Hairy cell leukemia (HCL) most often involves cells of B lineage. However, abnormal cells in HCL frequently exhibit phagocytic activity suggestive of a monocytic origin. In addition, cases of HCL with apparent T cell characteristics have also been reported. This line diversity is also seen in chronic lymphatic (CLL) and prolymphocytic leukemias (PLL). Indeed, clinical findings and induced characteristics have also been reported. This lineage divergently exhibit phagocytic activity suggestive of a monocytic surface immunoglobulins (slgs). Monoclonality was confirmed by clonal karyotype abnormality demonstrated in cell line HK. HCL parentage suggested by cytochemistry and electron microscopy was confirmed by the immunophenotypic observation that HCL lines expressed antigens aS-HCL1, aS-HCL3, and cCLL1. While aS-HCL1 and aS-HCL3 are nonspecific, their co-expression is characteristic of HCL cells. The cCLLs is a novel 69-kd membrane–HCL-associated polypeptide antigen not shared by circulating normal T or B lymphocytes nor by malignant cells from unrelated lymphoid or nonlymphoid malignancies. The doubling time of EH and HK was 24 and 36 hours, respectively. While HK included a small subset of Epstein-Barr virus (EBV) nuclear antigen-positive cells, EH cells were homogeneously negative for the presence of this antigen. Both cell lines were consistently implantable in irradiation-preconditioned immunodeficient mice giving rise to primary tumors and widespread metastasis.

**METHODS**

**Patients.** Mr Hkre is a 66-year-old male who on routine examination (August 1982) was found to have a spleen palpable 4 cm below the costal margin without lymphadenopathy. He was asymptomatic. His hemoglobin was 14 g/dL, the WBC count was 16,500/μL with 85% lymphocytes, and the platelets were 115,000/μL. CLL was suspected. He was observed untreated and remained asymptomatic, although the WBC count rose steadily. A biweekly regimen of chlorambucil was initiated in May 1984. At that time the WBC was 64,400/μL with 90% lymphocytes. Hemoglobin and platelet counts were unchanged. The spleen was now 8 cm below the costal margin. The observation of “shaggy” lymphocytes on blood smears led to a diagnostic reevaluation, including bone marrow aspirate/biopsy and cytochemistry studies, which revealed Hairy cell leukemia. The bone marrow was heavily infiltrated with large, tartrate-resistant acid phosphatase (TRAP)-positive lymphocytes. Splenectomy was performed in November 1984. In April 1985 the patient developed severe anemia (6.2 g/dL) with reticulocytopenia that responded partially to alpha interferon (Burroughs Wellcome study No. 49) but that required addition of prednisone (October 1985 to January 1987) to abrogate his transfusion requirement. Currently the patient is asymptomatic with (untransfused) normal hemoglobin level and platelets but with a WBC count of 18,500/μL with 56% lymphocytes. Hairy cells are easily identifiable on both blood and marrow smears.

Mrs Ehat is a 68-year-old female found to have massive splenomegaly on routine examination (November 1980). She was referred to our hematology/oncology clinics where a clinical diagnosis of HCL was made on the basis of splenomegaly (10 cm below the costal margin), pancytopenia (WBC, 4,600/μL with 41% lymphocytes; hemoglobin, 11g/dL; platelets, 143,000/μL), and characteristic hairy, TRAP-positive lymphocytes on blood smear. The bone marrow showed moderate numbers of large, TRAP-positive lymphocytes. She remained asymptomatic until early 1982, when because of increasing fatigue she underwent splenectomy (May 1982). She became asymptomatic post-splenectomy, and her blood counts reverted to normal. She has remained clinically stable since. Currently she exhibits a WBC count of 7,300 with 80% lymphocytes; a hemoglobin of 13.5 g/dL; and 281,000/μL platelets. Characteristic hairy cells are demonstrable on blood smears.

**Establishment of cell lines.** Mononuclear cell suspensions were prepared from heparinized venous blood obtained from patients Ehat and Hkre by a modified Ficoll-Hypaque (FH) gradient. Contaminating red cells were lysed with Tris- ammonium chloride. Cells were washed three times with alpha medium (Flow Laboratories, Inglewood, CA) supplemented with 20% fetal calf serum (FCS), 10⁻⁴ mol/L mercaptoethanol, 2 x 10⁻⁴ mol/L glutamine, and 100 μg/mL gentamycin (Schering Pharmaceutical Corp, Kenil-
CHARACTERIZATION OF HCL CELL LINES

worth, NJ). Cells were adjusted to 3 × 10⁶/mL, inoculated into 75-cm² tissue culture flasks (Corning Glass Works, Corning, NY), and incubated in a 5% CO₂ incubator (Forma Scientific, Marietta, OH) at 37°C. After 2 weeks the culture medium was replaced with fresh medium not containing mercaptoethanol. After initial growth was noted, cells were propagated in vitro with medium replacement twice weekly. Seeds were stored in liquid nitrogen.

**Electron microscopy and cytochemical staining.** Electron microscopy was performed on cultured cell pellets fixed overnight in 3% glutaraldehyde and osmium tetroxide. Sections were examined with a Siemens model 1A transmission electron microscope. Slides from cytocentrifuged cell line specimens were stained for May-Grünwald-Giemsa, sudan black, α-naphthyl acetate esterase, periodic acid shift (PAS), methyl green pyronine (MGP), and TRAP.¹⁰,¹¹

**Viability and phagocytic activity.** Cell viability was determined by dye exclusion method using 0.4% trypan blue in phosphate-buffered saline (PBS). Phagocytic activity was assessed as follows: 20 μg of iron particles, 0.2 μ average size (GAF Corp, New York), or of polystyrene particles, 0.86 μ average size (Pandex Laboratories, Mundelein, IL), preincubated (one hour at 22°C) or not with human IgG (1 mg/mL) or with normal human serum (1 mL) were washed twice and added to 10⁶ cells in 1 mL of RPMI 1640 media (GIBCO, Grand Island, NY) supplemented with 10% FCS. The mixture was rotated five minutes at room temperature and incubated 30 minutes at 37°C prior to counting cells showing ingested particles. At least 200 cells were counted in triplicate for each assay.

**DNA synthesis and doubling time.** DNA synthesis was ascertained as follows: cell aliquots in RPMI 1640 media supplemented with 10% FCS but containing no mitogen were added to 96-well filters (MA Bioproducts, Walkensville, MD) using a Mash II cell harvester (Cappel Laboratories, Cochranville, PA) and a panel of mouse monoclonal antibodies (Coulter Immunology, Hialeah, FL); αHC1 and αHC2 (a gift from Dr D.N. Posnett, The Rockefeller University, NY); MoAbs against human I cell leukemia virus (HTLV-1) viral core proteins p19/p24 (a gift from D. Bolognesi, Duke University, Durham, NC), and xenogeneic (aCLL) antiserum (Amgen, Thousand Oaks, CA); My4, Bl, B2, M02, and s (Coulter Immunology, Vista, CA); and 36 hours, respectively (Fig 1). Spontaneous DNA synthesis by either cell line was equivalent on exceeded that of Leitz epifluorescence microscope (E. Leitz, Inc. Rockleigh, NJ). Nonimmune mouse and rabbit antisera and irrelevant antibodies and target cells were included as negative controls.

**Detection of Epstein-Barr virus.** The presence of the Epstein-Barr virus (EBV) genome in cell lines was assessed by the Epstein-Barr nuclear antigen (EBNA) anticomplement immunofluorescence (ACIF) assay using a commercially available kit (Granbio, Inc, Temecula, CA). Smears of cell lines EH, HK, Raji, and Molt-4 were fixhed in acetone-methanol. Smears of admitted EBNA-positive and EBNA-negative cells provided with the kit were also tested. Human serum known to contain antibody against the EBNA and negative control sera both included in the kit were used as positive and negative substrate. Serum (10 μL of a 1:5 and 1:10 dilution) were added to each slide, incubated in a 5% CO₂ incubator for 20 minutes at 37°C, and washed. Slides were similarly processed after addition of 10 μL of guinea pig complement and 10 μL of FITC-conjugated polyclonal anti-guinea pig C-3 antibody. After the final wash nuclear fluorescence was assessed by fluorescence microscopy.

**Cytogenetics.** Chromosome preparations were made from Hkre blood and from EH and HK cell lines according to a standard air-dried technique. Briefly, Colcemid (Grand Island Biological Co, Grand Island, NY), 0.04 μg/mL was added to aliquots of 10⁶ cells one hour prior to harvest. The cells were treated with hypotonic KCL solution (0.074 mol/L) for ten minutes at room temperature, fixed, and washed in freshly prepared mixture of methanol and acetic acid (3:1, vol/vol), dropped on cold wet slides, and air dried overnight at 60°C before performing trypsin G-banding. At least 20 optimally banded metaphases were analyzed for each cell line. An abnormal clonal karyotype was defined as the presence of an extra chromosome or structural rearrangement in two or more cells or as similar missing chromosome in three cells, according to guidelines by the International System for Human Cytogenetic Nomenclature.¹²

**Tumorigenicity studies.** Transplantation studies are reported elsewhere. Briefly, EH and HK cell line aliquots (10⁶/0.1 mL) were inoculated subcutaneously in 8-week-old, immunodeficient nu/nu mice preconditioned ¹³,¹⁴ by total body irradiation (200 rad weekly × 3). Following inoculation tumor volume was calculated from three-dimensional tumor measurements obtained twice weekly. At death or when animals were killed, primary tumors were dissected, weighed, and sectioned to assess histology. Cell suspensions were also prepared from teased fresh tumor specimens for assessing cytochemistry and immunophenotype. All visceral organs were examined visually and microscopically to ascertain presence of metastasis.

**RESULTS**

**Growth characteristics.** At least one attempt was made to grow mononuclear cells derived from each of seven patients with clinically proven HCL. Cell lines could be developed from only two (Ehat and Hkre) of the seven HCL patients. Initially these cultures showed little growth. After approximately 6 weeks small clumps became apparent, and gradual proliferation was noted. At that stage subcloning was done by limiting dilution technique. Subcloned cells grew well in suspension. The established lines, identified as EH and HK, exhibited a doubling time of approximately 24 and 36 hours, respectively (Fig 1). Spontaneous DNA synthesis by either cell line was equivalent or exceeded that of phytohemagglutinin (PHA)-stimulated normal venous lymphocytes.¹³,¹² supporting their relatively high turnover rate. The viability of EH and HK cell lines was consistently >95%.
HK cells were not phagocytic. However, 11% to 22% of EH cells were capable of ingesting Ig-coated particles (Table 1).

**Morphology, cytochemistry, and electron microscopy.** Both cell lines (EH and HK) exhibited characteristic cytoplasmic projections when observed with either phase or light microscopy. Both cell lines exhibited positive α-naphthyl acetate esterase and TRAP reactivity but showed negative Sudan black, PAS, and MGP stains. Ultrastructural examination confirmed the projections and demonstrated stratified rough endoplasmic reticulum, ribosome lamella complex, abundant mitochondria, numerous lysosomes, and a monocytoid nucleus with large nucleoli (Fig 2).

**Immunophenotype.** As shown on Table 2, 71% and 73% of the total circulating mononuclear cells of patients Ehat and Hkre, respectively, expressed slgs. Likewise, EH and HK cell lines were of B cell derivation, as judged by their expression of slgs (61% and 62%, respectively) and, to a lesser extent, of antigens recognized by MoAbs B1, B2, LEU-12, LEU-14, and LEU-M5 (51%, 48%; 4%, 33%; 29%, 41%; 39%; 31%; and 47%, 22%, respectively). The monoclonality of parent cells and of cell lines is supported by the expression of slgs (61% and 62%, respectively) and, to a lesser extent, of antigens recognized by MoAbs B1, B2, LEU-12, LEU-14, and LEU-M5 (51%, 48%; 4%, 33%; 29%, 41%; 39%; 31%; and 47%, 22%, respectively).

<table>
<thead>
<tr>
<th>Particles</th>
<th>EH</th>
<th>HK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>NHS</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Polystyrene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncoated</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Coated with IgG</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>Coated with NHS</td>
<td>19</td>
<td>3</td>
</tr>
</tbody>
</table>

Iron or polystyrene particles (20 μg/mL) precoated or not with human IgG (1 mg/mL) or with normal human serum (1 mL) were incubated with 10⁸ cells/well for one hour at 37°C. Phagocytosis was assessed by light microscopy from 200 cell counts done in triplicate and is expressed as percent of total.

**EBV.** The adequacy of the system was ascertained using slides of 50% admixture of EBNA-positive and EBNA-negative cells and sera containing or not anti-EBNA antibody, included in the kit. As shown in Table 4, 45% to 46.1% of admixed cells were positive for the EBNA when assayed with 1:20 and 1:5 dilutions of anti-EBNA antibody containing serum, respectively. As expected, admixed cells were unreactive when assayed with anti-EBNA-antibody-negative serum or with PBS. All subsequent assays were done using 1:5 dilutions of anti-EBNA-antibody-positive and negative sera. Only 0.4% of EH and 16% of HK cells were EBNA reactive. Raji and MOLT-4 cells used as additional EBNA-positive and negative controls showed 65% and 0% reactivity, respectively. Of interest is the observation that 16% and 14% of EH cells exhibited characteristic spotty membrane fluorescence when assayed with anti-EBNA-antibody-positive and negative sera, respectively. Membrane fluorescence was not observed in any other cell preparation.

**Cytogenetics.** Both EH and HK cell lines exhibited diploid chromosome number. In EH cells all 20 metaphases analyzed had normal female, 46 XX chromosome complement. In HK cells 11 of 20 metaphases examined consistently showed one normal chromosome 14 and a small acrocentric marker (M1, Fig 3) resulting from deletion in the long arm of the other 14 (del(14)(q22)). One metaphase, in addition to the del(14q) marker, showed what appears to be reciprocal translocation between chromosome 12 and 17 (M2, Fig 3), ie, t(12;17)(q12;q23) plus a small metacentric element (M3, Fig 3), presumably representing the deleted segment of chromosome 12.

**Transplantation in the nude mice.** HK cell line has been transplanted subcutaneously in over 100 radiation-preconditioned, immunodeficient mice with nearly 100% success rate. The same success rate has been achieved with EH, although fewer mice have been transplanted. Tumors exhibited histologic features of lymphoid malignancies, and the teased cells demonstrated cytochemical and immunophenotypic characteristics similar to those of the parent cell lines. Tumors attained an average weight of 5 g over a 56-day average survival time and metastasized hematogenously to lymphoid and nonlymphoid organs.¹⁸
CHARACTERIZATION OF HCL CELL LINES

Fig 2. Electron microscopy of HK cell line. Several cells with characteristic “hairs,” prominent nucleoli, and abundant mitochondria are depicted in panel A. Panel B shows cell with well-developed “hairs.” Panel C illustrates a lamellar complex characteristic of hairy cells.
Table 3. Crossinhibition of αHC1, αHC2, and CLL-1 Binding by Purified cCLLa

<table>
<thead>
<tr>
<th>MoAbs</th>
<th>Cells</th>
<th>EH</th>
<th>HK</th>
<th>CLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>αHC1:</td>
<td>Unabsorbed</td>
<td>14</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Absorbed</td>
<td>13</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(Inhibition)</td>
<td>7.1</td>
<td>8.7</td>
<td>0.0</td>
</tr>
<tr>
<td>αHC2:</td>
<td>Unabsorbed</td>
<td>22</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Absorbed</td>
<td>21</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(Inhibition)</td>
<td>4.5</td>
<td>6.7</td>
<td>0.0</td>
</tr>
<tr>
<td>CLL-1:</td>
<td>Unabsorbed</td>
<td>44</td>
<td>49</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Absorbed</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(Inhibition)</td>
<td>93.2</td>
<td>95.9</td>
<td>98.5</td>
</tr>
</tbody>
</table>

Cell aliquots (10^6/well) were incubated 60 minutes at 22°C with the MoAbs (10 µg/well) in RPMI 1640 before and after absorption with column-purified cCLLa (10:1 wt/wt ratio). After washing three times, FITC-conjugated rabbit antimouse IgG (10 µg/well) was added and was incubated one hour at 22°C, and reactive cells were assessed by IFA. All assays were done in triplicate. Results are expressed as percent reactive cells or as percent inhibition.

cell lines derived from HCL patients. EBNA positivity has been alternatively suggested to represent in vitro transformation by EBV or in vitro infection of already established cell lines. The observation that most HCL cells are EBV-insusceptible B cells would tend to support the former view. However, a small subset of HCL cells appear clearly EBV-susceptible and transformable. Finally, changes in phenotype, cytochemistry, and even cytogenetics observed in HCL and other cell lines over time further cloud the issue of cell line parentage.

EH and HK cell lines were of B cell lineage, as shown by their expressing B differentiation markers, notably slgs, B1, B2, and LEU-12, with exclusion of T lymphocyte, myeloid, and monocytoid markers. Results of cytochemistry studies supported this observation. Expression of single heavy and light Ig chains suggested the monoclonality of both lines.

Table 4. EBNA Detection in EH and HK Cells

| Slides provided with the kit and cell line smears were layered with 10 μl/slide of the reagents shown in the indicated dilutions. After 20-minute incubation at 37°C, slides were incubated one hour at 22°C, and reactive cells were assessed by IFA. All assays were done in triplicate. Results are expressed as percentage of stained cells from at least 500 cells counted per assay.

<table>
<thead>
<tr>
<th>Kit Cells</th>
<th>EBV+ Serum</th>
<th>EBV- Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:5</td>
<td>1:20</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>1:20</td>
</tr>
<tr>
<td>KIT Cells</td>
<td>0.0</td>
<td>46.1</td>
</tr>
<tr>
<td>EH</td>
<td>0.4</td>
<td>45.0</td>
</tr>
<tr>
<td>HK</td>
<td>10.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>RAJI</td>
<td>65.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Abbreviation: Poly-V, polyvalent.

Immunophenotype of blood mononuclear cells (MNC) from patients Ehat and HKre and that of cell lines EH and HK derived from these patients were assessed by IFA. Results represent percent reactive cells from at least 600 cells examined per assay.

**DISCUSSION**

Attempts by previous investigators to demonstrate the HCL lineage of cell lines derived from blood or spleens of HCL patients were based mainly on cytochemistry, electron microscopy, and cytogenetic comparisons with the parent cells. However, none of the features characteristic of fresh HCL cells used diagnostically in the clinical setting are unique or pathognomonic. Indeed, well-established light and electron microscopy features of HCL can be seen in lymphoblastoid cell lines spontaneously or after exposure to tetradecanoylphorbol acetate (TPA) or other agents. Likewise, positive TRAP reactivity, like expression of p35, a 35-kd marker protein for HCL, also occurs in classical B cell lines. Thus, although cell lines with many characteristics of HCL have been described, none has been conclusively proven to originate from HCL cells. Additionally, a most vexing problem relates to detection of herpes-type virus or EBNA in...
Monoclonality was confirmed in both cell lines by Ig gene rearrangement (courtesy of Dr. D. Capra, University of Texas Southwestern Medical School, Dallas) and in HK by clonal karyotype abnormality. The absence of cytogenetic abnormalities in EH cell lines and in HK fresh cells is not surprising given the failure of previous investigators to demonstrate such abnormalities in HCL cell lines and the observation that only a small subset of HCL patients, particularly those with more aggressive disease, exhibit such abnormalities.

Furthermore, cytogenetic studies on fresh HK cells were conducted after the patient had undergone splenectomy and had received a course of interferon and chronic prednisone administration, reducing the subset of circulating malignant cells. The HCL parentage of EH and HK is supported by (1) cytology; (2) cytochemistry; and (3) electron microscopy and is confirmed by the immunophenotype results. Indeed, both cell lines showed reactivity with MoAbs aHC1, aHC2, aS-HCL1(LEU-14), aS-HCL3(LEU-M5), CL-1, and CL-2. While attempts have been made to identify disease-associated surface determinants in HCL or to delineate a unique HCL immunophenotype, markers thus far reported are also expressed by normal lymphoid and nonlymphoid cells or by other neoplastic cells.

In contrast to several other HCL-derived cell lines, EH was EBNA-negative. The membrane fluorescence exhibited by these cells was most likely a nonspecific Fc-related phenomenon. This is supported by the following observations: (1) the fluorescence was characteristically speckled and associated with the cytoplasmic membrane; (2) the reaction was not dependent on the presence of anti-EBNA antibody; and (3) MOLT-4 cells, a T cell line devoid of Fc receptors, exhibited negative membrane fluorescence. HK included a small subset of cells seemingly bearing the EBNA.
and HK cells in nonirradiated immunodeficient mice failed. Indeed, total body irradiation (TBI) greatly reduced NK cell activity, which is felt to be responsible for rejection of xenografts in nude mice. The relatively shorter doubling time exhibited by HK seemed to confer no appreciable tumor growth advantage. Implanted HCL cells gave rise to primary tumors and widespread metastasis. Hematogenous dissemination was demonstrated by flow cytometry analyses of DNA content of blood cells obtained from sequential retro-orbital vein punctures (manuscript in preparation). A xenotransplantation model involving cCLLa-positive HCL cell lines such as ours should prove of value for assessing the potential of cCLLa-specific MoAbs as immunotherapeutic agents. Such preliminary information might find clinical application for future management of HCL and the lineage-related disorders PLL and CLL.

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Cytochemical, cytogenetic, immunophenotypic and tumorigenic characterization of two hairy cell lines

GB Faguet, KL Satya-Prakash and JF Agee