Characterization of the Continuous, Differentiating Myeloid Cell Line (HL-60) From a Patient With Acute Promyelocytic Leukemia

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In a preliminary communication,\(^1\) we described the establishment of a continuous human myeloid cell line (HL-60). Here we report the detailed properties of this cell line and document its derivation from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia. As characterized by light and electron microscopy, the predominant cell type in both the fresh and cultured sources is a neutrophilic promyelocyte with prominent nuclear/cytoplasmic asynchrony. Up to 10% of the cultured cells spontaneously differentiate beyond the promyelocyte stage, and the proportion of terminally differentiated cells is markedly enhanced by compounds known to stimulate differentiation of mouse (Friend) erythroleukemia cells.\(^1\) The HL-60 cells lack specific markers for lymphoid cells, but express surface receptors for Fc fragment and complement (C3), which have been associated with differentiated granulocytes. They exhibit phagocytic activity and responsiveness to a chemotactic stimulus commensurate with the proportion of mature cells. As characteristic of transformed cells, the HL-60 cells form colonies in semisolid medium and produce subcutaneous myeloid tumors (chloromas) in nude mice. A source of colony-stimulating activity stimulated the cloning efficiency in soft agar 5-30-fold. Despite adaptations to culture, the morphological phenotype and responsiveness to chemical induction of differentiation is essentially unchanged through at least 85 passages. Cytogenetic studies reveal aneuploidy. Metaphases with 44 chromosomes predominated in vivo and in early culture passages; however, clones with 45 or 46 chromosomes became predominant with continued passaging. The most consistent karyotypic abnormalities were the deletion of chromosomes 5, 8, and X and the addition of a marker resembling a D-group acrocentric and of a submetacentric marker, most likely an abnormal E-group chromosome. No DNA herpesvirus or RNA retrovirus was isolated in the fresh or cultured cells. The HL-60 cultured cell line provides a continuous source of human cells for studying the molecular events of myeloid differentiation and the effects of physiologic, pharmacologic, and virologic elements on this process.

IN MICE, the availability of sustained cultures of erythroid\(^1\) and myeloid\(^2,3\) cells has fostered experiments to determine regulatory mechanisms involved in cell replication and differentiation and to study external factors that may affect these processes.\(^4,5\) In man, in vitro culturing of myeloid cells has primarily been restricted

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to growth of small colonies for short periods of time in semisolid medium,\textsuperscript{6} which imposes severe experimental limitations. We previously reported that human leukemic myeloid cells can be carried in an actively replicating and differentiating state for periods up to several months when cultured in the presence of conditioned medium from certain human embryo cells.\textsuperscript{7,8} However, there have been difficulties obtaining adequate quantities of active conditioned medium for extensive studies.\textsuperscript{8} In one case, after initiation with conditioned medium from a human embryonic lung culture, the leukemic myeloid cells continued to grow and differentiate in the absence of any exogenous growth stimulator. The basic characteristics of this cell line (HL-60) and its ability to respond to pharmacologic inducers of differentiation have been reported elsewhere.\textsuperscript{9} Here we report more detailed information regarding the HL-60 line and the patient with acute promyelocytic leukemia from whom it was derived. Recently, Koeffler and Golde\textsuperscript{10} also established a differentiating myeloid cell line from a patient with erythroleukemia. Prior to these reports, there had been numerous successes in establishing human lymphoblast cell lines, mostly B cell lines engendered by Epstein Barr virus (EBV).\textsuperscript{11} However, there had been only two successes with the extended propagation of human myeloid cells, and in neither case was differentiation observed in suspension culture.\textsuperscript{12,13} Recently, Karpas et al.\textsuperscript{14} have also reported the establishment of human leukocyte cell lines with a few myeloid features, but these lines primarily have the properties of B-lymphoblast cells, including the presence of EBV and surface immunoglobulins.

\section*{MATERIALS AND METHODS}

\subsection*{Case Report}

S.G., a 36-yr-old white female, was referred to the M. D. Anderson Hospital and Tumor Institute with a diagnosis of acute promyelocytic leukemia. She had been well until 2 mo previously when following an upper respiratory infection, she developed easy bruisability, swollen gums, and bone pain. On the admission physical examination, she had multiple small nodules, 1 cm or less in diameter, over her scalp, cervical lymphadenopathy, and a 6 x 8 cm nodule in the left lateral vaginal wall. Numerous ecchymotic spots and petechiae were present over the arms, trunk, and legs. The liver extended 10 cm below the right costal margin, the spleen 3 cm below the left costal margin. Admission peripheral blood counts were as follows: hemoglobin 9.3 g/dl, hematocrit 27.4%, platelets 80,000/cu mm, white blood cells 35,700/cu mm with 70% myeloblasts and promyelocytes, 14% myelocytes, 2% bands, and 14% lymphocytes. The bone marrow showed 4.0% blasts, 85% promyelocytes, 2.8% myelocytes, 0.8% metamyelocytes, and 1% normoblasts. The cells were heavily granulated, and special stains showed peroxidase positivity and PAS negativity. The serum lactic dehydrogenase (LDH) activity was elevated to 650 \textmu U/ml (normal 30–110 U); other values of the SMA 12 autoanalyzer were within normal limits. Coagulation parameters, including prothrombin time, partial thromboplastin time, fibrinogen, and fibrin split products were within normal limits. Treatment was initiated with intravenous C-parvum, systemic prophylactic antibiotics, and chemotherapy with adriamycin, vincristine, prednisone, and cytosine arabinoside. There was a partial response to two initial courses of this therapy, with a reduction in the WBC to less than 2000/cu mm and a decrease in the marrow leukemic infiltration and in size of the subcutaneous nodules. However, disease progression was noted during a third course of chemotherapy. On the 64th hospital day, the patient complained of the sudden onset of abdominal pain and succumbed to intraabdominal hemorrhage and shock. The coagulation profile, which up to this time had been normal, showed evidence of a consumptive coagulopathy with marked prolongation of the prothrombin time, an increase in the partial thromboplastin time, and a reduction of the serum fibrinogen level to 36 mg/100 ml. At autopsy, there was evidence of leukemic infiltrates in the mediastinum, lymph nodes, uterus, vagina, and ovaries, and there was a massive hemoperitoneum, splenomegaly, and multiple petechial hemorrhages. The source of bleeding was the spleen, which had a subcapsular infarct and capsular tears.
Establishment of the Promyelocytic HL-60 Cell Line in Suspension Culture

Prior to treatment of the patient, peripheral blood leukocytes were obtained by leukopheresis, using a blood cell separator. The cells were shipped on wet ice to the NCI and on arrival were 98% viable, as determined by trypan blue dye exclusion. As previously detailed, the leukocytes were cultured in the presence and absence of conditioned medium from human embryonic lung cultures. After a 3-wk lag period, active growth of cells in suspension was observed in flasks supplemented with conditioned medium from one human embryonic lung culture. Subsequently, it was found that continued growth of the leukocytes did not require conditioned medium supplements.

Cytogenetic Studies

Cytogenetic examinations were done on the following samples: (1) fresh bone marrow prior to chemotherapy; (2) cultured HL-60 cells from passages 6, 8, 15, 28, 35, 45, and 55, grown on RPMI 1640 medium with 10% heat-inactivated fetal calf serum, or from passages 21 and 41, grown for the last 6 passages on Ham's F-10 medium with 20% fetal calf serum; and (3) after passage through nude mice. For analyses, the cells were incubated in the presence of colcemid, 0.02 μg/ml, for 1 hr at 37°C, then treated with 0.075 M KCl for 20-30 min at room temperature, and fixed in three changes of methanol-acetic acid (3:1). Chromosome preparations were made using air-drying or flame-drying techniques. Chromosomes were banded by the trypsin-Giemsa method; slides were treated with 0.01%-0.03% trypsin in phosphate-buffered saline (Ca and Mg free) for 3-6 min at room temperature and stained with 4% Giemsa stain in Sorenson's buffer at pH 6.8.

Histological Staining Procedures

Published procedures were followed for Naphthol ASD chloroacetate and α-methyl acetate esterases, Sudan black B, myeloperoxidase, alkaline and acid phosphatases, and the periodic acid Schiff (PAS) reaction.

Electron Microscopic Preparation

The initial bone marrow and subsequent cultured HL-60 cells were mixed in 2.5% gluteraldehyde, pH 7.2, in Sorenson's phosphate buffer, 300 mosmol, for 1 hr at 25°C. Diaminobenzidine tetrahydrochloride was utilized to demarcate endogenous peroxidase activity. The tissue was post-fixed in 1% osmium tetroxide, pH 7.2, for 1 hr. Following dehydration in graded acetones, the cell pellets were embedded in Epon 812. Sections were cut on an LKB ultramicrotome and examined with a Siemens Elmiskop 102 at 80 kV.

Cell Surface Studies

Erythrocyte rosette tests were performed by slight modifications of established techniques, as detailed elsewhere. The E-rosette assay utilized sheep erythrocytes. The EA and EAC-rosette tests were performed with both sheep and bovine erythrocytes. Tests for surface immunoglobulins IgG, IgM, and IgA utilized immunobeads (Bio-Rad Laboratories), as detailed elsewhere. Direct testing of HLA antigens was done at Duke University by the standard two-stage microcytotoxicity method of Amos et al. and by the method of Mittal et al. with a panel of sera used to define HLA-A, B, and C locus antigens at past HLA Histocompatibibility Testing Workshops. Direct testing for human Ia-like antigens was done by complement dependent microcytotoxicity using rabbit and monkey antisera known to specifically detect Ia antigens by cytotoxicity and by radioimmunoprecipitation and polyacrylamide gel electrophoresis analysis.

Chemotaxis Studies

Cells were added to the upper well of a blind well chamber (model F013 WLB 00101 Neuro Probes, Inc., Bethesda, Md.) at a concentration of 2.5 x 10^6 ml of Gey's balanced salt solution containing 2% bovine serum albumin and 0.01 M Herpes. The dipeptide N-formylmethionyl-leucine (Sigma Corp., St. Louis, Mo.), a potent chemoattractant, was added to the lower well at a concentration of 10^-7 M. A 13-mm chemotactic membrane (Nucleopore Corp., Pleasanton, Ca.), containing an average pore diameter of 5.0 μ, separated the two compartments. The chamber was then incubated at 37°C in a humidified 5% CO₂ atmosphere for 1 hr. The filters were then removed, stained with Wright-Giemsa,
and examined microscopically for cells that had migrated to the underside. Results were expressed as the number of cells counted in four successive low power fields. All experiments were performed in triplicate and the results averaged.

**Phagocytosis Studies**

Cells were suspended at 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% FCS. *Candida albicans*, grown overnight in Sabouraud's medium (Difco, Detroit, Mich.), were washed twice with saline and added to the above HL-60 cell suspension at 4 x 10⁵ organisms/ml. The suspension was then incubated for 30 min at 37°C, and the percent of cells that had phagocytosed yeast particles was determined by light microscopy on Wright-Giemsa stained slides.

**Lysozyme Studies**

Cells, at a concentration of 10⁷/ml, were lysed by incubating with 1% NP-40 in PBS, pH 6.2, for 10 min at room temperature. The cell lysate was then spun at 3900 rpm at 4°C for 10 min, and the lysozyme assay was performed on 1 ml of the supernate with egg white lysozyme (Sigma) used as the standard. In addition, lysozyme assays were performed on 1-ml aliquot samples of the cell-free supernate of actively proliferating cells harvested at a time when cells were at a concentration of 1.5 x 10⁶/ml.

**Growth and Cloning in Semisolid Medium**

To assay for colony formation, various concentrations of HL-60 cells were suspended in 1.2% methylcellulose (Dow Chemical Corp.) or 0.3% agar (Difco) in McCoy's 5A medium containing 10% fetal calf serum (FCS) and 5% horse serum. One milliliter of this mixture was plated in 35-mm culture dishes (Falcon) and incubated in a humidified 5% CO₂ chamber at 37°C. Colony counts (≥30 cells) were performed after 7, 14, and 21 days of incubation. For cloning studies, the HL-60 cells were suspended at a concentration of 10⁷ cells/ml in 1.2% methylcellulose in McCoy's 5A medium containing 10% FCS and cultured as above. After 5–6 days, visible colonies containing 20–30 cells were individually transferred to wells of a 16-mm multiwell dish (Linbro Chemical Co., New Haven, Conn.) containing 0.5 ml of RPMI 1640 medium supplemented with 10% FCS. The cells were then recloned using the same procedure. As cell growth continued, cells were transferred to larger wells and ultimately to Falcon T/60 flasks.

**Growth in Nude Mice**

Athymic nude mice (BALB/c nu/nu) were obtained from ARS/Sprague Dawley (Madison, Wisc.). HL-60 cells at different passage numbers were injected subcutaneously into the mice. Tumors that developed were dissected, stained with Wright-Giemsa and myeloperoxidase, and analyzed for karyotype. A separate cell line (designated HL-60 Nu) was established by mincing a tumor obtained from injection of HL-60 at passage 45, incubating the tumor mince with 0.25% trypsin for 30 min at 37°C, resuspending the resultant cell suspension in RPMI 1640 plus 15% heat-inactivated FCS, and continuing the leukocyte suspension cultures under standard conditions.

**Test for Herpesviruses**

The Epstein-Barr virus nuclear antigen (EBNA) test was performed by a modification of the Reedman-Klein technique. Herpes simplex type I and cytomegalovirus (Town strain) probes were prepared by labeling the DNA with tritium in vitro by “nick translation,” using all four ³H-labeled nucleoside triphosphates to a specific activity of 1.5 x 10⁶ cpm/μg as detailed by Maniatis et al. These probes were hybridized to excess cellular DNA up to a final Ecot of 8000, and the hybrids were processed by hydroxyapatite chromatography.

**Tests for Retroviruses**

To test for possible extracellular virus, up to 100-ml samples of HL-60 cell culture fluid were concentrated and assayed for RNA-directed DNA polymerase (reverse transcriptase) activity, as previously reported. In addition, cultures were tested for release of RNA-containing particles by
pulse-labeling up to 50-ml culture samples with \(^{3}H\)-uridine, concentrating the labeled particles by ultracentrifugation, and centrifuging them to equilibrium on a 20%-60% sucrose gradient.\(^{33}\)

Attempts to induce virus production followed published procedures, including treatments with 5'-iododeoxyuridine,\(^{34}\) dimethylsulfoxide,\(^{26}\) and arginine-deprived medium.\(^{35}\) 5-Iododeoxyuridine was tested with and without the addition of hydrocortisone, \(10^{-7}M\).

The possible presence of subviral components within fresh and cultured HL-60 leukocytes was examined by a variety of tests. From 2 to 25 g of viable, nonfrozen cells were used per test. Methods to search for intracellular reverse transcriptase included the preparation and purification of the cytoplasmic microsomal-membrane fraction\(^{32}\) and detergent extraction of whole cytoplasmic extracts followed by sequential column chromatography.\(^{37}\) Tests for cellular antigens related to p30 proteins from known retroviruses were performed on whole cell extracts using a competition radioimmunoassay capable of detecting \(1\) ng of competing virus-related antigen.\(^{33}\) Attempts were made to demonstrate high molecular weight RNA associated with intracytoplasmic virus-like particles by direct pulse-labeling with \(^{3}H\)-adenosine.\(^{36}\) Also, cytoplasmic RNA was tested for possible relatedness to RNA from known retroviruses by two methods: (1) direct hybridization assays with \(^{3}H\)-cDNA viral probes\(^{33}\) and (2) indirect competition assays in which cytoplasmic RNA is tested for possible relatedness to viral nucleic acids by its ability to interfere with the hybridization of homologous viral RNA to proviral DNA.\(^{33}\) Finally, the HL-60 cellular DNA was tested for viral-related proviral sequences by direct hybridization with tritium-labeled type-C viral cDNAs\(^{35}\) and with \(^{125}I\)-labeled whole viral RNAs.\(^{33}\)

RESULTS

Growth Kinetics in Suspension Culture

As previously reported,\(^{10}\) the HL-60 blood leukocytes initially grew only in the presence of culture fluid supplemented with conditioned medium harvested from cultured human embryonic lung cells. However, after a few weeks, the cultured leukocytes grew as well in the absence as in the presence of conditioned medium supplements. At passage 9, the saturation density of the cultured cells was \(2.3-3.0 \times 10^6\) cells/ml, and the cell-doubling time was \(55-60\) hr.\(^{10}\) With continued passage, the HL-60 cells have continued to adapt to in vitro culture conditions. As shown in Fig. 1, the growth of the cells has remained absolutely dependent on the presence of FCS through 68 passages. Maximum growth stimulation was achieved with 10%-20% FCS. However, at a given serum concentration, the saturation
Fig. 2. Light microscopic appearance of fresh and cultured HL-60 leukocytes. (A) Cells from passage 38 growing in single-cell suspension as viewed in an inverted microscope (Leitz Diavert, Germany; ×160). (B) Smear of fresh, uncultured bone marrow from patient S.G. (HL-60) prior to chemotherapy, illustrating predominant leukemic promyelocytes. (Wright-Giemsa stain). (C) Cytocentrifuge preparation of cultured HL-60 leukocytes, illustrating predominant promyelocytes. (Wright-Giemsa stain.) (D) Strongly positive myeloperoxidase stain of cultured cells. (E) Positive ASO chloroacetate esterase stain of cultured cells. (F) Positive Sudan black B stain of cultured cells. (G) Wright-Giemsa stain of cultured HL-60 cells after treatment with DMSO for 6 days, showing differentiation of cells to myelocytes and predominant metamyelocytes. (H) Hematoxylin and eosin stained section of nude mouse tumor produced by subcutaneous inoculation of cultured HL-60 (×160). Nuclei are predominantly undifferentiated but eosinophilic cytoplasmic granules are present, which stain heavily with myeloperoxidase. Photomicrographs of cultured cells were taken with a LEITZ Ortholux II, Germany, at ×1000.
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density has increased and the doubling time has decreased as a function of passage level. At passage 17, the saturation density was $5.5 \times 10^6$ cells/ml, with a cell-doubling time of 36 hr during the logarithmic phase of growth; at passage 68, these values were, respectively, $7.6 \times 10^6$ cells/ml and 30 hr. Continued passage was also accompanied by some shift in the cytologic and karyotypic properties of the cultured cells (see below).

**Cytologic Characteristics of Fresh and Cultured Cells**

The cells grow in single-cell suspension without any tendency to clump or to adhere to plastic or glass (Fig. 2A). The cells are generally round or ovoid, and occasional cells have blunt pseudopods. The modal cell diameter is 13μ; however, there is considerable variation in cell size, ranging from 9μ to 25μ in diameter. The larger cells are frequently binucleate.

Figures 2B and 2C illustrate typical leukemic myeloid cells observed in Wright-Giemsa-stained preparations from the patient's fresh blood specimen and from the cultured HL-60 cells. In both instances, the predominant cell is an abnormal promyelocyte. This cell typically contains a large round nucleus with regular, distinct margins, fine chromatin, and 2–4 nucleoli. The cytoplasm is deeply basophilic and contains multiple prominent azurophilic granules. Despite an intensive search, Auer rods were not observed in either the fresh or cultured specimens. These cells stained heavily with stains specific for myeloid cells, including myeloperoxidase (Fig. 2D), ASD chloroacetate esterase (Fig. 2E), and Sudan black B (Fig. 2F). However, no positivity was noted with alkaline phosphatase, a stain characteristically positive in normal neutrophilic granulocytes. The promyelocytes showed slight diffuse staining of the cytoplasm with the periodic acid-Schiff reagent, and occasional cells stained positively for acid phosphatase. The more mature granulocytes stained strongly positive with PAS and were negative for acid phosphatase. Stains for α-naphthol AS-D acetate (nonspecific) esterase were uniformly negative. This staining pattern is characteristic of myeloid cells.37

As summarized in Table 1, 5%–10% of the myeloid cells spontaneously differentiate into more mature granulocytes, including myelocytes, metamyelocytes, and banded and segmented neutrophils. This process is accompanied by diminished cell size, decreased nuclear–cytoplasmic ratio, increased nuclear pycnosis and segmentation, decreased cytoplasmic basophilia, and replacement of the coarse azurophilic

| Table 1. Differentiation of HL-60 Cells as a Function of Culture Passage |
|---------------------|---------------------|---------------------|---------------------|
| Percent of Myeloid Cells | Percent of Myeloid Cells | Percent of Myeloid Cells | Percent of Myeloid Cells |
| Passage Number | Myeloblasts to Promyelocytes | Myelocytes | Metamyelocytes to Segmented Neutrophils | Percent Inducible by 1.25% DMSO |
| Fresh* | 81 | 16 | 2 | Not tested |
| 0† | 95 | 4 | 1 | Not tested |
| 9 | 89 | 7 | 4 | 70–90 |
| 18 | 92 | 4 | 4 | 70–90 |
| 35 | 97 | 1 | 2 | 70–90 |
| 63 | 96 | 2 | 2 | 70–90 |

*From differential WBC on fresh blood specimen at M.D. Anderson Hospital.
†From differential WBC on leukopheresis specimen prior to culture at NCI.
Fig. 3. Electronmicroscopic appearance of fresh and cultured HL-60 cells. (A) This promyelocytic HL-60 cell displays peroxidase-positive cytoplasmic granulation. The granule morphology and enzymatic content is, in general, more variable than that observed in similarly staged cells in vivo. The nuclear development is asynchronous, and the structure bears a nucleolus and a nuclear bleb (arrow) on its surface. This latter morphological abnormality is associated in high frequency with aneuploid acute leukemias (×9600). (B) Asynchronous development in the nuclear/cytoplasmic areas is apparent in this promyelocyte from the initial bone marrow specimen. The cytoplasm demonstrates evidence of both peroxidase-positive azurophilic and peroxidase-negative specific granules. Bundles of fibrils, a feature associated with leukemic cells, are noted (arrow) in the juxtanuclear area of the cytoplasm. Some dilated cisternae of the endoplasmic reticulum are also evident (×6400). (C) Low magnification micrograph illustrating the overall morphology observed in
the initial bone marrow specimen of this patient. The developing nuclei are primarily blastic in appearance, whereas the cytoplasmic areas are promyelocytic (x 5000). (D) This “band” or “stab” HL-60 cell displays evidence of nuclear and cytoplasmic differentiation. Although the granules are malformed, both peroxidase-positive azurophilic (large arrow) and peroxidase-negative specific (small arrow) forms are present (x 20,000). (E) Following dimethyl formamide (50 mM) incubation for 6 days, the nuclei of HL-60 display dramatic nuclear segmentation and increased definition of heterochromatic areas. Cytoplasmic granules are of both the azurophilic (large arrow) and specific (small arrow) type (x 39,000).
granules by smaller specific granules. As reported in detail elsewhere, the proportion of differentiating promyelocytes could be greatly enhanced by adding certain compounds known to induce differentiation of mouse (Friend) erythroleukemia cells, e.g., DMSO, to the cultured HL-60 cells (Fig. 2G). Differentiation was limited to the neutrophilic series in suspension culture, since no eosinophilic or basophilic granules were detected in HL-60 cells after either spontaneous or induced differentiation. In addition to the neutrophilic granulocytes, a minor population of cells resembled monocytes with folded nuclei, pale-staining blue cytoplasm, and less distinct and regular cytoplasmic margins. No cells resembling immature erythroid cells were observed, and benzidine stains for hemoglobin were negative. With continued passaging of the HL-60 cells, the percentage of myeloid cells showing spontaneous terminal differentiation has diminished slightly. This did not substantially affect the induction of differentiation by DMSO (Table 1).

Electron Microscopy of Fresh and Cultured Cells

Ultrastructurally, the HL-60 line consists of a heterogeneous population of cells with the predominant morphology varying from myeloblastic to promyelocytic. Nuclear/cytoplasmic asynchrony is present, the cytoplasm representing the more mature element. The nuclear chromatin is primarily euchromatic, and well-developed nucleoli are situated adjacent to the nuclear membrane. Cytoplasmic granulation in the cultured cells is mostly of the azurophilic type. The size, shape, and density of these peroxidase-positive granules (Fig. 3A) is more diverse than those observed in the patient's original bone marrow (Fig. 3B). In this initial specimen, 85% of the cells are characterized by blast-like nuclei and progranulocytic cytoplasm (Fig. 3C). In addition to the nuclear/cytoplasmic asynchrony, nuclear blebs and bundles of cytoplasmic fibrils are present in leukemic cells both in vivo and in vitro. Under routine culture conditions, a small percentage of these cells demonstrate "stab" or "band-like" nuclei and evidence of both azurophilic and specific granulation of the cytoplasm (Fig. 3D). The addition of the differentiating agent, dimethyl formamide, to the HL-60 culture medium induces further segmentation of the nuclei and increased specific granule formation in the cytoplasm (Fig. 3E).

Cytogenetic Analysis of Fresh Bone Marrow

A total of 40 metaphases were analyzed from the initial bone marrow sample. The analysis showed 31 cells with 44 chromosomes (approximately 75%), 8 with 45 (20%) and 1 with 46 (5%) (Table 2). Examination of these clones, with the help of Giemsa-banding techniques, indicated that the common alterations in all of the

<table>
<thead>
<tr>
<th>Passage Number</th>
<th>Metaphases Counted</th>
<th>Chromosome Number</th>
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<tbody>
<tr>
<td>0 (Fresh)</td>
<td>40</td>
<td>43 44 45 46 47 48</td>
</tr>
<tr>
<td>6 and 8</td>
<td>145</td>
<td>6 87 18 26 6 2</td>
</tr>
<tr>
<td>15 and 28</td>
<td>105</td>
<td>2 1 47 51 4</td>
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<tr>
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<td>50</td>
<td>3 7 29 8 3</td>
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<tr>
<td>45</td>
<td>90</td>
<td>2 5 64 8 9 2</td>
</tr>
<tr>
<td>55</td>
<td>71</td>
<td>2 4 47 7 8 3</td>
</tr>
</tbody>
</table>
cells studied were as follows: −5, −8, and + a submetacentric marker chromosome resembling an E-group chromosome (M3) (Fig. 4A). Additional changes in one metaphase included the presence of a marker acrocentric chromosome resembling a D-group chromosome (M2) and an extra chromosome 18. The pseudodiploid karyotype shown in Fig. 4B illustrates these additional changes. Other marker

![Karyotype analysis of fresh, uncultured HL-60 bone marrow specimen. (A) Hypodiploid karyotype with 44 chromosomes, representing the main clone (75%) found in the initial bone marrow sample of the patient (44, XX, −5, −8, −17, + M3). (B) Pseudodiploid karyotype found in the initial bone marrow sample of the patient (5%) in the in vitro leukemic cell lines, passage 28 (60%) (44, XX, −5, −8, −17, + 18, + M2, + M3). The two markers most likely represent a D-group chromosome (M2) and a 17p+(M3).]
chromosomes, double minute chromosomes, and structural alterations involving other C-group chromosomes (numbers 7, 9, 10) were also occasionally observed in a few hypodiploid metaphases. These variant karyotypes differed from cell to cell and were difficult to categorize even with the use of Giemsa-banding techniques.

Cytogenetic Analysis of Cultured HL-60 Cells

During the early passages in vitro, the modal chromosome number was 44, as in the fresh leukemic bone marrow (Table 2). However, with continued passaging, the modal chromosome number shifted to a mixed population with 45 or 46 chromosomes. Using the Giemsa-banding technique, considerable variation was noted with respect to individual chromosome composition. However, as in the fresh marrow, the following karyotypic alterations were commonly found: \(-5, -8, +\) an abnormal E-group chromosome, \(+\) 18, and \(+\) an acrocentric D-group marker. Although inconsistent, other alterations included missing C-group chromosomes 9 and 10, loss of chromosome 16, loss of chromosome X, the presence of a variable number of double minute chromosomes, and translocation of additional chromatin material to chromosome 7.

Figure 5 illustrates a frequently observed karyotype with 45 chromosomes that emerged after passage 35. It includes 7 recognizable abnormalities as follows: (1) \(-5\); (2) \(-8\); (3) \(-16\); (4) \(-X\); (5) \(+\) an A-group marker (M1); (6) \(+\) an
acrocentric D-group marker (M2); and (7) + a submetacentric E-group marker (M3). All alterations except no. 5 were also noted commonly in the earlier passages. The M1 marker was only rarely observed in metaphases from the fresh bone marrow. The M3 marker appears to correspond to the abnormal E-group chromosome, but its identity remains to be determined with certainty.

**Cell Surface Markers**

The results of surface marker tests, which have been generally used to characterize human leukocyte cell lines, are summarized in Table 3. The E-rosette test, characteristically positive with T lymphocytes, and tests for surface immunoglobulin, characteristically positive with B lymphocytes, were uniformly negative with the HL-60 cells. The EA test for Fc receptors was negative using sheep erythrocytes, but was quite positive using bovine erythrocytes, with over 50% of the cells forming rosettes. Conversely, in the EAC test, no rosetting was observed using bovine erythrocytes, but 2%-9% of the cells formed EAC rosettes using the sheep erythrocyte assay. The percentage of sheep cell EAC-rosette-forming HL-60 cells increased up to sixfold after induction of differentiation by DMSO. Control cultured T lymphocytes (Molt-4) and undifferentiated myeloid cells (K-562) usually formed no EAC rosettes using sheep erythrocytes.

Microcytotoxicity testing of HL-60 cells with high-titered rabbit and monkey antisera to human Ia-like antigens was negative. Moreover, no Ia-like antigens could be immunoprecipitated from 125I-labeled, deoxycholate-solubilized HL-60 membranes with these antisera. More detailed studies on the nature of other membrane antigens of HL-60 cells and the characterization of rabbit and chimpanzee antisera to the cell line will be published separately (Metzgar et al., in preparation).

HLA typing revealed that HLA-Bw17 was a major marker at all passage levels. HLA-A1 (predominant) and HLA-A2 were also detectable but varied somewhat quantitatively at different passage levels. In addition, there were reactions with some individual typing sera defining other minor HLA-A, B and C antigens.

**Cellular Enzymes**

In addition to enzyme assays by histochemical staining procedures, extracts of HL-60 cells were tested for lysozyme (muramidase), terminal deoxynucleotidyl transferase, and reverse transcriptase.

<table>
<thead>
<tr>
<th>Test</th>
<th>HL-60 Cell Results</th>
<th>Reported Leukocyte-Receptor Association</th>
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<tbody>
<tr>
<td>E rosette</td>
<td>-</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>EA rosette</td>
<td>+</td>
<td>Fc receptor lymphocytes and differentiated granulocytes</td>
</tr>
<tr>
<td>EAC rosette</td>
<td>± *</td>
<td>Complement (C3) receptor: B lymphocytes, differentiated granulocytes</td>
</tr>
<tr>
<td>Surface immunoglobulins (IgG, IgM, IgA)</td>
<td>-</td>
<td>B lymphocytes</td>
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<tr>
<td>Ia-like antigen</td>
<td>-</td>
<td>B lymphocytes, immature granulocytes</td>
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</tbody>
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*Some 2%-9% of HL-60 cells formed EAC rosettes with sheep cells versus 0%-2% of control T lymphocytes (Molt-4) or undifferentiated myeloid cells (K562).
Lysozyme, an enzyme marker for leukocytes of the neutrophilic-monocytic cell lineage, was detected at a concentration of 4.7 μg/10⁷ HL-60 cells. It was also detected at a concentration of 2.3 μg/ml in the cell culture fluid after growth of HL-60 cells to a density of 1.5 × 10⁶ cells/ml. No intra- or extracellular lysozyme was found in a myeloid blast cell line (K562) or in a B-lymphoblast cell line (NC37). The values obtained with the HL-60 cultured cells are similar to those reported for a differentiating mouse myelogenous leukemic cell line and for fresh and cultured myelogenous leukemia cells; they are substantially less than values reported for fresh or cultured leukemic leukocytes with prominent monocytic elements.

Terminal deoxynucleotidyl transferase is an enzyme that may be specific for immature T lymphocytes but which has been detected in some cases of human myelogenous leukemia lacking a prominent lymphocyte cell population. This enzyme was not detectable in extracts of fresh or cultured HL-60 cells, i.e., incorporation of ³H-dGMP was less than 0.1 n mole/hr/10⁹ cells using a primer of oligo (dA),₂.₅

Reverse transcriptase, an enzyme specific for RNA retroviruses, has been partially purified from the blood leukocytes of a few patients with acute myelogenous leukemia or, in one case, from the spleen of a patient with myelofibrosis prior to conversion to acute myelogenous leukemia. To date, this enzyme has not been identified in any human culture cell line. In the present studies, up to 10 g of fresh and cultured HL-60 cells were extracted and analyzed for reverse transcriptase-like activity. A cytoplasmic DNA polymerase activity was identified that transcribed a synthetic RNA template better than a synthetic DNA template, i.e., incorporation of ³H-TMP was 152 pmole/hr/10⁹ cells with oligo (dT)₁₂₋₁₈ poly(A) and was 43 pmole/hr/10⁹ cells with oligo (dT)₁₂₋₁₈ poly(dA). However, this polymerase failed to transcribe more specific primer templates for RNA-directed DNA polymerase, oligo(dG)₁₂₋₁₈ poly(C) or oligo(dG)₁₂₋₁₈ poly(MeC), and it was not at all inhibited by antisera that inhibit (>90%) reverse transcriptase from the woolly monkey sarcoma-gibbon ape leukemia virus group or from the baboon endogenous virus group. This pattern of results is most consistent with human cellular DNA polymerase γ activity rather than reverse transcriptase.

Cell Functional Studies

Cultured HL-60 cells were tested for their ability to phagocytize yeast particles and to respond to a chemotactic stimulus, as described in Materials and Methods. On repeated testing, 5%-10% of the cultured cells were capable of ingesting one or more particles of Candida albicans. This corresponds to the percentage of terminally differentiated granulocytes in the culture (Table 1).

As presented in Table 4, the ability of the cultured HL-60 cells to migrate through a 5μm pore size membrane was stimulated about fourfold by the powerful chemoattractant dipeptide N-formylmethionyl-leucine. In the absence of chemoattractant, the number of migrating HL-60 cells was the same as cultured leukocytes that fail to respond to chemotactic stimuli. This corresponds to the anticipated properties of HL-60 cells as partially differentiated neutrophils vis-à-vis K-562 cells (undifferentiated myeloid cells), NC37 cells (B lymphoblasts), and Molt-4 cell (T lymphoblasts).
Table 4. Chemotaxis Assays of Cultured Leukocytes

<table>
<thead>
<tr>
<th>Cell Culture</th>
<th>Migrating Cells/4LPF*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Chemoattractant</td>
</tr>
<tr>
<td>HL-60</td>
<td>3 ± 2†</td>
</tr>
<tr>
<td>K-562</td>
<td>0</td>
</tr>
<tr>
<td>NC-37</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Molt-4</td>
<td>0</td>
</tr>
</tbody>
</table>

*4LPF, 4 low-power microscope fields (160x).
†Numbers equal the average number of migrating cells ± the observed range from triplicate experiments.

Both the phagocytic and chemotactic functions of the HL-60 cells were greatly enhanced by induction of further differentiation with DMSO. The detailed properties of the induced HL-60 in comparison to normal mature neutrophils will be presented in another report (S. Collins et al. in preparation).

Growth and Cloning in Semisolid Medium

The HL-60 cells formed colonies in both methylcellulose and agar. At culture passage 16, the plating efficiency was 0.5% ± 0.3%, while at passage 85, the plating efficiency was 8.0% ± 3.2%. When the HL-60 cells were stimulated with various sources of colony-stimulating activity (CSA), the plating efficiency was increased 5–30-fold, as will be presented in detail elsewhere (Ruscetti et al., in preparation). In addition, over 150 clones of HL-60 cells, presumably representing single-cell clones, have been picked and propagated from methylcellulose. Although these have not as yet been analyzed in detail, only minor variations in differential cell counts of cultures derived from the clones have been observed in comparison to the uncloned culture.

Tumor Formation in Nude Mice

The number of mice developing tumors after injection with different passages of HL-60 cells is shown in Table 5. All tumors developed at the site of injection and in no instance did postmortem examination show any evidence of metastases to a distant site. Macroscopically, all the tumors had a greenish hue. Microscopically, the tumor consisted predominantly of promyelocytes and myeloblasts (Fig. 2H), which were strongly peroxidase positive. Karyotypic markers confirmed the derivation of the tumor from HL-60 cells. The cell culture HL-60 Nu, established from one such tumor was most tumorigenic, with all mice injected developing tumors (3/3) and with as few as 10³ cells required to produce tumors (Table 5). Unfortunately, it has not been possible so far to serially transplant the nude mouse

Table 5. Formation of Tumors by HL-60 Cells in Athymic Nude Mice

<table>
<thead>
<tr>
<th>Cell History</th>
<th>Tumors/Mice Injected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>Not done</td>
</tr>
<tr>
<td>Passage 15</td>
<td>1/8</td>
</tr>
<tr>
<td>Passage 45</td>
<td>3/8</td>
</tr>
<tr>
<td>Nu†</td>
<td>3/3</td>
</tr>
</tbody>
</table>

*3–5 × 10³ cells inoculated subcutaneously.
†Nu†, suspension culture derived from an explant of a subcutaneous tumor originally formed by inoculation of passage 15 HL-60 cells.
Table 6. Tests of Cultured HL-60 Cell DNA for Herpesvirus-Related Nucleic Acid Sequences

<table>
<thead>
<tr>
<th>Source of Unlabeled DNA</th>
<th>DNA Probe Hybridized (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-1*</td>
</tr>
<tr>
<td>HL-60</td>
<td>4.0</td>
</tr>
<tr>
<td>Uninfected human cells</td>
<td>3.9</td>
</tr>
<tr>
<td>Infected human cells</td>
<td>85.0</td>
</tr>
</tbody>
</table>

*Herpes simplex virus type I (courtesy of Dr. Alvaro Puga).
†Cytomegalovirus (Town strain).

tumors for more than three generations either by direct in vivo passaging or by reestablishment of the HL-60 Nu<sub>i</sub> cells in tissue culture between inoculations. Also, no success has been encountered in attempts to develop an ascites tumor by intraperitoneal injection. The HL-60 Nu<sub>i</sub> culture has not been productively infected by a mouse retrovirus, as determined by reverse transcriptase assays or electron microscopic observation.

Tests for DNA Herpesvirus Information

The HL-60 cultured cells were repeatedly demonstrated to be negative for Epstein-Barr virus (EBV) by the nuclear antigen (EBNA) test. This assay has been reported to be 100% effective in detecting intracellular EBV DNA. Nucleic acid hybridization analyses were performed to test for two other herpesviruses that have been associated with transformation of human cells, cytomegalovirus, and herpes simplex virus. Tests for both viruses were negative at a sensitivity capable of detecting less than 0.1 virus genome copy per cell (Table 6).

Tests for RNA Retrovirus Information

Extensive tests were performed on the fresh and cultured HL-60 leukocytes in attempts to detect retrovirus information according to procedures described or referenced in Materials and Methods. No evidence for the release of extracellular virus-like particles was observed from the cultured leukocytes either spontaneously or after procedures known to induce or stimulate retrovirus production in animal cell culture systems, including treatment with DMSO, 5'-iododeoxyuridine, corticosteroid, and arginine deprivation. An intracellular cytoplasmic DNA polymerase activity was observed that banded on a sucrose equilibrium density gradient at 1.16 g/ml, the characteristic density of type-C retroviruses. However, as described in the section on cellular enzymes, this polymerase activity had properties of DNA γ, not reverse transcriptase. No other evidence of intracytoplasmic virus-like particles was observed by electron microscopy or by 3H-adenosine-labeling of cellular RNA. Finally, in tests for possible relatedness to specific retrovirus molecules, including p30-related protein and nucleic acids, no positive results were observed in systems designed to detect homology to probes from woolly monkey (simian) sarcoma virus, baboon endogenous virus, murine leukemia virus, and feline leukemia virus.

DISCUSSION

This article documents the derivation of the myeloid cell line HL-60 from the leukemic blood cell population of a 36-yr-old female with acute promyelocytic
leukemia (APL) by demonstrating virtual cytologic identity of the fresh and cultured leukocytes and by defining distinctive chromosomal markers in the fresh and cultured cells.

In contrast to cultures of peripheral blood leukocytes from other cases of myelogenous leukemia that we have studied, the HL-60 cells were able to sustain replication in suspension culture in the absence of a continuous added source of conditioned medium (i.e., after culture initiation). This does not appear to be due to the secretion of an endogenous growth stimulator, since conditioned medium from the cultured HL-60 cells did not stimulate the growth of leukemic leukocytes or normal bone marrow in liquid suspension or semisolid medium. Similar autonomous growth was noted with all clones of HL-60 cells derived from colonies picked from methycellulose. Further, the HL-60 cells formed colonies in either methycellulose or agar in the absence of any added source of supplemental growth stimulator. Nevertheless, like fresh myeloid progenitors from some cases of acute myelogenous leukemia, the cultured HL-60 cells can respond to an exogenous source of colony-stimulating activity (CSA), since the plating efficiency and colony size are greatly enhanced by CSA (Ruscetti et al., in preparation). Finally, the HL-60 cells formed localized subcutaneous tumors in athymic nude mice as early as the 15th in vitro passage (when first tested; Table 5). These properties are characteristic of progressed malignant leukocytes and establish the oncogenicity of the HL-60 cells in vivo.

The cultured HL-60 cells have certain cytologic properties that have been associated with leukemic promyelocytes. These include nuclear/cytoplasmic asynchrony, nuclear bleb formation, and heavy azurophilic granulation (Figs. 2 and 3). However, both the fresh and cultured HL-60 cells lack other features that have been described for promyelocytes from some cases of acute promyelocytic leukemia, including morphologically abnormal granules with Auer rod and extensive intracytoplasmic fibril formation and deformation and dilatation of the cisternae of the endoplasmic reticulum. The latter cytologic features have particularly been associated with cases of APL in which hemorrhage due to disseminated intravascular coagulation (DIC) has been prominent. This, in turn, has been related to the presence of high amounts of tissue thromboplastin in leukemic promyelocytes. Although the present patient ultimately died from an intraabdominal hemorrhage associated with DIC, her coagulation parameters were essentially normal until terminally. Our preliminary studies indicate that some thromboplastin activity is present in the HL-60 leukocytes; however, further studies are required to quantitatively relate this thromboplastin activity to that present in leukocytes from other patients with APL and DIC.

In addition to specific cytologic alterations, APL has been associated with a common karyotypic abnormality involving a reciprocal translocation between chromosomes 15 and 17. In both the fresh bone marrow and cultured HL-60 cells, evidence was obtained in most metaphases for the presence of a submetacentric E-group-like chromosome. Although the identity of this chromosome could not be certified, it could be a chromosome 17 with extra chromosomal material on its short arm. This chromatin did not appear to arise by a translocation from chromosome 15. Possibly, it could have originated from one of the heavy bands of the short or long arms of the missing chromosome 5. The loss of chromosome 8 in the fresh and
cultured HL-60 cells is also interesting, since abnormalities involving this chromosome, particularly trisomy, have frequently been associated with human acute leukemia.\textsuperscript{62,63}

DNA herpesviruses have been associated with the transformation of human leukocytes and immortalization of lymphoblast cells in vitro.\textsuperscript{13,64} RNA retroviral information has also been associated with some fresh and cultured human leukemic cells, especially those of myeloid cell type,\textsuperscript{9,65-68} and recent experiments in our laboratory suggest that some oncogenic primate retroviruses may have the potential of transforming human leukocytes.\textsuperscript{69} However, no evidence of information related to known herpesviruses or retroviruses has been found in fresh or cultured HL-60 cells.

A remarkable feature of the cultured HL-60 cells is their capacity to terminally differentiate despite their aggressive malignant growth potential in vitro and in vivo. This suggests a dissociation of genetic control elements for replication and commitment to differentiation. Such a dissociation has also been suggested for murine (Friend) erythroleukemia cells.\textsuperscript{4} In mouse myelogenous leukemia cultured cells, the capacity for myeloid differentiation has been related to alterations in numbers of specific chromosomes.\textsuperscript{70} So far, the differentiated phenotype of the HL-60 cells has remained quite stable (see Table 1) despite progressive adaptation of the cells to continued culture and despite clonal selection either spontaneously in suspension culture or by deliberate cloning in methylcellulose. This suggests that the observed cytogenetic abnormalities are not directly involved with the differentiative capacity of the cells. Certainly, however, phenotypic variations could be present in different clones that have not been detected by the limited methods so far utilized to characterize the state of differentiation of the HL-60 cells. In this regard, the differentiative capacity of HL-60 cells appears to differ from normal neutrophils, since completely differentiated segmented neutrophils are rarely seen even after chemical induction\textsuperscript{11} and since at least one enzyme characteristic of normal granulocytes, alkaline phosphatase, was not histochemically detectable in HL-60 cells. Thus, this human cell line seems of unusual interest for studying intrinsic and extrinsic elements that may affect or perturb the processes of myeloid growth and differentiation.

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Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia

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