Peripheral Blood Mononuclear Cells of a Patient With Advanced Hodgkin’s Lymphoma Give Rise to Permanently Growing Hodgkin-Reed Sternberg Cells

By Jürgen Wolf, Ursula Kapp, Heribert Bohlen, Martin Kornacker, Claudia Schoch, Bettina Stahl, Susanne Mücke, Christof von Kalle, Christa Fonatsch, Hans-Eckart Schaefer, Martin-Leo Hansmann, and Volker Diehl

A novel Hodgkin’s disease (HD) derived cell line, L1236, was established from the peripheral blood of a patient with advanced Hodgkin’s disease. Analysis of immunoglobulin (Ig) gene rearrangements revealed a biallelic Ig heavy chain and a monoallelic Ig kappa light chain gene rearrangement, pointing to a B-lymphoid origin of these cells. No DNA of Epstein-Barr virus was detected in L1236. The cells expressed the HD-associated surface antigens CD30 and CD15 as well as the transferrin receptor (CD71). Cytogenetic analysis of early passages of L1236 cells revealed a grossly disordered karyotype including cytogenetic aberrations described previously in other HD-derived cell lines. The Hodgkin/Reed-Sternberg (H-RS) cell origin of L1236 cells is further confirmed by Kanzler et al. (Blood 87:3429, 1996), who found identical Ig gene rearrangements in L1236 cells and H-RS cells of the same patient’s bone marrow. L1236 cells expressed antigens necessary for efficient antigen presentation to T cells including HLA class I and II, B7.1 and B7.2, as well as adhesion molecules ICAM 1 and LFA 3. The cells secreted the interleukins (IL)-6, -8, -10, tumor necrosis factor (TNF) α, interferon (IFN), γ, transforming growth factor (TGF) β, and the granulocyte-macrophage colony stimulating factor (GM-CSF). After subcutaneous inoculation into SCID mice, a necrotic regression of initially growing tumors at the injection site was followed by disseminated intralymphatic growth. Our findings, together with the results of Kanzler et al, demonstrate that H-RS cells of B-lymphoid origin were present in the peripheral blood of a patient with advanced HD. These cells exerted a malignant phenotype with regard to their in vitro and in vivo characteristics. © 1996 by The American Society of Hematology.

HODGKIN’S LYMPHOMA is unique among malignant lymphomas in that the putative malignant cells, the mononucleated Hodgkin cells, and their bi- or polynucleated Reed-Sternberg cell derivatives, represent only a minority of 0.1% to 1% of the total cell population in affected lymphatic tissue. They are surrounded by reactive T lymphocytes, histiocytes, eosinophils, and stromal cells. Due to the scarcity of the Hodgkin/Reed-Sternberg (H-RS) cells and the resulting technical problems of their in situ characterization, the cellular origin and the clonality of these cells has been a matter of debate in the past decades.1 Immunophenotyping of H-RS cells yielded a heterogeneous pattern of lineage specific marker expression disallowing the determination of the normal cellular counterpart.2 Cytogenetic analysis revealed the presence of numerous structural and numerical chromosomal aberrations, which are neither consistent nor specific.3 A search for gene expression at the single cell level showed a heterogeneous pattern, which also did not elucidate the origin of these cells.4

In numerous human neoplasms the establishment of permanent cell lines, with limitations, has allowed biologic characterization of the tumor cell population. By comparison, the outgrowth of a permanent Hodgkin cell line from Hodgkin’s lymphoma-derived tissue cultured in vitro is an extremely rare event.5 Only 14 cell lines have been established to date, which may be considered to derive from H-RS cells.6-16 These cell lines have been extensively studied with regard to karyotype, immunophenotype, immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements and expression of cytokine genes, cytokine receptor genes, and oncogenes. Similar to in situ analysis of biopsy material, results from the analysis of Hodgkin’s disease (HD)-derived cell lines were heterogeneous. With the exception of the consistent expression of some surface antigens (CD30, CD15, CD71), no specific antigen expression pattern allowed the determination of the hematopoietic lineage derivation of H-RS cells. The cell lines either express T cell specific markers, B cell specific markers, both, or—in one case (HD-MyZ)—none of them. In analogy, Ig- and TCR-gene rearrangements were found. No specific cytogenetic aberration, consistent oncogene expression, or loss of tumor suppressor gene function could be identified in the cell lines.17 In addition, validity of results obtained with these cell lines was discussed controversially, since the H-RS cell origin of these cells could not be proven on the molecular level.

Recently, isolation of single H-RS cells from frozen tissue sections by micromanipulation and subsequent polymerase chain reaction (PCR) amplification of Ig gene sequences was used as an experimental tool to determine lineage origin of H-RS cells in biopsy specimen.18 In three of three cases clonal Ig gene rearrangements were found demonstrating unequivocally a B cell origin of these H-RS cells; however, no functional characterization of B cell-derived H-RS cells could be performed yet.

In this report we describe in vitro cultivation and characterization of H-RS cells with a B lymphoid origin from the peripheral blood of a patient with advanced HD. These cells expressed typical HD-associated surface markers. Cytogenetic analysis revealed a completely aberrant karyotype in-

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HODGKIN’S LYMPHOMA/B-CELL ORIGIN/CELL LINE L1236

Fig 1. CD30 staining of H-RS cells in lymph node tissue excised for primary diagnosis of HD in 1991. Cervical lymph node, paraffin section, streptavidin-biotin immunostaining, original magnification × 600. (A) In the middle of the picture a Reed-Sternberg cell strongly positive for CD30 surrounded by small lymphocytes and histiocytes; (B) a large Reed-Sternberg cell and a Hodgkin cell positive for CD30.

Fig 2. CD15 staining of H-RS cells in lymph node tissue excised for primary diagnosis in 1991. Cervical lymph node, paraffin section, streptavidin-biotin immunostaining, original magnification × 600. (A) A multinucleated Reed-Sternberg cell and a Hodgkin cell positively immunostained with CD15; (B) Hodgkin-infiltrate showing a Reed-Sternberg cell with intracytoplasmic and membrane bound positivity for CD15. In addition, histiocytes, epitheloid cells, and lymphocytes negative for CD15 are present.

Fig 3. Hematoxylin-eosin staining of lymph node tissue excised for diagnosis of Hodgkin’s lymphoma relapse in 1993. Abdominal lymph node, paraffin section. (A) Infiltration of lymph node tissue by HD, original magnification × 175; (B) numerous mononuclear Hodgkin cells, original magnification × 650; (C) polynucleated Reed-Sternberg cells, original magnification × 650.

including specific chromosomal rearrangements described previously in other H-RS cell lines. Analysis of Ig gene rearrangements showed a B lymphoid origin of this tumor cell population. The H-RS cell origin of L1236 cells was confirmed by Kanzler et al9 who detected identical Ig gene rearrangement sequences in L1236 cells and the H-RS cells in the bone marrow of the same patient. Analysis of L1236 cells may thus provide valid information on biologic characteristics of H-RS cells of B lymphoid origin.

MATERIALS AND METHODS

Case report. In 1991, HD of the mixed cellularity subtype, clinical stage IA (cervical lymph node involvement) was diagnosed in a 31-year-old patient (Figs 1 and 2). After radiation therapy (40 Gy), a complete remission was obtained. A first relapse occurred in 1992 with involvement of the spleen and one splenic hilar lymph node. A splenectomy was performed without any adequate specific therapy. In 1993, a second relapse was diagnosed with involvement of abdominal lymph nodes and bone marrow (Fig 3). The treatment included three cycles of chemotherapy (COPP/ABVD) followed by high-dose chemotherapy with autologous bone marrow transplantation. Three months later the patient again relapsed with extended involvement of bone marrow and the liver. In April 1994, the patient...
was admitted to our hospital for experimental treatment with Ricin-A coupled anti-CD25 immunotoxins. Before therapy, a bone marrow biopsy was performed showing pronounced infiltration of the bone marrow with Hodgkin’s lymphoma (Fig 4). After administration of the first course of immunotoxin therapy no response was observed. Subsequently, salvage chemotherapy (Dexa-BEAM, dose reduction 50%) was begun in April 1994, but had to be stopped due to severe liver toxicity. The patient’s condition worsened progressively. He developed fever, pulmonary infiltrations, and died in May of 1994.

Immunohistochemical investigations were performed using monoclonal antibodies against CD30, CD15, and CD80 (Becton Dickinson, Mountain View, CA). The streptavidin-biotin complex method (ABC) was applied. Briefly, the sections were digested with trypsin followed by incubation with the primary antibody (30 min). After a washing step (Tris-buffered saline) the slides were incubated with biotinylated rabbit antimouse F(ab)-fragments (30 min). After another washing step and incubation with streptavidin-biotin complex labeled with alkaline phosphatase (30 min), the enzyme reaction was developed with the Neufuchsin method and the slides were counterstained with haemalun and mounted.

Cell culture. Lymphocytes were separated from peripheral blood of the patient by density centrifugation (Ficoll-Hypaque). All cells (initial peripheral blood lymphocytes, established cell line L1236, control Burkitt lymphoma cell line BL60-P7) were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 50 U/mL penicillin, 50 μg/mL streptomycin, and 4 mmol/L L-glutamine in a 5% CO2 atmosphere at 37°C.

Analysis of immunophenotype. All monoclonal antibodies (MoAb) against surface antigens used in this study were obtained from Becton Dickinson. For staining, cells were incubated with the first antibody (5 μg/mL; 50 μL/1 × 10^6 cells) for 30 minutes at 4°C. The cells were then washed twice, stained with goat antimouse-FITC (15 min at 4°C) and, after another washing step, analyzed on a FACScan flow cytometer (Becton Dickinson). A minimum of 1 × 10^6 events was analyzed. Immunofluorescence data were displayed on a four-decade log scale. Data were evaluated by CellQuest software (Becton Dickinson).

Cytogenetics. After 3 months in tissue culture, L1236 cells were treated with colcemid (0.1 to 0.5 μg/mL medium) for 0.5 or 2 hours before harvesting. Then they were sedimented at 1,000 rpm, treated with hypotonic KCl solution (75 mmol/L at room temperature for
20 minutes, fixed in methanol:acetic acid (3:1), dropped on ice-cold slides, and air dried. A modified Giemsa-Acid-Saline-Giemsa (GAG) band staining of chromosomes was performed as previously described.

Fluorescence in situ hybridization. Dual color chromosome painting was performed. The air-dried slides were immersed in 70% formamide/2X SSC at 70°C for 2 minutes, transferred to ice-cold ethanol, 70%, 80%, 90%, and 100% sequentially, and air dried. Equal parts of one fluorescein isothiocyanate (FITC)- and one biotin-labeled whole chromosome probe readily prepared with hybridization buffer and competitor DNA were mixed. Whole chromosome probes of chromosomes 1, 2, 3, 4, 7, 8, 10, 11, 12, 14, 15, 16, 17, 20, and 21 were used (Angewandte Gentechnologie Systeme GmbH, Heidelberg, Germany and Oncor, Gaithersburg, MD). The probe mixture was heated in a water bath at 70°C for 5 minutes and then incubated at 37°C for 1 hour. Ten microliter of probe mixture was placed onto slides, covered with coverslips, sealed with rubber cement, and incubated overnight at 37°C. Posthybridization washes were performed at 42°C in 50% formamide/1X SSC and 2X SSC for 2 × 5 minutes each. Hybridization signals were detected and amplified using solution A containing mouse anti-FITC antibodies (0.4 pg/µL; Boehringer Mannheim, Germany) and Texas Red-conjugated streptavidin (5 pg/µL; Dianova, Hamburg, Germany), and solution B containing FITC-conjugated sheep antimouse (12 pg/µL) and biotinylated goat antistreptavidin antibodies (2.5 pg/µL; Vector Laboratories, Burlingame, CA). All reagents were made up in 4X SSC and 1% bovine serum albumin (BSA). Detection of hybridized chromosomes was achieved by covering the slides with a blocking solution (4X SSC, 3% BSA) followed by sequential incubations in the solutions A, B, and A for 20 minutes at 37°C each. Incubations were separated by washes in 4X SSC, 0.1% Tween 20 for 3 × 5 minutes at 42°C. After a final wash, the preparations were counterstained with 700 ng/mL 4,6-diamidino-2-phenylindol (DAPI) in Mellova’a’s buffer (5.6 mmol/L citric acid, 87.2 mmol/L disodium phosphate, pH 7.0) for 2 minutes and mounted in 90% glycerol, 10% phosphate-buffered saline, and 1 mg/mL p-phenylenediamine. The hybridized metaphases were photographed under a Zeiss epifluorescence microscope equipped with Zeiss filter combination 02 (DAPI), 10 (FITC), and 15 (Texas Red) using Kodak Ektachrome 400 films.

Southern blotting. Extraction of cellular DNA and restriction endonuclease digestion were performed using standard protocols. Briefly, 10 µg of cleaved cellular DNA were separated by agarose gel electrophoresis and transferred to a nylon filter (NEN; Gene Screen Plus, Boston, MA). Hybridization was performed in 50% formamide, 2X SSC at 42°C with 32P-labeled DNA probes. The following probes were used for detection of immunoglobulin gene rearrangements: a genomic 2.2 kb Sau3A fragment of the human immunoglobulin heavy chain joining region (IgH J), a 2.1 kb Sac I-EcoRI fragment of the human immunoglobulin κ light chain constant region, and a genomic 3.5 kb HindIII-EcoRI fragment of the human immunoglobulin λ light chain constant region. T-cell receptor β gene rearrangement was analyzed with an 800 bp cDNA fragment. To detect the presence of EBV DNA, the 3.2 kb BglII U fragment (nucleotides 13944-17016) specific for the EBV repeat 1 (IR 1) was used.

Enzyme-linked immunosorbent assay (ELISA). Production of human cytokines interleukin (IL)-2, -4, -6, -7, -8, -10, interferon (IFN)γ, tumor necrosis factor (TNF)α, transforming growth factor (TGF) β, and granulocyte-macrophage colony stimulating factor (GM-CSF) by the cell line L1236 was determined by an ELISA (Quantikine, for IL-2, -4, -6, -8, -10, IFNγ, TGFβ, GM-CSF, and TNFα; Bio-kinine for IL-7, Biermann Diagnostica GmbH, Bad Nauheim, FRG). ELISA was performed according to the manufacturer’s instructions. The sensitivity thresholds were: 88 pg/mL (IL-2), 3 pg/mL (IL-4), 0.35 pg/mL (IL-6), 4.1 pg/mL (IL-7), 4.7 pg/mL (IL-8), 1.0 pg/mL (IL-10), 3.0 pg/mL (IFNγ), 0.17 pg/mL (TNFα), 1.5 pg/mL (GM-CSF).

Xenotransplantation. SCID mice were initially obtained from W. Schuler, Basel Institute of Immunology, Switzerland, under licensing of Melvin Bosma, Fox Chase Center, Philadelphia, PA. The animals were propagated under specific pathogen-free (SPF) conditions. Leakiness of the animals was excluded by measurement of their serum Ig levels as described. Mice at the age of 4 to 8 weeks were used for transplantation experiments. 2 × 107 viable cells from exponentially growing cultures in a total volume of 0.2 mL RPMI 1640 without FCS and antibiotics were inoculated subcutaneously (SC) or intraperitoneally (IP) in each flank of an animal. Diameters of the grafts were measured twice weekly.

RESULTS

Peripheral blood mononuclear cells were obtained from the patient the day before the Dexa-BEAM salvage chemotherapy was started in April 1994. The patient’s blood count showed 300 leukocytes/µL. In the differential count 60% atypical lymphocytes were described, but no H-RS cells were identified. These atypical lymphocytes with an almost double-size diameter compared with normal lymphocytes were characterized by irregular nuclear profiles and basophilic agranular cytoplasm (Fig 5). To discriminate them from monocytes, esterase-enzyme reaction was performed and showed negative results (data not shown). Lymphocytes were separated from peripheral blood by density centrifugation (Ficoll-Hypaque) and transferred into RPMI tissue culture medium as described earlier. Culture medium was exchanged twice weekly, and dead cells were removed by gentle centrifugation. The cells grew in suspension, forming small clumps up to a maximal density of 6 × 107 cells/mL before growth arrest occurred. Heterogeneity with regard to size and form between the cells was observed. Most of the cells were mono- or binucleated with round to irregularly shaped large nuclei and a medium-sized basophilic cytoplasm, partly with vacuoles (Fig 6A). A minority of approximately 10% of the cultures consisted of multinucleated giant cells with vacuolated cytoplasm (Fig 6B).

Surface antigen expression of L1236 cells was analyzed on a FACSsort. The cells showed surface expression of the HD-associated activation antigens CD30 (HD-associated antigen), CD15 (X-Hapten), and CD71 (transferrin receptor), while they did not express CD25 (IL2-receptor). L1236 cells also expressed CD23 (B-cell associated activation antigen), CD80 (B7-1 molecule), CD86 (B7-2 molecule), the adhesion molecule CD54 (ICAM-1), CD58 (LFA-3), HLA class I as well as class II (HLA-DP, -DR) antigens. With the exception of CD23 no expression of B-lineage antigens (CD19, CD20, CD38, s-Ig κ and λ light chain) was found. The cells were also negative for CD10 (CALLA), T-lineage antigens (CD3, CD4, CD5, CD8, CD45, CD45RO, CD45RA, TCR γ delta), the myeloid-lineage associated antigen CD33, the natural killer cell marker CD16, the monocyte antigen CD14, and the hematopoietic stem cell antigen CD34. Figure 7 shows representative FACS analysis of surface antigen expression on L1236 cells for CD30, CD15, CD71, CD58, CD54, CD23, CD80, HLA class I, and HLA class II.

Immunohistochimical analysis of the H-RS cells in the
patient's bone marrow revealed, in concordance with FACS analysis of in vitro cultured L1236 cells, expression of the antigens CD30 (Fig 4), CD15, and CD80. Expression of further antigens was not tested due to the scarcity of the available bone marrow material.

Cytogenetic analysis of the L1236 cells revealed a near-triploid grossly disordered karyotype with numerous structural and numerical aberrations. Nearly all chromosomes were affected by duplications, deletions, inversions, and mainly translocations (Fig 8). To identify the origin of chro-
mosome segments in marker chromosomes, FISH analysis with painting probes was used. Two examples for the characterization of marker chromosomes using FISH are given in Fig 9A and B. Table 1 summarizes the cytogenetic aberrations identified in L1236 cells.

Absence of EBV DNA in cell line L1236 was demonstrated by Southern blot analysis. L1236 DNA was probed with the BgIII-U 1 fragment of the EBV genome, which hybridizes to the internal repeat 1 (IR 1) of EBV. In contrast, DNA of the EBV positive BL60-P7 cell line harboring about 10 genome copies of integrated EBV,26 yielded a strong positive hybridization signal even after dilution with EBV-negative placenta DNA in a 1:50 ratio confirming the sensitivity of Southern blot analysis to be below 1 copy of the EBV genome per cell (data not shown).

DNA of L1236 cells was analyzed by Southern blot hybridization for rearrangements of Ig and TCR genes. After restriction enzyme digestion with either EcoRI or HindIII and subsequent hybridization with an Ig heavy chain joining (IgH J) region fragment, two rearranged fragments were detected, indicating a biallelic Ig heavy chain gene rearrangement. Hybridization with an Ig κ light chain probe after digestion with EcoRI, HindIII, and BamHI showed each one rearranged and one germline Ig κ light gene. Only germline fragments were detected after hybridizing EcoRI or HindIII digested L1236 DNA with an Ig λ light chain probe. Figure 10 shows Southern blot analysis for Ig heavy and light chain gene rearrangements with each one representative restriction enzyme. No TCR rearrangements were detected using Southern blot analysis (data not shown).

Cytokine concentrations in the supernatant of exponentially growing L1236 cells were measured using ELISA. L1236 cells produced detectable amounts of the IL-6, -8, -10, INF γ, TGF β, GM-CSF and, with strikingly high amounts, TNF α. No secretion of IL-2 and IL-4 was detected (Table 2).

Native SCID mice (n = 3) were inoculated SC with 2 × 10⁷ L1236 cells each. Another group of 3 native SCID mice were inoculated IP with 2 × 10⁷ L1236 cells each. At SC inoculation sites after a latency period of 6 to 8 weeks, initial tumor growth could be observed. When tumors reached a size of 0.5 to 1 cm in diameter, all SC tumors underwent extended necrosis and regressed completely within about 2 weeks. After 4 months the overall condition of all animals (SC and IP inoculations) worsened and they were killed. Autopsy revealed disseminated intralymphatic tumor growth in three of three animals of the SC group and in two of three animals of the IP group. Axillary, inguinal, mesenterical, and portal lymph nodes were enlarged to about 5 mm. Infiltration of extralymphatic tissue was not observed. Figure 11 shows the histology of an enlarged inguinal lymph node. Massive infiltration with L1236 cells exerting a basophilic cytoplasm and irregularly shaped nuclei with one or more nucleoli has taken place resembling the histologic picture of anaplastic large cell lymphoma (ALCL).

DISCUSSION

In the present study we have shown that malignant H-RS cells of B-lymphoid origin are present in the peripheral blood of a patient with advanced HD. These cells gave rise to the permanent cell line L1236 after in vitro cultivation. L1236 cells exerted an H-RS cell morphology and expressed HD-associated activation antigens CD30 (Ki 1), CD71 (transferrin receptor), as well as CD15. They had a grossly disordered karyotype including clonal chromosomal aberrations previously described in HD-derived cell lines L428 and L540.31,32 In addition to these historically accepted criteria for defining an H-RS cell line, the H-RS cell origin of this
Fig 11. Dissemination of L1236 cells into an inguinal SCID mouse lymph node. Paraffin section, hematoxylin-eosin staining, original magnification × 600. The lymph node infiltration shows features of anaplastic large cell lymphoma (ALCL) with numerous large tumor cells with round to irregularly shaped nuclei and a broad basophilic cytoplasm and some multinucleated cells.
Table 1. Marker Chromosomes in L1236 Identified by Giemsa Banding and FISH Analysis

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Description</th>
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<tr>
<td>I</td>
<td>der(1)(1;14)(p34;?)</td>
</tr>
<tr>
<td>II</td>
<td>der(1)(1;8)(p22;?)</td>
</tr>
<tr>
<td>III</td>
<td>der(1)dup(1)(q21q44)add(1)(p31;32)</td>
</tr>
<tr>
<td>IV = V</td>
<td>dup(2)(p15p23)</td>
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<td>VI</td>
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<td>der(7)I</td>
</tr>
<tr>
<td>XIV</td>
<td>der(8)I</td>
</tr>
<tr>
<td>XV</td>
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<tr>
<td>XVI</td>
<td>add(11)(p13)</td>
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<td>del(11)(q13)</td>
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<td>del(12)(q22)</td>
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<tr>
<td>XXVI</td>
<td>del(19)(7;19)(7;p13)</td>
</tr>
<tr>
<td>XXVII</td>
<td>add(20)(15;20)(q22;q11)(2x)</td>
</tr>
<tr>
<td>XXVIII</td>
<td>mar1</td>
</tr>
<tr>
<td>XXIX</td>
<td>mar2</td>
</tr>
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</table>

* Material of chromosomes 14 and 10 is added to the short arm of der(8).
† Pericentric inversion of XI with additional material of chromosome 11 at the short arm.
‡ Material of chromosomes 1 and 14 is added to 8q24.
§ Material of chromosomes 17 and Y is added to 12p11.
¶ Material of chromosomes 14q and 10q is added to 16q24.

isolated H-RS cells from fresh lymph node suspensions of 13 patients with various subtypes of HD and reported the absence of Ig rearrangements in all of them. Delabie et al. analyzed resuspended single cells from formalin-fixed paraffin-embedded tissue of lymphocyte predominant HD (LPHD). In four cases analyzed, the CDR3 region of the Ig heavy chain gene rearrangement was amplified from the L&H cells, the putative malignant H-RS cell equivalents of LPHD. Since the amplified regions differed in length and sequence within each case, these experiments suggested a polyclonal B-cell nature of the tumor cells in LPHD. Tamaru et al. analyzed HD cases with a B-cell phenotype by PCR amplification of IgH gene rearrangements in whole tissue sections and also found evidence for a B-cell origin of H-RS cells. However, in this analysis it remained unclear whether the clonal Ig gene rearrangements detected originated from the H-RS cells. The question of clonality of H-RS cells was further addressed using interphase cytogenetic and FISH analysis. These authors found evidence for the clonality of H-RS cells in seven of seven cases analyzed. At present it remains unclear whether methodological aspects or sample diversity account for these discrepancies. Possibly in HD different subentities with different lineage origin of H-RS cells exist. In addition, HD may start as a polyclonal disorder and progress to a monoclonal neoplasm in the course of the disease. More cases will have to be analyzed to answer these questions. Nevertheless, the results of Küppers et al. clearly demonstrated that at least in a portion of HD-cases the H-RS cells derive from B lymphocytes at various stages of differentiation. The in vitro cultivation of L1236 cells carrying a biallelic heavy chain and a monoallelic light chain gene rearrangement provides evidence that in HD of B-cell origin in advanced stages, the H-RS cells can be present in the peripheral blood even if they are not identified as H-RS cells. In addition, the H-RS cell origin of the L1236 cell line is proven not only by analysis of

Table 2. Cytokine Production of L1236 Cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>ELISA</th>
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<tbody>
<tr>
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<td>neg</td>
</tr>
<tr>
<td>IL-4</td>
<td>neg</td>
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<td>516</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>505</td>
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</table>

Cytokine production was measured in the supernatant of exponentially growing L1236 cells (4 × 10⁵ cells/mL). The values given represent cytokine concentrations (pg/mL). Each value is a mean of two independently measured values. The sensitivity thresholds of ELISA for each cytokine is given in Materials and Methods. Negative (neg) means beyond the indicated sensitivity threshold.
morphology, surface antigen expression, and cytogenetics, but also on the molecular level by detection of identical Ig gene rearrangement sequences in the H-RS cells of the patient's bone marrow. Further genetic probes as well as specific monoclonal antibodies against L1236 may be developed. This cell line, thus, represents a valid biological model for the study of biology and homing pattern of HD and its possible relation to B-cell differentiation.

HD shares many clinical and biological characteristics with an inflammatory process, such as, eg, fluctuating fever, nightsweats, and elevated levels of IL-2 receptor in the patients serum. In affected lymphatic tissue H-RS cells are surrounded mostly by T lymphocytes. Expression of CD4, CD45Ro, and CD45RB characterizes these lymphocytes as T helper cells, expression of CD38 may indicate their previous activation. These observations led to the hypothesis, that in HD an atypical, ie, non–self-limited immune response takes place. L1236 cells express HLA class I and class II molecules, the B7.1 and B7.2 molecules (CD80, CD86) and the adhesion molecules ICAM-1 (CD54) and LFA-3 (CD58). All these molecules have been found to be crucial for physiological T-cell recruitment and activation; the B7 molecule via ligation to the CD28 molecule on the T cell and the adhesion molecules ICAM-1/LFA-3 by binding to their T-cell counterparts CD2/LFA3. It is tempting to speculate that expression of these antigens on L1236 cells and other HD-derived cell lines (authors’ own unpublished data) indicates an original function as antigen-presenting cells in an (unsuccessful) T-cell response against a still unknown viral or cellular target antigen.

Although no specific chromosome aberration has been delineated in HD up to now, cytogenetic peculiarities can be observed that differ from other lymphomas. In most cases near triploid to tetraploid chromosome numbers and an excess of structural aberrations were observed. The chromosome bands 1p13-21, 2p16-22, 4q25-q28, 6q15-q21, 7q11.2-23q, 11q13-q23, 12p11-p13, 12q22-q23, and 19p13 are nonrandomly involved in rearrangements in HD. Moreover, the short arms of acrocentric chromosomes, harboring genes for the ribosomal RNA, the so-called nucleolus organizer regions, seem to be affected by chromosome aberrations. The new established cell line L1236 is characterized by a near-triploid karyotype with multiple structural rearrangements involving chromosome bands that have been reported to be consistently rearranged in HD. As observed in the HD-derived cell lines L428 and L540, chromosomes 1, 2, 6, 7, 11, and 12 are involved in structural aberrations in L1236, too. A deletion in the long arm of chromosome 11—del(11)(q13-q14)—was observed in all three cell lines, a tetrasomy of chromosome 2 occurred in L540 and L1236, a rearrangement of the short arm of chromosome 2 could be identified in L428 as well as in L1236, the marker chromosome XX[del(12)(q15)] of L1236 was also found in L428. By comparison, the very complex composition of marker chromosomes VIII and XXIV in L1236 cells suggests that these markers developed during in vitro cultivation and do not represent HD specific anomalies. In other malignant diseases with less complex aberrant karyotypes specific so-called primary chromosome aberrations were identified that are thought to cause malignant transformation, such as, eg, Ig-gene/c-myc translocations in Burkitt’s lymphoma or the bcr/abl translocation in chronic myeloid leukemia. In contrast, in HD cytogenetic analysis of primary tumor material as well as of HD-derived cell lines show complex chromosome anomalies, so that no primary chromosomal aberration could be delineated up to now. It might be conceivable that in HD several karyotype changes have to be acquired before the disease becomes clinically apparent and before cultivation for karyotype analysis is possible. In late stages of the disease, a complex aberrant karyotype as present in L1236 cells might then correspond to an aggressive growth of H-RS cells no more restricted to lymphatic tissue and resistant to radiation and polychemotherapy.

The malignant growth potential of L1236 cells is reflected by their intralymphatic dissemination in SCID mice after SC and IP inoculation. While HD-like lesions were only exceptionally observed after transplantation of HD biopsy material into SCID mice, disseminated intralymphatic growth of HD-derived cell lines has been described. The dissemination pattern of L1236 cells resembles that of the HD-derived cell lines L540 and its subline L540Cy with involvement of axillary, mediastinal, mesenteric, and inguinal lymph nodes. Because of the similarity with the spread of HD in humans, L1236 represents a suitable tool for studying in vivo growth characteristics of H-RS cells as well as for preclinical testing of new treatment modalities. After SC inoculation into SCID-mice, HD-derived cell lines formed progressively growing tumors at the injection site. In contrast, L1236 cells inoculated SC only initially formed small tumors that underwent necrosis and regression. This resembles the in vivo growth pattern of EBV-immortalized lymphocytes in SCID mice. Despite intralymphatic dissemination of LCL cells after SC inoculation, tumors at the injection site regressed with necrosis. In nude mice there is evidence that regression of LCL tumors after SC inoculation is caused by a cytokine-induced host response. It remains to be established whether one of the numerous cytokines secreted by L1236 cells is responsible for a locally restricted anti-tumor host response in nonlymphatic SCID mouse tissue.

The cultivation of L1236 H-RS cells from the peripheral blood of a patient with advanced stage disease might also have clinical implications. After failure of first line chemotherapy, an increasing number of HD patients are treated by high-dose chemotherapy followed by autologous bone marrow transplantation or blood stem cell transplantation. This therapeutic procedure has been reported to improve rates of complete remissions and disease free survival. Up to 50% of the patients, however, suffer from lymphoma relapse. Recently, a case of NHL relapse with extended pulmonary infiltrations early after autologous bone marrow transplantation was determined to be due to contamination of the infused bone marrow with tumor cells. Similarly, a fulminant course of HD relapse after autologous peripheral stem cell transplantation was observed in our clinic (unpublished observation). At present, it remains an open question whether early relapse in these cases reflects survival of H-RS cells during high-dose chemotherapy, or, alternatively, tumor cell contamination of the grafted cells and fulminant
spread due to the missing T-cell control after intensive cytotoxic therapy. The results presented here, together with the data of Kanzler et al., for the first time formally demonstrate patients includes the risk of reinfusing malignant cells. Ab-
toxic therapy. The results presented here, together with the presence of H-RS cells in the peripheral blood of a cell transplantation after high-dose chemotherapy in these spread due to the missing T-cell control after intensive cyto-plantation possibly represents an efficient purging procedure for H-RS cells.

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