate fluorescence, and red fluorescence (ethidium bromide). Nonviable cells could be excluded by gating out fluorescence cells. Platelets, debris, and erythrocytes were excluded by threshold limitation for high forward scatter intensities. One argon laser (514 nm) was used to excite the ethidium-labeled cells; the second argon laser (488 nm) was used to analyze anti-B1, anti-B2 goat anti-mouse fluorescein isothiocyanate-positive cells.

It was found that 12%–27% and 8%–19% of the analyzed unfractionated bone marrow cells were positive for B1 and B2, respectively, whereas <1% of cells derived from each individual supernatant after the panning procedure were found to be positive for B1 and B2.

These subcellular fractions are very crucial to confirm that the depletion experiments yielded marrow-cell preparations without any contamination by B cells that contribute significantly to mixed colony formation.

Colony Assay for Hematopoietic Progenitors. Mixed hematopoietic colonies (1–5), erythroid bursts (12), and granulo-
cytic colonies (13) were grown as described. All experiments were performed with 1.2 × 10^6 mononuclear (B-cell deple-
ted) cells per culture plate. At this cell density, basically all mixed colonies were found to originate from one single cell rather than being generated by cell aggregates. To obtain a larger number of mixed colonies, cultures were plated in

Abbreviations: sgl, surface immunoglobulin; BCGF, B-cell growth factor.

*Present address: Division of Hematology, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, PQ, Canada H3A 1A1.

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quadruplicate. Cells were admixed with Iscove’s modified Dulbecco’s medium/30% human plasma/0.9% cellu-
lose/5% leukocyte conditioned medium. Aliquots of 0.9 ml were placed in 35-mm Petri dishes and incubated at 37°C in
humidified 5% CO2. Fifty microliters of the monoclonal
growth factor for B cells (BCGF) was added to each plate on
day 0 (14). Erythropoietin (1 unit/ml) (step III, Connaught
Laboratories, Willowdale, ON, Canada) was added on day 4
of culture in a vol of 100 µl. Each dish was examined after an
additional 10 days of incubation for the presence of hematopoietic colonies—i.e., erythroid bursts, granulocytic col-
ones, and multilineage colonies. Mixed hematopoietic col-
ones were identified at day 14 by their characteristic morpho-
logical appearance. These colonies contained cells with the
red color typical for hemoglobin admixed with colorless and
translucent cells of various sizes. For further analysis, mixed
hematopoietic colonies, erythroid bursts, and granulocytic colonies were removed from the cultures by micropipette.

Examination of Hematopoietic Colonies for slgM and slgD and for B-Cell-Associated Antigen-Positive Cells with the Use of the Peroxidase-Antiperoxidase Slide Technique. Individual multilineage colonies, erythroid bursts, and granulocytic colonies were aspirated by micropipette from the cultures and washed in Iscove’s modified Dulbecco’s medium (5 min; 300 g). Aliquots were transferred onto poly-l-lysine (Sigma)-
coated wells on glass slides. Poly-l-lysine allows firm attach-
tment of viable cells to the positively charged surface of
the wells; the area between the wells was coated with a com-
ponent that repels protein-containing solutions (Rain X, Un-
elko, S. Holland, IL). The slide preparation and details of
the peroxidase-antiperoxidase assay have been described
(10, 11). Cells derived from each individual colony that were
attached to the charged surface of the well were fixed with
0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) to
block Fc receptors and to preserve cell morphology. Cells
were stained either with the monoclonal antibodies anti-hu-
man IgM and anti-human IgD (Bethesda Research Labora-
tories) or with the B1 antibody (Coulter). Control experi-
ments were performed with β2-microglobulin and the differ-
sent sandwich antisera—i.e., swine anti-rabbit and rabbit
anti-mouse.

RESULTS

slg and B-Cell-Associated Antigen-Positive Cells in Multiline-
age Hematopoietic Colonies. Mixed hematopoietic colonies,
granulocytic colonies, and erythroid bursts were grown from
nonadherent and B-cell-depleted marrow cells of eight pa-
tients with non-Hodgkin lymphoma in the presence of leuko-
cyte conditioned medium, erythropoietin, and a monoclonal
BCGF. Individual hematopoietic colonies were aspirated from

Table 1. Identification of cells positive for slgM and B-cell-associated antigen (B1) in hematopoietic colonies derived from human bone marrow

<table>
<thead>
<tr>
<th>Patient</th>
<th>Erythroid bursts</th>
<th>Granulocytic colonies</th>
<th>Mixed hematopoietic colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive for slgM and slgD/B1</td>
<td>Positive for slgM and slgD/B1</td>
<td>Positive for slgM and slgD/B1</td>
</tr>
<tr>
<td></td>
<td>No. analyzed</td>
<td>No. analyzed</td>
<td>No. analyzed</td>
</tr>
<tr>
<td>P.M.</td>
<td>0/0</td>
<td>12</td>
<td>0/0</td>
</tr>
<tr>
<td>K.N.</td>
<td>ND</td>
<td>ND</td>
<td>0/0</td>
</tr>
<tr>
<td>R.U.</td>
<td>ND</td>
<td>ND</td>
<td>0/0</td>
</tr>
<tr>
<td>T.S.</td>
<td>0/0</td>
<td>15</td>
<td>0/0</td>
</tr>
<tr>
<td>B.G.</td>
<td>0/0</td>
<td>10</td>
<td>0/0</td>
</tr>
<tr>
<td>D.F.</td>
<td>0/0</td>
<td>15</td>
<td>0/0</td>
</tr>
<tr>
<td>M.U.</td>
<td>0/0</td>
<td>10</td>
<td>0/0</td>
</tr>
<tr>
<td>G.S.</td>
<td>0/0</td>
<td>15</td>
<td>0/0</td>
</tr>
</tbody>
</table>

ND, not done.

*No. in parentheses is mean no. of slgM- and slgD-positive cells per colony.
†No. in parentheses is mean no. of B1-positive cells per colony.

DISCUSSION

A culture assay for human multilineage hematopoietic pro-
genitors has been established (1, 2). These primitive progeni-
tors form mixed colonies containing lymphopoietic and my-
eloid cells of different lineages (3–5). Recloning experiments of multilineage colonies indicated that some fulfill criteria of
stem cells (15)—i.e., they display self-renewal capacity and
pluripotency and are subject to regulatory control mecha-
nisms. The single cell origin of multilineage hematopoietic
colonies was confirmed by the following evidence. (i) The
cultures and were examined for the coexistence of lymphopoietic progeny—i.e., B cells (Fig. 1). slg and B1 posi-
tive cells could be identified in multilineage colonies from six
of eight patients (Table 1). The frequency of B cells observed
in the examined mixed colonies ranged from 0% to 37%. The
number of positive cells per colony ranged from 0 to 340 cells
(173 ± 46, mean ± SD). In contrast to multilineage colonies,
cells derived from individual erythroid bursts and granulo-
cytic colonies did not stain for slg and for antibodies to B-
cell-associated antigens. Bone marrow cells of five healthy
individuals were cultured under identical culture conditions
as described. Individual multilineage colonies, erythroid
bursts, and granulocytic colonies were examined for the
presence of cells reacting with the monoclonal anti-B-cell
antibodies. Unlike colonies from non-Hodgkin lymphoma,
none of 65 examined mixed colonies from normal bone mar-
row revealed cells that stained positive with B-cell-associated
antigens. The same results were obtained for erythroid
bursts and granulocytic colonies.
assay was found to be linear with increasing cell number, with extrapolation through the origin. (ii) Determination of the sedimentation velocity of cells responsible for the formation of mixed colonies. (iii) Cocultivation experiments of marrow samples of female and male origin (1). (iv) Recloning experiments of primary mixed colonies, to assess their self-renewal capacity (15–17). (v) Examination of mixed colonies derived from individuals who were heterozygous for the X-linked glucose-6-phosphate-dehydrogenase locus (18). In this study, bone marrow cells were depleted of B-cell-associated antigen-positive cells using the monoclonal antibodies B1, B2, and BA1 sequentially. The supernatants containing nonadherent and B-cell-depleted cells were analyzed for B cells by the peroxidase-antiperoxidase slide technique and with the use of a fluorescence-activated cell sorter. Both examination procedures revealed that the aliquots prior to plating contained <1% of cells that stained for B1, B2, or BA1, respectively. These subcellular fractionations are very crucial to confirm that the depletion yielded cell preparations without contaminants that contribute meaningfully to mixed colony formation. The lymphopoietic component in mixed hematopoietic colonies consisted of T cells of various phenotypes—i.e., OKT 3, OKT 4, OKT 8, or E 2-22 (3-5).

Until now, B cells could not be identified in multilineage hematopoietic colonies derived from marrow cells of healthy volunteers or from patients with hematological disorders. The availability of an azaguanine-resistant human T-cell line-degenerated monoclonal BCGF facilitated mixed colony formation containing sIgM and sIgD and B-cell-associated antigen-positive cells derived from bone marrow of patients with lymphocytic lymphoma. Preliminary data suggest that B cells within individual colonies produce a single type of immunoglobulin when assessed for κ or λ light chains, but approximately equal numbers of colonies containing each light-chain type were observed.

In the murine system, cyogenetic evidence for the coexistence of a pluripotent stem cell capable of differentiating into myeloid and lymphoid progeny including both B and T lymphocytes had been provided by Abramson et al. (19). The data presented suggest a stem cell capable of differentiating into myeloid cells and cells that are positive for B-cell-associated antigens and sIg in patients with lymphocytic lymphoma.

The lack of B cells in multilineage hematopoietic colonies derived from healthy volunteers so far might be explained by a different state of responsiveness and thus can be seen in lymphoma; perhaps improved culture conditions through the addition of other growth factor(s) would elicit normal B cells.

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