Characterization of Malignant Peripheral Blood Cells of Juvenile Chronic Myelogenous Leukemia

Zeev Estrov, Barry Zimmerman, Tom Grunberger, Judy Chao, Ikuko E. Teshima, Helen S. Chan, and Melvin H. Freedman

Divisions of Hematology/Oncology and Allergy, Departments of Genetics and Pathology, Research Institute, The Hospital for Sick Children, Toronto, Canada M5G 1X8

ABSTRACT

Characterization studies were performed on the malignant peripheral blood cells from three patients with juvenile chronic myelogenous leukemia (JCML) by allowing the cells to increase numerically in liquid cultures. JCML cells proliferated rapidly and excessively in the absence of an added humoral growth factor, whereas control peripheral blood cells declined in number when cultured under identical conditions. Clonality of JCML cells was proven by cyogenetic analysis of the proliferating population. JCML cells were exclusively of monocytic lineage as determined by morphology, staining characteristics, and monoclonal antibody identification of cell-specific surface antigens, but cytochemical and functional studies identified aberrant properties indicating defective differentiation. Striking differences from control cells in ultrastructure and topography were also observed by transmission and scanning electron microscopy. These data provide new information on the cellular origin of JCML and form the basis for further study of leukemic cell biology in this disease.

INTRODUCTION

JCML is a severe progressive hematopoietic malignant disorder of early childhood characterized by an acute course with a median survival of less than 10 mo (1, 2). When cultured in a semisolid system marrow or peripheral blood mononuclear cells from patients with JCML yielded excessive numbers of monocyte-macrophage colonies suggesting primary involvement of the monocytic lineage in the pathogenesis of this disease (3). Because the disorder is uncommon, there has been limited opportunity for further study of the biological characteristics of the malignant lineage in JCML. Therefore, on diagnosis of 3 new cases, we transferred patients’ PB cells to liquid cultures to allow clonal expansion of the cell lineage. The increase of abnormal cells in culture allowed the detailed characterization studies reported herein.

MATERIALS AND METHODS

Subjects

The 3 children with JCML all fit the classical clinical and laboratory description of this disorder (1, 2) (Table 1). All presented with varying combinations of lymphadenopathy, skin manifestations, variable splenomegaly, pallor, and hemorrhagic signs due to thrombocytopenia.

For control studies, PB was obtained from healthy, hematologically normal volunteers. These studies were performed with informed consent and were approved by the Human Experimentation Committee of our institution.

Preparation of Cells

Heparinized PB cells from JCML patients and from normal donors were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (200 × g, 4°C) for 20 min to remove neutrophils and red cells. Adherent cells were removed by incubating post-Ficoll mononuclear cells in 10% FCS in α-medium at 37°C for 2 h. Washed nonadherent cells were incubated in α-medium containing 10% FCS at 5 × 10⁶ cells/ml in 16-mm-diameter, 24-well, plastic tissue culture plates (Costar, Cambridge, MA) at 37°C, 5% CO₂ in air, in a humidified atmosphere. After 10 days in culture, a new adherent layer was formed. The supernatants were removed, and the wells were washed with Dulbecco’s modified Eagle’s medium to remove residual nonadherent cells. The adherent cells that remained were incubated for 3 min in cold medium containing 0.1% bovine serum albumin and 0.2% sodium EDTA, and they were then removed from the dishes by gentle scraping with a rubber policeman. The cell suspensions of adherent as well as nonadherent cells were harvested separately and pooled, cytocentrifuged, and spread on glass slides for morphological analysis. All cell preparations used for morphology, surface markers, cytochemistry, cytogentic, electron microscopy, and functional studies were tested separately for viability using trypan blue dye exclusion. More than 90% of the cells in each preparation were viable.

Surface Markers

The cell surface phenotype was determined by indirect cell-surface fluorescence on the cells adherent to the plastic wells after 10 days in culture, using the following monoclonal antibodies: OKIa (Ortho Diagnostic Systems, Inc., Raritan, NJ) that detects HLA-D framework antigens; Leu-M1, Leu-M3, OKM1 (Becton-Dickinson, Mountain View, CA), and Mo2 (Coulter Immunology, Hialeah, FL), and HLA-A,B, and C (Becton-Dickinson) that detect T- and B-cells, respectively. A fluorescein-conjugated goat anti-mouse IgG (Fab)2 was used as secondary antibody. The labeling was performed at room temperature in the presence of 0.2% sodium azide. The percentage of cells with surface fluorescence was determined by counting cells stained in 3 replicate wells using an inverted phase-fluorescent microscope.

Cytochemical Enzymatic Analysis

Slides prepared from cytocentrifuged cellular pellets of adherent cells were studied following Wright stain and cytochemical staining for: myeloperoxidase; specific CAE; nonspecific esterase (ANAE) with or without fluoride inhibition (4); acid phosphatase (5); and β-glucuronidase (6). Cellular lysozyme was detected according to a previously described method (7).

Cytogenetic Analysis

Cytogenetic studies on BM cells were performed by the direct method of Hozier and Lindquist (8) and on PB cells by the standard technique of 72-h incubation in the presence of phytohemagglutinin. For the study of chromosomes of nonadherent cultured cells, Colcemid (0.1 μg/ml) was added for 1 h to the culture wells on Day 10 of incubation and then removed by washing the nonadherent cells with phosphate-buffered saline. Chromosome analysis was performed on these cytogen-
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Electron Microscopy

Both normal and JCML cultured cells were prepared for EM examination at 4, 7, and 10 days of culture for ultrastructural and topographical characteristics.

TEM. Cultured cells obtained from the adherent fraction were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 2 h at 4°C. Cells were washed 3 times with 0.1 M phosphate buffer, pH 7.4, then fixed with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, dehydrated in graded ethanol solutions, and embedded in Epon-Araldite epoxy resin. Ultrathin sections were cut on a Porter-Blum ultramicrotome, stained with lead citrate and uranyl acetate, and examined in a Philips EM 400 transmission electron microscope at 60 kV.

SEM. Cultured cells adherent to coverslips which were placed in culture plates prior to incubation were fixed with 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 2 h at 4°C. Cells were washed 3 times with 0.1 M phosphate buffer, pH 7.4, dehydrated in graded ethanol solutions, and critical point dried in a Polaron Jumbo E 3100 critical point dryer. The coverslips of cultured cells were then mounted on aluminium stubs with conductive silver paint, gold coated in a Denton Vacuum Desk-1 sputter coater, and examined under a JEOL 35 scanning electron microscope at 20 kV.

Procoagulant Activity Assay

The procoagulant activity of the control and JCML PB adherent cell layer was tested according to the assay described by Levy et al. (11). Following the removal of the supernatant, the adherent cells were covered for 3 min with Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin and 0.2% EDTA. The adherent cells were then removed from the dishes by gentle scraping with a rubber policeman, and viable cells (more than 90% by trypan blue exclusion) were killed with 1% trypsin-EDTA solution after 5 min. The remaining monolayer was then washed with a medium, and the cells were examined under the microscope.

Results

JCM and control adherent cells (10⁶ cells/ml) were subjected to 3 cycles of freeze-thaw and sonication. To 0.1 ml of the cellular homogenate at 37°C were added 0.1 ml of citrated normal human platelet-poor plasma (Helena Laboratories, Beaumont, TX) and 0.1 ml of 25 mm CaCl₂ to start the reaction. The time in s for the appearance of a fibrin gel was recorded. The clotting time with control cells was defined as 100%, and the JCML results were expressed as a percentage of the control.

Plasminogen Activator Assay

JCM and control adherent cells were studied for their ability to activate plasminogen using the method described by Cole et al. (12). The cells for study (10⁶ cells/ml) were converted to homogenates by freeze-thaw and sonication as before. Plastic, flat-bottomed, 24-mm-diameter 24-well plates (Linbro Plastics, McLean, VA) were coated with 250 μl of Tris-buffered fibrinogen and dried at 22°C for 3 days. Fibrinogen was then converted to fibrin by adding 1 ml of 10% acid-treated PCS in 0.1 M Tris-buffered saline, pH 7.4, to each well for 4 h at 37°C in a 5% CO₂ environment. After the incubation period, the following reaction mixture was added to each well: 0.1% gelatin in 0.1 M Tris-buffered saline; 10 μg of plasminogen; and an aliquot of the cellular homogenate for study (20 to 100 μl/well). The final volume was 1 ml/well. The wells containing the reaction mixture were then incubated at 37°C for 45 min. For positive assay controls, homogenate of mouse staphylococcal proteinase (Sigma Chemical Co., St. Louis, MO) and 0.25 units of human plasmin (Sigma) were used. Results with control adherent cells were expressed as the mean of triplicate samples of the maximum 125I released per 10⁶ cells. The control results were defined as 100%, and the JCML results were expressed as a percentage of the control.

Latex Ingestion Assay

A dilution of 1:1000 latex beads (average diameter, 0.3 μm; Sigma) in α-medium was added to adherent cells in 24-well plates (2 x 10⁶ cells/ml) in various amounts (10 μl, 50 μl, 100 μl, 200 μl). Following 2 h of incubation (37°C, 5% CO₂), beads were removed, the wells were washed with α-medium, and the cells were examined under the microscope (13). The number of control cells ingesting latex beads was defined as 100%, and the JCML results were expressed as a percentage of control.

PB cells from JCML patients showed an extremely high proliferative rate in liquid culture compared to control PB cells. Daily inspection of the cultures revealed an obvious increase in JCM cells numbers by Day 3 of incubation. Concurrently, a population of JCM cells became adherent to the plastic wells and quickly formed a monolayer. If removed from cultures, the nonadherent cells continued to increase in number, and a new adherent fraction formed within 3 days. After 10 days of incubation, JCM cell numbers increased spontaneously in Patient 1 by approximately 80%, in Patient 2 by 100%, and in Patient 3 by 150%, whereas control cell numbers decreased by about 7%. Trypan blue exclusion testing at this time showed that more than 90% of adherent and nonadherent JCM cells were viable, and 80% of control cells were viable. If the cultures were "refed" with new medium twice a week, JCM cells continued to proliferate in vitro for several months.

PB cells from JCM patients on Day 10 from the nonadherent layer, were examined by light microscopy, and consisted of monoblasts, early and mature monocytes, and premature and fully differentiated macrophages; the adherent fraction was composed primarily of mature and differentiated macrophages. Control PB cells in culture contained mature monocytes, macrophages, and cells of other lineages. JCM cells obtained from the adherent layer expressed surface markers of monocyteic origin but not those of B-cells or T-cells (Table 2). BM cells from Patient 1 at diagnosis had an abnormal karyotype (Fig. 1). The finding was used to prove clonality of the spontaneously proliferating JCM cells in culture. In 100% of 18 BM cells in metaphase, there were 2 distal deletions from the long arms of chromosomes 1 and 13 and additional terminal bands on the long arms of chro-
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Table 2 Surface markers of cells obtained from PB liquid cultures of 3 JCML patients compared to control.

OKIa was used to detect HLA-D framework antigen; Mo2, Leu-M1, Leu-M3, and OKM1, to identify monocytes and macrophages; Leu-1 and Leu-4, to detect T-cells; and B1 and B4, to detect B-cells. Results are expressed as the percentage of cells expressing the antigen of interest. Positive results are scored as weak, moderate, or strong.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKIa</td>
<td>&gt;95% strong</td>
<td>&gt;95% strong</td>
<td>90% strong</td>
<td>94% strong</td>
</tr>
<tr>
<td>Mo2</td>
<td>80% weak</td>
<td>70% moderate</td>
<td>0%</td>
<td>50% moderate</td>
</tr>
<tr>
<td>Leu-M1</td>
<td>66% moderate</td>
<td>0%</td>
<td>0%</td>
<td>90% strong</td>
</tr>
<tr>
<td>Leu-M3</td>
<td>94% strong</td>
<td>90% strong</td>
<td>100% strong</td>
<td>70% strong</td>
</tr>
<tr>
<td>OKM1</td>
<td>79% strong</td>
<td>83% strong</td>
<td>90% strong</td>
<td>87% strong</td>
</tr>
<tr>
<td>Leu-1</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Leu-4</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>B1</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>B4</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Control</td>
<td>94% strong</td>
<td>50% moderate</td>
<td>90% strong</td>
<td>70% strong</td>
</tr>
</tbody>
</table>

Table 3 Cytochemical analysis of adherent peripheral blood mononuclear cells from 3 patients with JCML harvested from liquid culture

Results are expressed as "++" indicating positive staining or "-".

<table>
<thead>
<tr>
<th>Assay</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANAE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ANAE + FIa</td>
<td>+ Weak</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>+</td>
<td>NDa</td>
<td>+</td>
</tr>
<tr>
<td>CAE</td>
<td>+</td>
<td>ND</td>
<td>+ Weak</td>
<td>+</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>+ Weak</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>+ Weak</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

aN ANE + FIa, ANEA with fluoride inhibition; ND, not done.

1 13q− 13q− 5 5q+ 12 12q+

Fig. 1. Partial karyotype from JCML bone marrow (Patient 1). There are deletions of the long arms of chromosomes 1 and 13 and additional terminal bands of 5q and 12q. The identical abnormal karyotype was identified in cells derived from liquid culture of the patient's PB.

Table 4 Functional studies performed on adherent peripheral blood mononuclear cells from 3 patients with JCML harvested from liquid cultures.

In these studies, the results obtained with control cells were defined as 100%, and the results with JCML cells were expressed as the percentage of control. In the latex ingestion assay, the data shown are from studies using 200 μl of the 1:1000 latex bead suspension.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Patient 1 (%)</th>
<th>Patient 2 (%)</th>
<th>Patient 3 (%)</th>
<th>Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procoagulant activity</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Plasminogen activator</td>
<td>1</td>
<td>0</td>
<td>NDa</td>
<td>100</td>
</tr>
<tr>
<td>Latex ingestion</td>
<td>79</td>
<td>60</td>
<td>2</td>
<td>100</td>
</tr>
</tbody>
</table>

* ND, not done.

Fig. 2a. Transmission electron micrograph of normal cultured macrophage. Note the round cell with central nucleus (N), rough surface with a few short cytoplasmic projections (arrow), abundant cytoplasm with mitochondria (m), sparse endoplasmic reticulum (er), Golgi apparatus (g) near the nucleus region, and some dense granules (d). x 8400.

Fig. 2b. Scanning electron micrograph of normal cultured macrophage showing the round cell with multiple foldings on surface which correlate with the rough surface in TEM (Fig. 2a). x 7000.

Fig. 2. a, transmission electron micrograph of normal cultured macrophage. Note the round cell with central nucleus (N), rough surface with a few short cytoplasmic projections (arrow), abundant cytoplasm with mitochondria (m), sparse endoplasmic reticulum (er), Golgi apparatus (g) near the nucleus region, and some dense granules (d). x 8400. b, scanning electron micrograph of normal cultured macrophage showing the round cell with multiple foldings on surface which correlate with the rough surface in TEM (Fig. 2a). x 7000.

Characterization studies of JCML adherent cells on Day 10 by cytochemistry and by functional properties are shown in Tables 3 and 4. Whereas control peripheral blood monocyte-macrophages grown in culture under identical conditions gave positive results for the 7 staining procedures that were tested, JCML cells did not stain for specific (chloroacetate) esterase and showed heterogeneity among the 3 patients in staining for fluoride inhibition of nonspecific esterase, β-glucuronidase, and lysozyme. Compared to control cells in the functional studies, JCML cells displayed marked deficiencies as well as heterogeneity in the panel of tests performed. Of the 3 patients, cellular homogenates from 2 showed no ability to convert plasminogen to plasmin, and homogenates from 2 patients failed to induce a plasma clot in a one-stage clotting assay. Interestingly, cells from 2 patients demonstrated moderate ability to ingest latex beads.

EM evaluation of cells harvested from the cultures of PB from Patients 2 and 3 at 3 time points (Days 4, 7, and 10 of culture) revealed some typical features of monocytes-macrophages, but also showed striking morphological abnormalities that were similar in both patients compared to cells obtained from control cultures. The abnormalities were present in thousands of cells examined. As shown in Fig. 2a, control macrophages examined by TEM were mainly round or oval shaped...
Fig. 3. a, transmission electron micrograph of JCML cell at Day 4 of culture showing the small round shape with central nucleus (N), abundant cytoplasm with mitochondria (m), and numerous cytoplasmic projections (arrow) on the surface. × 8640. b, transmission electron micrograph of JCML cell at Day 4 of culture. Note the oval-shaped cell with slightly eccentric nucleus (N'), abundant cytoplasm with distinct long narrow rough endoplasmic reticulum (er), the dense (d) and opaque (o) granules, and also numerous cytoplasmic projections (arrow) on the surface. × 8640. c, scanning electron micrograph of JCML cell at Day 4 showing the numerous cytoplasmic projections on the cell surface. × 7000. d, transmission electron micrograph of JCML cell at Day 7 of culture. Note the elongated shape, the eccentric nucleus (N), and the numerous vacuoles (V). × 6580. e, scanning electron micrograph of JCML cell at Day 7 of culture showing the elongated shape and the smoother cell surface. × 6330. f, transmission electron micrograph of JCML cell at Day 10 of culture. Note the large round empty cell filled with vacuoles (V') and also some stubby cytoplasmic projections (arrow) on the cell surface. × 5000. g, scanning electron micrograph of JCML cell at Day 10 of culture showing the large round rough-looking cell. × 6000.
with central or eccentric nuclei. The cytoplasm was abundant with mitochondria, had sparse endoplasmic reticulum, and showed obvious dense granules. The Golgi apparatus was near the nucleus. The cell surfaces were rough with a few short cytoplasmic projections, which correlated with the multiple foldings seen on the cell surfaces when studied by SEM (Fig. 2b). These findings for control macrophages were unchanged at the 3 time points studied.

Initially on Day 4, JCMl cells showed two characteristics. They were either small, round with central nuclei and short, sparse endoplasmic reticulum (Fig. 3a), or oval shaped with eccentric nuclei and long narrow rough endoplasmic reticulum (Fig. 3b). The cytoplasm in all cells was abundant with normal mitochondria and dense and/or opaque granules. The cells also had numerous "hairy" cytoplasmic projections or pseudopodia (Fig. 3, a and b). The surface structure under SEM showed slightly oval-shaped cells with numerous long microvilli (Fig. 3c). The appearance was different from the control macrophages (Fig. 2b).

On Day 7, most cells became elongated with irregular eccentric nuclei and developed a highly vacuolated cytoplasm (Fig. 3d). The mitochondria were normal, but the long narrow rough endoplasmic reticulum and cytoplasmic projections became less prominent. By SEM, the changes were similar to those seen by TEM, especially the elongation and smoother cell surface with a few projections (Fig. 3e).

Lastly on Day 10, the cells became larger and rounder with eccentric nuclei (Fig. 3f). The cytoplasm was almost completely replaced by large vacuoles which gave the cells a "hollow ball" appearance. There were some cytoplasmic projections, but these were short and stubby. On SEM (Fig. 3g), these cells looked large, rough, round, and barely viable. Compared to control cells (Fig. 2b), and to JCMl cells on Days 4 and 7 (Fig. 3, c and e), their appearance differed markedly.

DISCUSSION

To date, JCMl has been a highly lethal hematopoietic malignancy of infancy with refractoriness to conventional chemotherapy, and there has been intense interest in learning more about the cellular biology of the disease. Thus, we studied the PB mononuclear cells from 3 newly diagnosed, untreated patients with JCMl to characterize the disorder in terms of proliferative patterns in vitro, cell lineage, and degree of differentiation and function.

Our first observation was the excessive degree of cellular proliferation when PB mononuclear cells from JCMl patients were put in liquid culture. The culture system contained only nutrients and lacked specific humoral growth factors, such as colony-stimulating activity, which are usually essential for replication of myeloid cells in vitro. There were clear differences in the rate of cell proliferation between JCMl and control PB mononuclear cells. After 10 days, control cells declined in number, underscoring the need for a humoral growth stimulus for normal cell replication in culture. The spontaneous cellular proliferation seen with JCMl cells was aberrant and suggested a "neoplastic" property. Since the finding was striking and reproducible in all 3 cases studied, the abnormal growth pattern appears to be a cardinal hallmark of JCMl. Indeed, we observed a similar, exuberant, colony-forming ability of JCMl PB and BM cells when cultured in a semisolid clonogenic assay (14).

Abnormal karyotypes of BM cells at diagnosis have been described in some patients with JCMl (3, 15–17). The detection of an abnormal karyotype in BM but not PB lymphocytes from Patient 1 was a convincing argument for the single cell origin of JCMl. The identification of the same marker in PB cells grown in liquid culture proved that the proliferating elements originated from the abnormal clone.

Our data support previous observations (1–3) that suggested a monocytic origin of the JCMl cells. In our studies, the cells adherent to the plastic culture plates as well as the nonadherent cells had the morphological features of monocytic elements in various stages of maturation when examined by light microscopy. Monoblasts, early and mature monocytes, and primitive and fully differentiated macrophage elements could all be clearly recognized when removed from the culture mix after 10 days of incubation. The liquid culture system that was used appeared to favor growth of JCMl cells in a selective way. The cells that proliferated stained intensely with nonspecific esterase and, for the most part, the stain was inhibited by fluoride. Moreover, JCMl cells in culture expressed antigens of monocytic lineage exclusively, whereas no antigens of lymphoid origin could be detected using monoclonal antibodies.

A number of aberrant properties of JCMl cells were identified in cytochemical staining and in functional studies. The findings were not uniform in the 3 patients and indicated heterogeneity, probably in degree of differentiation as well as functional maturation. Functionally, JCMl cells were primarily defective, although some normal properties were also observed, such as the ability to phagocytize foreign matter. Some of the differences from control cells in cytochemistry and in function were very obvious and may reflect a "primitive" quality of JCMl cells.

The EM studies were quite revealing and produced new information on the ultrastructure of the malignant cells. At various stages of culture, JCMl cells, although having monocyte-macrophage features, consistently demonstrated clear-cut abnormalities. Some of the abnormalities, such as the vacuolation seen on Day 7, could have been artificial, possibly due to culture conditions. Alternatively, the changes could have been due to "cells in distress." Nevertheless, the JCMl cells had a different appearance than control cells. Also, the TEM and SEM finding of macrophages in various stages of maturation ruled out the hypothesis proposed by Shannon that JCMl cells are in "maturation arrest" (17).

Our data suggest that juvenile chronic myelogenous leukemia is not "myelogenous," but a disorder that involves the monocytic lineage. Morphologically and functionally, JCMl cells display a number of properties both mature and primitive. The finding of spontaneous and excessive cell proliferation in vitro reflects the malignant nature of this disorder.

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