Simian Virus 40–Transformed Adherent Cells From Human Long-term Marrow Cultures: Cloned Cell Lines Produce Cells With Stromal and Hematopoietic Characteristics

By Jack W. Singer, Pierre Charbord, Armand Keating, John Nemunaitis, Greg Raugi, Thomas N. Wight, Jose A. Lopez, Gerald J. Roth, Lois W. Dow, and Philip J. Fialkow

Adherent cells from long-term marrow cultures from 23 individuals were transformed with wild-type simian virus 40 (SV40). After transformation, cloned cell lines were developed that even after rigorous subcloning invariably produced both stromal cells and round cells. The stromal cells expressed cytoskeletal filaments similar to those of long-term marrow culture adherent cells and produced interstitial and basal lamina collagen types. The round cells had the electron microscopic appearance of primitive hematopoietic cells and when examined with cytochemical stains and monoclonal antibodies to hematopoietic differentiation antigens had reaction patterns suggestive of cells from several lineages. Most round cells expressed the pan-hematopoietic T-200 determinant, and lesser percentages expressed the early T cell antigens CD-1 and CD-3, HLA-DR determinants, the monocytic antigen recognized by Leu M3, and the myeloid antigens detected by monoclonal antibodies 1G10 and 12.8. In addition, when plated in semisolid medium in the presence of a source of colony-stimulating activity, up to 11% of the cells formed colonies consisting of blastlike cells that also expressed hematopoietic cell surface determinants. The data suggest that adherent cells in long-term marrow cultures contain a cell that after transformation by SV40 obligately produces cells with hematopoietic as well as stromalike features.

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THE HUMAN LONG-TERM MARROW culture (LTMC) system permits the proliferation of hematopoietic progenitors for several months in the presence of a layer of adherent or stromal cells that act as an in vitro microenvironment. The adherent layer is heterogeneous in its cellular content and contains variable numbers of fat-containing cells and macrophages, a minor population of round hematopoietic cells, and a major population of flat angulated cells of fibroblastic appearance. Nonhematopoietic properties of stromal cells include the synthesis of a complex extracellular matrix similar to that synthesized by cultured smooth muscle cells.

Attempts to grow clones of human stromal cells similar to those developed from murine stromal cells have had limited success. In the present study, we used wild-type simian virus 40 (SV40) to transform cells and generate stromal cell lines on 25 marrow samples from 23 individuals. Seven of the resultant cell lines, including three from females heterozygous for the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD), were cloned at limiting dilutions. All clones produced both adherent cells with stromal characteristics and round, loosely adherent cells with morphological and phenotypic characteristics of hematopoietic cells of several lineages. Moreover, when transformed cells were plated in semisolid medium in the presence of a source of colony-stimulating activity (CSA), colonies of blastlike cells were formed that expressed hematopoietic determinants.

MATERIALS AND METHODS

Subjects. LTMC were generated from clinical marrow aspirates obtained after informed consent from subjects with the diagnoses listed in Table 1 under protocols approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. Three patients with acute lymphoblastic leukemia were heterozygous for G6PD.

Cell cultures. LTMC were grown as described. Confluent adherent layers from 4-week-old cultures were treated with trypsin (1:250; Difco, Detroit) in EDTA and passaged into fresh flasks. SV40 (SV40/VERO 7; Meloy Laboratories, Springfield, VA) was added (ten particles per cell), and after 12 hours at 37°C, the adherent cells were harvested and reseeded in microtiter plates (Costar, Cambridge, MA) at 1,000 cells/well in medium supplemented with 30% conditioned medium from the KG-1 cell line. The KG-1 cell line produces a factor that stimulates proliferation of cultured, passaged adherent cells from LTMC (unpublished data). Between 50% and 95% of the wells gave rise to passagable cell lines. After two to three passages, the KG-1 conditioned medium, the horse serum, and hydrocortisone were deleted, and the concentration of fetal calf serum in the medium was gradually decreased to 5%. Some cell lines were later adapted to serum-free conditions in HL-1 medium (Ventrex; Portland, ME). The absence of serum did not alter the morphology of the cells.

Clonal growth. Limiting dilution cloning was performed on cell lines from seven of the patients including the three who were heterozygous for G6PD. After treatment of the cell lines with trypsin, a single-cell suspension was obtained by vigorously passing...
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A series of mixed samples was then prepared that contained cultures, loosely adherent cells and nonadherent cells were harvested amounts of G6PD activity in round cells and stromalike cells in these were available for characterization studies. Only wells with a single and wells with growth were serially passaged until sufficient cells were available for characterization studies. Only wells with a single focus of growth were considered to contain potential clones. G6PD in the putative clones from the three G6PD heterozygous patients was assayed by starch gel electrophoresis. To estimate the relative amounts of G6PD activity in round cells and stromalike cells in these cultures, loosely adherent cells and nonadherent cells were harvested by gentle agitation. This population contained predominantly round cells that bore hematopoietic determinants. Adherent cell layers with the most of the round cells removed by agitation were separately harvested from a culture of the opposite G6PD type from the round cells. A series of mixed samples was then prepared that contained between 5% round cells and 95% stromal cells or between 5% stromal cells and 95% round cells and tested for G6PD activity by starch gel electrophoresis.

Characterization with monoclonal antibodies. For characterization with monoclonal antibodies, cells from both native and subcloned cell lines were passaged into chamber slides (Lab-tek; Miles Scientific, Naperville, IL), and when confluent, the cells were fixed with absolute methanol for examination with monoclonal antibodies to the "T" translational product of SV40 (Oncogene Sciences, Mineola, NY) and to cytoskeletal proteins or in 2% formaldehyde and 0.1% glutaraldehyde (4°C; 30 minutes) for assays with antibodies to cell surface epitopes. The cells were then exposed to an appropriate dilution of the test antibody or an irrelevant isotype control antibody for 30 minutes at room temperature. After washing, a goat fluorescein isothiocyanate-conjugated, F(ab')2 antismouse IgG or IgM (Cappel Laboratories, West Chester, PA) was applied for 30 minutes before examination under a fluorescence microscope. Test antibodies included the anticytoskeleton antibody CGA-7, which recognizes smooth muscle actin; HHF, which reacts with smooth muscle, skeletal, and cardiac muscle; and 4G8E8, which reacts with vimentin in mesenchymal cells but not in hematopoietic cells (kindly donated by A. Gown). The monoclonal antibodies to hematopoietic determinants included 9.4, which recognizes the T-200 common hematopoietic determinant, 7.2, which reacts with an HLA-DR framework determinant; 6.1, which recognizes CD-3; 7.6, which reacts with CD-2 (all kindly donated by Paul Martin); 12.8, anti-Leu 6, an antibody to the CD-1 determinant of immature thymocytes; Leu M3, an antibody that reacts with nearly all monocytic cells (Becton Dickinson, Mountainview, CA); 12.8 and 1G10, which react with early and late myeloid cells, respectively (kindly donated by Irwin Bernstein); and 60.1 and 60.3, which recognize the α and β subunits of the CB3B1 receptor complex, respectively (kindly donated by Patrick Beauty).

The presence of collagenous proteins and thrombospondin was tested by labeling stromal cells with 125I-methionine and extracting them in SDS before electrophoresis on 7.5% polyacrylamide gels. The antithrombospondin antibody was prepared by immunizing rabbits against purified thrombospondin from human platelets as described. An IgG fraction was obtained by repeated precipitation with ammonium sulfate. Contaminating antibrinogen antibody activity was removed by affinity chromatography using Sepharose 4B linked to human fibrinogen. The resulting preparation bound to thrombospondin but not to fibronectin, fibrinogen, and human collagen types I, III, IV, and V. The anti-type IV collagen antisemur IgG fraction was similarly prepared. It reacted with type IV collagen but not with types I and III and only slightly with type V collagen. For radiolabeling, washed cells were cultured for 18 hours.
in methionine-deficient medium. Immunoprecipitations were performed as described\(^4\) and analyzed by autoradiography after gel electrophoresis.

Platelet glycoprotein lb (GpIb) was measured by placing cell lysates in wells with adsorbed rabbit polyclonal anti-GpIb affinity-purified antibody in the presence of EDTA, N-ethyl maleimide, and leupeptin.\(^5\) A mouse monoclonal anti-GpIb (C7E10, kindly donated by Diane Nugent) was added after washing and was followed by alkaline phosphatase–labeled goat antimouse antibody (Cappel) and phosphatase substrate. The amount of GpIb present was estimated by using a standard of purified human platelet glycopacin. The reactivity of each of the other antibodies was similarly confirmed by using an enzyme-linked immunosorbent assay (ELISA).\(^6,7\)

RESULTS

Initially, the cell lines consisted predominantly of spindle-shaped and flat-angulated cell similar to those observed in nontransformed adherent cell layers from LTMC. Increasing numbers of round cells, some of which were loosely adherent, appeared after six to nine passages (Fig 1A). Scanning electron microscopy of the cultures revealed small, round cells adhering to flat, stromal cells (Fig 1B). Transmission electron microscopy of these same cultures demonstrated the presence of undifferentiated hematopoietic-appearing cells admixed with an adherent cell population that appeared similar to stromal cells found in untransformed LTMC (Fig 1C).

Cloning studies. Seven of the cell lines were cloned at limiting dilutions between passages 6 and 13 (Table 2). Most wells seeded with fewer than ten cells had only a single focus of growth when examined at 1 week. Wells with growth were serially passaged three to four times until sufficient numbers of possessing growth when examined at 1 week. Wells with growth were serially passaged three to four times until sufficient numbers of cells were available for characterization. The linearity of the semilogarithmic plots of the percentage of wells without growth vs the number of cells plated allowed calculation of the cloning efficiency of the lines.\(^8\) This varied between 5\% and 50\% as shown in Table 2. G6PD was used as a cell marker to determine whether the putative clones originated from single cells. Limiting dilution cloning was performed on cell lines from the three G6PD heterozygous patients at times when the native cell lines expressed both A- and B-type G6PD. All but one of the 68 cell lines initiated with ten or fewer cells per well expressed only either A- or B-type G6PD, further attesting to their clonal nature (Table 3). Each of the cell lines that manifested only a single G6PD enzyme appeared morphologically identical to the parent line and consisted of both round cells and stromalike cells. When G6PD, type A, round cells were mixed in various proportions with G6PD, type B, stromal cells and the relative activities were determined, the amount of G6PD activity per cell was approximately equal. Five percent G6PD A-type round cells were detectable when mixed with 95\% type-B stromal cells, and conversely, 5\% added type-B stromal cells were detectable when mixed with 95\% type-A round cells. Since the proportion of round cells in the tested cloned cell lines was at least 30\%, these data suggest that they should have been detectable by G6PD if they had been of the opposite enzyme type. Therefore, the G6PD data suggest that these putative clones were true clones.

Cell line characterization. Subsequent characterization studies were performed on both cloned cell lines initiated with fewer than ten cells per well from the limiting dilution cultures and the native cell lines. Most of the cell lines grew rapidly for 20 to 30 passages, with a doubling time of three to six days, and then entered a crisis. Only one line was rescued after entering crisis (line 21). This cell line has been maintained in continuous culture for more than 48 passages (over 16 months). All characterization studies were performed with cell lines between the tenth and 20th passages. Examination of the cell lines by indirect immunofluorescence with a monoclonal antibody to the large T antigen of SV40 demonstrated the presence of the antigen in nearly all nuclei of cells.

Fig 1. (A) Phase-contrast photomicrograph of subcloned transformed stromal cell line 10. Note the confluent adherent cell layer and the loosely adherent round cells. (B) Scanning electron microscopic appearance of transformed subcloned stromal cell lines. (Original magnification \(\times\) 320; current magnification \(\times\) 176.) A number of round cells can be seen associated with adherent stromal cells. (C) Transmission electron micrographs of the same cultures. (Original magnification \(\times\) 13,000; current magnification \(\times\) 8,880.) Round cells are present above the adherent stromal cells (s). a refers to the location of the bottom of the culture flask.
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from native and subcloned lines (data not shown).\(^1^7\) Southern blotting hybridization analysis of DNA from cell lines 3 and 5 demonstrated integration of SV40 DNA (data not shown).

**Stromalike cells.** The cell lines were examined with monoclonal antibodies recognizing cytoskeletal filaments found in stromal cells from LTMC.\(^8\) Reactivity of round cells was rarely seen. In contrast, nearly all elongated, adherent cells expressed actin and vimentin as recognized by the antiactin antibodies CGA-7 and HHF and the antivimentin antibody 43E8.\(^8\) Unlike the filamentous actin network found in untransformed stromal cells from LTMC,\(^8\) the SV40-transformed cells displayed diffuse cytoplasmic fluorescence. Even the vimentin network was not as well delineated as in nontransformed cells. However, Western blot analysis confirmed the presence of both actin and vimentin in the transformed cells (Fig 2A). These data suggest that, as in other SV40-transformed cell lines, actin did not polymerize normally in the transformed stromal cells.\(^34\) Analysis of collagenous proteins synthesized by the transformed cell lines demonstrated the presence of basal lamina collagen (type IV), a collagen synthesized by both smooth muscle and by LTMC adherent cells but not by narrow fibroblasts.\(^4\) (Fig 2B).

**Round cells.** A series of monoclonal antibodies recognizing myeloid and lymphoid differentiation antigens was also used to characterize the cells from native and subcloned cell lines. Only the round cells and not the elongated stromalike cells reacted with these antibodies (Table 4). In indirect immunofluorescence assays, 55% ± 4% (mean ± SEM) of the round cells reacted with a monoclonal antibody with a specificity for the common leukocyte T-200 antigen (9.4) (Fig 3A and B); 15% ± 8% reacted with antibody Leu 6, which recognizes the CD-1 determinant on immature thymocytes\(^2^5\); 22% ± 5% reacted with antibody 64.1, which recognizes the T cell receptor complex (TPI9-29, CD-3) (Fig 3C and D); and lower frequencies of cells reacted with antibodies recognizing CD-2, the sheep RBC receptor (35.1),\(^2^1\) the monocyte-macrophage antigen identified by Leu M3,\(^2^4\) and the myeloid antigens recognized by antibodies 12.8 (Fig 3E and F) and IG10.\(^2^5\)^2\(^6\) In addition, 15% ± 7% of the round cells expressed HLA-DR framework determinants (antibody 7.2). Approximately 3% of the round cells reacted with antibody 60.3, which recognizes the \(\beta\) subunit of the C3Bi receptor, an antigen expressed by lymphoid and myeloid cells.\(^2^7\) The cells did not react with antibodies recognizing the \(\alpha\) subunit of the C3Bi complex (60.1), but small numbers of round cells reacted with both a monoclonal antibody and an affinity-purified polyclonal antibody to platelet Gp1b.\(^3^5\) By using an enzyme-linked immunosorbant assay, the cellular content of GP1b was estimated to be 13, 23, and 2 ng/mg protein in subcloned cell lines 7, 2, and 19, respectively. The assay is capable of detecting <1 ng of glycoalamin/mg of protein (unpublished data).

Analysis of five subclones from different patients by using cytochemical stains showed that most cells contained acid phosphatase,\(^3^9\) some cells contained large amounts of PAS-positive material,\(^2^9\) and between 5% and 42% reacted with

**Table 1. Limiting Dilution Cloning of Cell Lines From Three G6PD Heterozygous Patients**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Passage Cloned</th>
<th>G6PD A:B</th>
<th>No. Cells/Well A+B</th>
<th>No. Wells With G6PD</th>
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<tr>
<td>10</td>
<td>6</td>
<td>1:1</td>
<td>1</td>
<td>3</td>
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<td>10</td>
<td>5</td>
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<td>0:1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>11</td>
<td>1:1</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>9:0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>11:2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*The numerator is the number of wells with passageable cell lines. The denominator is the number of wells seeded.*
antiactin (A) and antivimentin (v) monoclonal antibodies.

Incubation of line I with phorbol myristate acetate (PMA) at 10^{-7} mol/L for four days increased the frequency of nonspecific esterase–positive cells from 7% ± 3% (mean ± SD) of cells to 24% ± 6%. Neither peroxidase nor chloroacetate esterase was detectable in any of the cell lines. Between 3% and 25% of trypsin-harvested cells formed rosettes with AET-treated sheep RBC when subclones from patients 2, 4, and 5 were tested.

Colony formation. To determine whether cells in the SV40-transformed adherent layers formed colonies in semisolid medium, the cultures were treated with trypsin, and single-cell suspensions were prepared and plated in semisolid medium with and without PHA-LCM or recombinant GM-CSF. No evidence of cell division was seen until day 5 when doublets were first noted. Thereafter growth was rapid, and colonies of up to 1,000 cells formed by day 14 (Fig 4A). Each of five tested cell lines formed colonies. Only a single type of colony was observed. It consisted of a large, tight ball with occasional loose cells around its periphery. Colonies grew only in the presence of a source of CSA. There was a dose-response relationship with PHA-LCM (Table 5). As with CFU-GM, optimal colony formation was observed when 20% PHA-LCM was added (Fig 5A). The number of colonies grown was linearly related to the number of cells plated (Fig 5B). In the presence of 20% PHA-LCM, 1.1%, 2.3%, and 11.0% of the cells seeded formed colonies in lines 10A, 11, and 9 respectively. When tested with recombinant GM-CSF, cluster formation (defined as round cell aggregates of between 20 and 50 cells) was observed at concentrations as low as 10 U/mL; some large colonies were present in plates containing 1,000 and 10,000 U/mL (Table 6). However, even if the numbers of colonies and clusters that grew with 10,000 units of GM-CSF are combined, the cloning efficiency was only 23% of that observed with 20% PHA-LCM.

When colonies were individually plucked and placed in suspension culture, some cells became adherent and assumed a stromalike configuration. Cell division continued, and within 2 weeks, these suspension cultures reassumed the appearance of the native, SV40-transformed cell lines in suspension culture. When the colonies were plucked, disaggregated with trypsin to obtain single-cell suspensions, and reseeded in semisolid medium with PHA-LCM, secondary colonies were formed with a reseeding efficiency of 1.1% with 20% PHA-LCM and 0.5% with GM-CSF (10,000 U/mL).

Characterization of colonies. Most cells in the colonies appeared to be undifferentiated, but occasional cells had monocytoid or lymphocytoid features (Fig 4B). The majority of cells in the colonies reacted with nonspecific esterase stains, and most ingested latex beads. The cells did not react with peroxidase. Indirect immunofluorescence assays with monoclonal antibodies on colonies from three different cell lines demonstrated that 50% ± 5% of the cells reacted with 9.4, an antibody to the common hematopoietic T-200 determinant; 39% ± 4% reacted with 64.1, an antibody that reacts with the CD-3 complex; 12% ± 4% reacted with antibody Leu 6, which recognizes the CD-1 determinant; 12% ± 2% reacted with antibody 12.8, a 115-kd cell surface complex present on immature hematopoietic cells; and 18% ± 4% reacted with 7.2, an antibody to an HLA-DR framework determinant (Fig 4C). Although the native cell lines from which the colonies were grown expressed actin and vimentin as detected by antibodies CGA-7 and 43B/E8, the cells in the colonies, like the round cells in native cultures, did not react...
<table>
<thead>
<tr>
<th>Antibody Reactivity</th>
<th>Antigen</th>
<th>Cell Lineages</th>
<th>References</th>
<th>Percentages of Round Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.4 (n = 25)†</td>
<td>T-200</td>
<td>Nucleated hematopoietic cells</td>
<td>15, 16</td>
<td>55 ± 4*</td>
</tr>
<tr>
<td>Leu 6 (n = 6)</td>
<td>CD-1</td>
<td>Immature thymocytes</td>
<td>46</td>
<td>15 ± 7</td>
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<tr>
<td>64.1 (n = 25)</td>
<td>CD-3</td>
<td>T cell receptor complex</td>
<td>22, 23</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>35.1, 9.6 (n = 4)</td>
<td>CD-2</td>
<td>T cells, sheep RBC receptor</td>
<td>22, 27</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Leu M3 (n = 4)</td>
<td></td>
<td>Monocytes</td>
<td>26</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>12.8 (n = 12)</td>
<td>115 kd</td>
<td>Immature myeloid cells</td>
<td>25</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>1G10 (n = 15)</td>
<td>Leα</td>
<td>Granulocytes-monocytes</td>
<td>24</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>7.2 (n = 4)</td>
<td>HLA-DR</td>
<td>Multiple lineages</td>
<td>26, 27</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>60.3 (n = 9)</td>
<td>β subunit, C3βi complex</td>
<td>Multiple lineages</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>60.1 (n = 6)</td>
<td>α subunit of C3βi complex</td>
<td>Granulocytes</td>
<td>34</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>C7E10 (n = 4)</td>
<td>Gp1b</td>
<td>Megakaryocytes</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: kd, kilodalton.

*Mean (± SEM) percentage of brightly fluorescent round cells on subcloned lines from multiple different subjects tested between the tenth and 20th passages.

†Cell lines derived from each of the 23 subjects were tested for expression of T-200 and CD-2. Expression of the other antigens was assessed on cell lines from between four and 15 of the subjects. Only minor differences in expression of these antigens were found between cell lines derived from different individuals. Not shown in the table are the results of analysis of subcloned cell lines from the limiting dilution clones from Tables 1 and 2. In each case, round cell antigen expression was nearly identical to that of the cell lines before cloning.

Fig 3. Immunofluorescence of transformed, subcloned cell lines. (A) Anti-T-200 or common leukocyte antigen with antibody 9.4. (C) Anti-CD3, the T cell receptor complex with antibody 64.1. (E) Anti–early myeloid antigen with antibody 12.8. (B, D, and F) Corresponding phase-contrast photomicrographs.
with these anticytoskeletal filament antibodies. However, when colonies were plucked from the semisolid medium, placed in suspension culture, and allowed to readhere for seven days, the adherent cells reexpressed actin and vimentin filaments.

**DISCUSSION**

Twenty-five cell lines from SV4O-transformed LTMC adherent cells from 23 patients each contained cells that by morphological, cytochemical, and antigenic criteria were from disparate cell lineages. Each line contained adherent cells with morphological and biosynthetic properties similar to those of nontransformed stromal cells. These cells synthesized proteins characteristic of marrow stromal cells such as interstitial and basal lamina collagens, muscle-type actin, vimentin, and thrombospondin. Moreover, even after rigorous limiting dilution cloning, cell lines, including those shown by G6PD analysis to originate from single cells, produced loosely adherent round cells that expressed cell surface and enzymatic determinants usually associated with hematopoietic cells. The round cells were unlikely to be simply stromal cells in mitosis for several reasons. Morphologically by both light and electron microscopy, the round cells were rarely observed in mitosis. Moreover, as the cultures became confluent and cell division slowed, the frequency of round cells increased rather than decreased. Last, when fluorescence-activated cell sorter analyses were done on trypsinized cell lines, the cells that expressed hematopoietic determinants such as CD-1, CD-3, and Leu M3 (data not shown) were among the cells with the lowest forward light scattering properties whereas the T-200 was expressed on both large and small cells.

The hematopoietic features of the round cells included expression of the common leukocyte antigen T-200 and other antigens associated with immature thymocytes such as CD-1 and CD-3. Differentiation markers of several other lineages were also detected. For example, some round cells expressed nonspecific esterase, were phagocytic for latex particles (data not shown), and expressed the antigen recognized by Leu M3 and the β subunit of the C3Bi receptor, which suggests a relation to monocytes. Platelet Gp1b was also detectable, which indicates that determinants associated with megakaryopoiesis were expressed by some cells. The increase in expression of nonspecific esterase activity observed after the addition of PMA suggested that differentiation of these cell lines could be further influenced. Surprisingly, despite their derivation from normal donors, patients with aplastic anemia, and patients with acute and chronic leukemias, the expression of cell surface antigens on all cell lines was similar. Each expressed CD-1, CD-3 and T-200 with similar frequencies on round cells. However, the expression of antigens recognized by antibodies to myeloid differentiation antigens and to platelet Gp1b was more variable. These observations suggest that SV40 transforms a primitive cell not involved by these disease processes.

Although each of the several hundred cell lines grown from these patients produced cells that were both stromalike and round, without using genetic markers, the possibility could not be excluded that each cell line arose from two or more intimately associated cells of different lineages. For this reason, studies were done of limiting dilution cloning of SV4O-transformed stromal cells from patients with cellular mosaicism for G6PD. The G6PD system has been used to demonstrate the clonal origin of hematopoietic colonies. When cell lines from three G6PD heterozygous patients were cloned at limiting dilutions, all but one of 64 cell lines originating at ten or fewer cells per well expressed either A or B G6PD, thereby indicating their single-cell origin. When lines originating at 100 cells/well from cell line 10 were tested, seven of 24 expressed both A and B G6PD, thereby indicating derivation from several cells. The sensitivity of the G6PD system is such that the addition of 5% of cells of the opposite enzyme type can be detected. Approximately 30% of cells in the transformed cultures are round, and the remainder appear stromal. The G6PD activity per cell of round and stromal cells from the transformed cultures is approximately equivalent. These data suggest that subcloned cell lines derived from wells seeded with fewer than ten cells are probably clones.

Two G6PD type B clones from cell line 10, one type A and one type B clone from cell line 14, and two type B clones from cell line 13 were characterized by immunofluorescence with monoclonal antibodies and were found to express muscle-type actin with antibodies CGA-7 and HHF in the stroma-
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Fig 4. (A) Phase-contrast photomicrograph of a colony cultured from an SV40-transformed stromal cell line in the presence of 20% PHA-LCM. (B) Wright stain of colony. (C) Indirect immunofluorescent staining with 7.2 recognizing an HLA-DR framework determinant. (D) Corresponding phase-contrast photomicrograph.

like cells and the hematopoietic determinants T-200 and CD-3 in the round cells. Thus, clonal cell lines appeared to produce cells with both hematopoietic and stromal characteristics.

Studies of other cell lineages transformed with SV40 virus have shown that, in general, the resultant cell lines were similar to the starting cell populations. For example, although SV40-transformed human epidermal cells differed from nontransformed cells in growth properties and had decreased expression of keratins and cross-linked envelopes, their growth remained anchorage independent, and they were not tumorigenic in athymic nude mice. Transformed human keratinocytes reexpressed certain characteristics of fetal cells including keratin polypeptides found in fetal epidermis. SV40-transformed marrow or skin fibroblasts do not produce round cells or react with any of the antibodies to hematopoietic determinants shown in Table 2 (unpublished data). Thus, the finding of obligate, apparent multilineage stromal and round cell differentiation in SV40-transformed cell lines arising from LTMC adherent cells suggests that this differentiative program is a normal property of the cells in LTMC susceptible to SV40 transformation.

The morphological appearance of the SV40-transformed...
lines described in the present report suggests that these are different from lines transformed by a recombinant plasmid of SV40. Unlike the present cells, the plasmid-transformed clones contained exclusively cells that morphologically were fibroblastic. It is possible either that different cell populations were transformed by the two methods or that intact virus is needed to effect the additional differentiative programs required for round cell expression.

Although the identity of the cell(s) transformed by SV40 remains unknown, such cells were apparently present in hematopoietically inactive LTMC from two patients with aplastic anemia. The cell lines derived from these cultures contained both round cells and stromal cells and were grossly indistinguishable from similar cell lines derived from normal donors. If SV40 virus causes a cell to revert to a fetal phenotype, as suggested for keratinocytes by Bernard et al., it is possible that in our system there is selective transformation of cells present in adult marrow analogous to the fetal mesenchymal cell thought to differentiate into blood island cells in the embryonic yolk sac. Moreover, the present data, like those reported previously, suggest that the stromal cells in human LTMC and some hematopoietic cells may originate from a common stem cell. In addition to implications for hematopoietic cell ontogeny, the results reported here indicate that SV40 transformation provides a useful method for immortalizing stromal cells from human LTMC so that these cells can be cloned and examined for their biosynthetic properties, their interaction with hematopoietic cells, and their origin in patients with neoplastic disorders.

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Simian virus 40-transformed adherent cells from human long-term marrow cultures: cloned cell lines produce cells with stromal and hematopoietic characteristics

JW Singer, P Charbord, A Keating, J Nemunaitis, G Raugi, TN Wight, JA Lopez, GJ Roth, LW Dow and PJ Fialkow