Clonal Relationship Between Lymphocytic Predominance Hodgkin’s Disease and Concurrent or Subsequent Large-Cell Lymphoma of B Lineage

By R.S. Wickert, D.D. Weisenburger, A. Tierens, T.C. Greiner, and W.C. Chan

The occurrence of a large-cell lymphoma (LCL) concurrent with or subsequent to lymphocytic predominance Hodgkin’s disease (LPHD) is well documented. Given the well-characterized B-cell nature of the Reed-Sternberg cell variants in LPHD, there may be a clonal relationship between the LPHD and the associated B-cell LCL. In this study, we adapted a highly sensitive, clonospecific assay to test whether the clone comprising the LCL exists in the corresponding LPHD tumor. Nine cases meeting the histologic criteria of nodular LPHD and B-cell LCL were identified, reviewed, and studied. Initially, clonality of both lesions was assessed using consensus primers to conserved regions in the IgH variable framework III and joining region genes in a polymerase chain reaction (PCR) assay. The PCR assay detected a clonal B-cell population in five of the LCLs, whereas analysis of eight cases of LPHD did not detect a dominant clone. Clonal products from the LCL were then sequenced, and clonospecific oligonucleotides were designed from the unique nucleotide sequence encoding the complementarity-determining region-III. These were then used as primers and/or probes in sensitive PCR-based assays on the corresponding LPHD tumors. In two cases, the clonospecific assay showed that the LPHD and LCL shared a common clone that was further confirmed by sequence analysis. This finding provides genotypic evidence that, at least in some cases, the LCL represents a clonal progression of LPHD.

© 1995 by The American Society of Hematology.

From the Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE.

Submitted March 6, 1995; accepted May 3, 1995.

Supported in part by National Institutes of Health Grant No. CA6727.

Address correspondence to Wing C. Chan, MD, Department of Pathology and Microbiology, University of Nebraska Medical Center, 600 S 42nd St, Omaha, NE 68198-3135.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1995 by The American Society of Hematology.

0006-4971/95/8606-0005$3.00/0

In 1944, Jackson and Parker described a subset of Hodgkin’s disease (HD), called paragranuloma, in which the lymph node structure was obliterated by an infiltrate with an abundance of lymphocytes. In 1966, Lukes and Butler renamed paragranuloma as HD of the lymphocytic and histiocytic (L&H) type and further divided it into two subtypes, nodular and diffuse, taking into consideration the growth pattern of the tumor. These two subtypes were later combined at the Rye conference into the lymphocytic predominance HD (LPHD), which comprises approximately 5% of newly diagnosed cases of HD.

Classical Reed-Sternberg (RS) cells are rare in LPHD, whereas the RS variant known as the L&H cell is relatively frequent and usually present singly or in small clusters. A nodular growth pattern is easily appreciated in about 80% of the cases, and the nodules bear a resemblance to the large, altered germinal centers called progressively transformed germinal centers (PTGC), which have been reported in lymph nodes biopsied before, after, or concurrent with the diagnosis of LPHD.

The L&H cells consistently express a number of B-cell-associated molecules, such as CD20, CD22, mb-1, and cytoplasmic J-chain. However, attempts to demonstrate clonality immunohistochemically by light chain restriction have generally been unsuccessful, although Schmid et al have reported light chain restriction in 5 of 19 cases of LPHD. Recent studies using in situ hybridization to detect cytoplasmic kappa and lambda mRNA have also yielded conflicting results. One recent study reported clonal immunoglobulin heavy chain (IgH) gene rearrangements in a substantial percentage of cases of LPHD by the polymerase chain reaction (PCR) analysis. However, we have not been able to confirm monoclonality in the L&H cells by single-cell analysis.

Over the years, small series of cases in which a large-cell lymphoma (LCL) occurred concurrent with or subsequent to a diagnosis of LPHD have been reported. The majority of such LCLs have been shown to belong to the B-lineage using immunohistochemical and/or molecular techniques. It has been suggested that LPHD represents a polyclonal lymphoproliferation that originates in the germinal centers and that the development of B-LCL represents a histologic progression.

We report our investigation of the relationship between LPHD and subsequent or concurrent B-LCL. The hypothesis that a small B-cell clone, most likely derived from an L&H cell, undergoes transformation and progresses to B-LCL was tested using sensitive clonospecific (CS) molecular techniques.

MATERIALS AND METHODS

Patient Population and Pathology Review

Nine patients with a diagnosis of LCL subsequent to or concurrent with LPHD were identified in the files of the Nebraska Lymphoma Registry and the consultation files of two of us (W.C.C., D.D.W.). All biopsies and pathology records were reviewed to confirm the diagnoses, and the paraffin blocks were obtained from the referring institutions. In one patient, no paraffin block representative of LPHD was available for study.

DNA Preparation

A modification of the method described by Wright and Manos was used to extract DNA from the paraffin-embedded, formalin- or B5-fixed tissue. Five-micrometer sections of tissue were dewaxed with xylene and washed in absolute ethanol. The sample was then digested overnight at 50°C in 1 × PCR buffer (50 mMm/L KC1, 10 mMm/L Tris-HCl pH 8.3, 0.1 mg/mL gelatin, 0.45% Nonidet P40, and 0.45% Tween 20) containing 200 µg/mL of proteinase K. Finally, the proteinase K was inactivated by a 10-minute incubation at 94°C. For tissues fixed in B5, the procedure was modified to
include a 4-minute incubation in 1% (wt/vol) iodine in xylene to remove the mercury introduced by the fixative.

High-molecular-weight (HMW) DNA was extracted from frozen tissue as previously described.26 The integrity of the isolated DNA from both frozen and paraffin-embedded materials was evaluated in a PCR-based assay using primers to the human beta tubulin gene.27 A 280-bp product, visualized on a 1.5% agarose gel (NuSieve 3:1 agarose blend; FMC BioProducts, Rockland, ME) stained with 0.2 µg/mL ethidium bromide, confirmed the presence of amplifiable DNA.

To prevent cross-contamination, either a new or an unused portion of the microtome blade was used to cut the paraffin blocks. The bath was cleaned, and the water was replaced before each sectioning. No LPHD and corresponding LCL specimens were cut or extracted on the same day. The DNA was physically extracted in an area free of previous PCR products, and gloves were changed frequently during the procedures.

Complementarity Determining Region-III (CDR-III)

Clonality in Tumor Specimens

To assess the clonality of the LCL and LPHD specimens, a semi-nested PCR approach based on the technique of Wan et al26 was used. The primers used are listed in Table 1. A hot start procedure was used to maximize the yield of specific product.28 Thirty PCR cycles were used, with 40 seconds at 94°C to denature, 40 seconds at 55°C to anneal, and 40 seconds at 72°C to extend the DNA. A second round of amplification using 5 µL of a 1:500 dilution of the initial reaction products was performed under similar reaction conditions with the JH nested primer replacing the initial JH primer (sequence in Table 1), but only 20 cycles were used.

The amplification products were analyzed in 8% nondenaturing polyacrylamide gels (Ambrosen, Solon, OH) in 1 X TBE buffer (0.089 mol/L Tris-borate, 0.002 mol/L EDTA pH 8.0). The positive control consisted of 1:10 and 1:100 dilutions of Namalwa DNA (a Burkitt’s lymphoma cell line known to have a rearranged IgH gene) in germline DNA.29 The negative controls consisted of either germline or polyclonal DNA from the myeloid leukemia cell line K562 or peripheral blood mononuclear cells (PBMC), respectively, and sterile water.

Sequencing the Clonal CDRIII Product

The PCR products from cases showing a clear monoclonal band with no detectable polyclonal background products on the polyacrylamide gel were directly sequenced using a 50-µL aliquot of the double-stranded PCR products. A clonal band among a smear of polyclonal products required further separation by denaturing gradient gel electrophoresis (DGGE).30 To prepare products for DGGE, a GC-clamped JH primer (Table 1) was used in the second amplification step of the CDRIII assay. After DGGE and staining in ethidium bromide, the band of interest was excised using a clean scalpel, and DNA was extracted by freeze-thawing the gel slice three times using a dry ice/ethanol slurry and a 37°C water bath. To prepare products without the GC-clamped end for sequencing, 5 µL of a 1:500 dilution of the extract in water was reamplified using the JH nested and Vp80 primers, as described above. Excess primers, deoxynucleotides, and buffer components were efficiently removed with Qiagen-spin columns (Qiagen, Inc, Chatsworth, CA) according to the manufacturer’s recommendations.

Sequencing reactions were performed in a Perkin Elmer-Cetus Model 480 thermocycler (Perkin Elmer-Cetus, Foster City, CA) using one of the amplification primers, JH nested or Vp80, along with Taq polymerase and four dye-labeled deoxyxynucleotide terminators supplied in the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Excess deoxyxynucleotide terminators were removed with a Centri-Sep column (Princeton Separations, Adelphia, NJ) before product analysis on an Applied Biosystems 373A automated DNA sequencer. Sequence data generated with each primer were aligned (Sequence Navigator; Applied Biosystems) to ensure correct base assignment.

Design of CS Oligonucleotide Primers and Probes

Computer analysis of the sequence was performed using the sequence analysis software package of the University of Wisconsin Genetics Computer Group, Release 7.3. The sequences obtained were compared with an indexed previously published D and JH genes provided by Dr Harry Schroeder, Jr (University of Alabama, Birmingham, AL). CS oligonucleotide primers and probes were designed with the help of a primer analysis program (Oligo version 4.1; National Biosciences, Inc, Plymouth, MN).

Confirmation of the Specificity of the CS Oligonucleotide Probes

To confirm the specificity of the CS probes, a dot blot assay was performed. Clonal CDRIII products derived from the LCL specimens were retrieved, as well as CDRIII products from peripheral blood lymphocytes (PBL) and the Namalwa cell line. After running an aliquot on an 8% polyacrylamide gel to confirm the presence of analytes, 5 µL and 2 µL were denatured in 200 µL of 0.4 N NaOH for 30 minutes. The DNA was blotted to a nylon filter using a dot blot apparatus (S&S Minifold I; Schleicher & Schuett, Keene, NH) and was ultraviolet (UV) crosslinked.

Ten picomoles of probe were radiolabeled with [γ-32P]-adenosine triphosphate (ATP) using T4 Polynucleotide Kinase (Life Technologies, Gaithersburg, MD). Hybridization with 5 pmol of the labeled probe proceeded for 4 hours at 10°C below Tm, as calculated from the formula Tm = 4 (G+C) + 2(A+T), with constant agitation maintained by a hybridization oven (Model 310; Robbins Scientific, Sunnysvale, CA). Filters were washed once in 2 X saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) for 15 minutes at room temperature and then washed for another 15 minutes in a pre-warmed portion of the same solution in the hybridization oven at the original hybridization temperature. Autoradiography commenced overnight at ~70°C in a light-proof cassette with intensifying screens.

Determining the Sensitivity of the CS Assay

To evaluate the sensitivity of the CS amplifications, serial 10-fold dilutions of the LCL DNA were made into carrier DNA (K562 or
Detection of products were used as template in the second step. Finally, either the reactions based on the estimation that a follow-up hybridization with the corresponding CS probes to determine the sensitivity of the CS probe without a specific primer amplification step. All products were run on 2% agarose gels, transferred to nylon filters (Turboblotter; Schleicher & Schuell), and probed as described above.

**Detection of the LCL Clone in the Corresponding LPHD**

Undiluted DNA samples extracted from the LPHD specimens were analyzed using consensus CS primers and were probed with CS probes as described in the sensitivity validation assays. A total DNA equivalent of 200,000 cells was assayed in six separate reactions based on the estimation that a 1 cm x 1 cm x 5 μm tissue section contains approximately 500,000 cells. Controls consisted of the 1:100 and 1:1,000 dilutions of LCL DNA and PBMC DNA, a 1:100 dilution of Namalwa DNA, and sterile water. Only the consensus primers were used in cases 6 and 9, in which a CS primer was not available. All PCR products were electrophoresed on 2% agarose gels, transferred to nylon filters, and probed as described earlier.

**RESULTS**

**Clinical and Pathologic Data**

The ages of the nine patients ranged from 21 to 70 years (median, 45 years), and the male-to-female ratio was 5:4. The most common localization of LPHD was the axillary lymph nodes (n = 4), with no LCL site predominating. In four of the five patients with concurrent disease, both processes presented at the same anatomic site. Of the four patients with subsequent diagnoses of LCL, all presented with LCLs at sites other than the initial LPHD. The time intervals in this second group were 6, 26, 209, and 269 months. The clinical data are presented in Table 2.

All cases of LPHD were classified as the nodular subtype, and the L&H cells in all seven cases studied had a B-cell phenotype (CD20+, with/without CD74+). The LCLs were also of the B-cell type in the eight cases studied. In all but one case, the LPHD and the LCL were taken from separate tissue blocks, and the LCL occurred as a large macroscopic nodule. In case 6, the LCL existed as a large nodule amid typical LPHD, and this nodule was microdissected for molecular study.

**Molecular Data**

**DNA integrity:** DNA from 15 paraffin-embedded tissue blocks was extracted, and its suitability for PCR analysis was evaluated by amplification of a 280-bp region of beta tubulin, a gene present in all nucleated cells. Successful amplification of this target was achieved in 66% (10 of 15) of the available materials (Table 3). DNA extracted from the two frozen tissues was shown to be intact by the same procedure. In cases 6 and 9, a clonal CDRIII rearrangement was demonstrable by PCR even though the β-tubulin gene was not amplifiable, probably because the small size of the CDRIII fragment made the assay less sensitive to DNA degradation.

**Clonality of LPHD and LCL.** Material representative of LPHD was analyzed with consensus primers to DNA sequences flanking the CDRIII of the IgH genes (Table 3). Of the eight cases that could be studied, an oligoclonal pattern consisting of many bands was observed in approximately

---

**Table 2. Clinical Findings**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yr)/Sex</th>
<th>Localization LPHD</th>
<th>Occurrence</th>
<th>Localization LCL</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Simultaneous</td>
<td></td>
<td>Subsequent (interval)</td>
</tr>
<tr>
<td>1</td>
<td>70/F</td>
<td>R femoral LN</td>
<td>Colon</td>
<td>+ (26 mos)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>38/M</td>
<td>L axillary LN</td>
<td>Spleen</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>47/M</td>
<td>R axillary LN</td>
<td>R axillary LN</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>45/F</td>
<td>Paraortic LN</td>
<td>Paraortic LN</td>
<td>+ (269 mos)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>21/M</td>
<td>Axillary LN</td>
<td>R femoral LN</td>
<td>+ (269 mos)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>52/M</td>
<td>Mesenteric LN</td>
<td>Mesenteric LN</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>51/F</td>
<td>Abdominal LN</td>
<td>L and R cervical LN</td>
<td>+ (209 mos)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25/F</td>
<td>SC LN</td>
<td>Breast mass</td>
<td>+ (6 mos)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>33/M</td>
<td>R axillary LN</td>
<td>R axillary LN</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** LN, lymph node; R, right; L, left; SC, supraclavicular.

---

**Table 3. Molecular Analysis**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Tubulin</th>
<th>CDRIII</th>
<th>Tubulin</th>
<th>CDRIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+ (w)</td>
<td>O</td>
<td>+</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>O</td>
<td>+</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>O</td>
<td>+</td>
<td>O</td>
</tr>
<tr>
<td>5</td>
<td>+ (w)</td>
<td>O</td>
<td>+</td>
<td>−*</td>
</tr>
<tr>
<td>6</td>
<td>+ (f)</td>
<td>O</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>P</td>
<td>+ (f)</td>
<td>C*</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>P</td>
<td>−</td>
<td>C</td>
</tr>
</tbody>
</table>

**Abbreviations:** +, amplification detected; −, no amplification detected; C, clonal (1 or 2 bands); O, oligoclonal (many discrete bands); P, polyclonal (smear of products); w, weak; f, frozen tissue used for analysis; NA, not available.

* IgH clonally rearranged by Southern analysis.
60% (five of eight) of the cases. Two cases yielded a polyclonal pattern with the amplification products forming a smear along the expected size range, and no particular sized product predominated. In case 2, with negative β-tubulin PCR, no amplification products were demonstrated.

In the corresponding LCLs, clonality was demonstrated in 56% (five of nine) of the cases, as shown in Fig 1. Of these five cases, a monoclonal band alone was observed in three. Two bands were amplified in case 9, and a single band among a dense polyclonal smear was seen in case 8. DGGE was used to resolve the complex banding pattern and to isolate the clonal products from cases 8 and 9 for further study. Results from the other four LCL cases included one case with an oligoclonal pattern and three cases with no amplification products. Southern analysis for IgH rearrangement was performed on cases 5 and 8, and, in each case, a clonal population was demonstrated. PCR analysis of these two cases, however, demonstrated a clonal product in case 8, whereas no amplification products were observed in case 5.

Studies with CS primers and probes. The sequence of CDRIII was obtained in five LCL samples, and CS primers and probes were designed (Table 4). The specificity of the five CS oligonucleotide probes was confirmed by dot blot analysis. In each case, a hybridization signal was detected only in the area blotted with the corresponding patient CDRIII products. No cross-hybridization with other patient- or PBL-derived CDRIII products was observed, as shown in Fig 2.

The sensitivity of amplification assays using both CS primers and probes consistently reached the 1:10,000 dilution in all three patients (cases 1, 3, and 8) studied, as shown in Fig 3. The sensitivity of the CS probe to targets generated by consensus primers was 1:10,000 in two cases, and 10-fold less (1:1,000) in one.

Five cases of LPHD associated with LCL were suitable for further analysis using CS primers and/or probes (Table 5). Only CS probing of the LPHD could be performed in two patients because size constraints of the clonal CDRIII products made it impossible to design good CS primers as well. In these two patients, the LCL clone was not detected in the corresponding LPHD tissue. However, CS amplification revealed the presence of the LCL clone in the LPHD component in two of the remaining patients. In case 3, LCL and LPHD were present in the same anatomic site, but in different tissue blocks, whereas case 8 presented with LCL at a different site 6 months after the initial LPHD diagnosis (Table 2). In all experiments, the negative controls (PBMC DNA, 1:100 Namalwa DNA, and sterile water) displayed no hybridization with the CS probe (Figs 4 and 5). Table 5 summarizes the results of these experiments.

The CDRIII products amplified with CS primers from the LPHD tissue of cases 3 and 8 were directly sequenced, and the sequences were compared with those obtained from the corresponding LCLs (Table 6). The sequences obtained from case 8 were identical. The sequence from the LPHD of case 3 contained one position that cannot be assigned definitively, while
Table 4. CDR III Sequence Data

<table>
<thead>
<tr>
<th>Case No.</th>
<th>V\textsubscript{\textalpha} Segment</th>
<th>NDN</th>
<th>J\textsubscript{\textgreek{n}} Segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TGGGTCACAC</td>
<td>GGGGGCACGGTAGTGACTCCCGT</td>
<td>TGGTACTTTCGATCTC</td>
</tr>
<tr>
<td>3</td>
<td>TGGAAGAGA</td>
<td>TTTTGGAAGAAGACTCTTACTTCggggatta</td>
<td>ttgattcc</td>
</tr>
<tr>
<td>6</td>
<td>TGCGCGA</td>
<td>GGCCTACTACTC</td>
<td>GACCTAT</td>
</tr>
<tr>
<td>8</td>
<td>TGCGAGA</td>
<td>GTCTGCAAAGTGTACCTTGATACAAAAaggacactctatgtact</td>
<td>tggACTCC</td>
</tr>
<tr>
<td>9</td>
<td>TGCTAGA</td>
<td>GGAAGCGTAGTGGCN</td>
<td>GATTAC</td>
</tr>
</tbody>
</table>

Abbreviation: NDN, D segment with franking N sequence.

Letters in upper case bold indicate sequence used as primer; lower case bold, reverse complement of sequence used as primer; underscore, sequence used as probe; *, sequence with homology to published D-region genes.

DISCUSSION

The cases of LPHD in this series had the classical morphology with a macronodular architecture. However, the definition of an LCL arising in LPHD is still somewhat controversial. We do not consider microscopic nodules of L&H cells as evidence of LCL, and all the LCLs in this study were macroscopically identifiable tumors that were clearly delineated from the LPHD areas. In all cases except one, the LCL was in a separate tissue block from the LPHD studied. In case 6, the two entities occurred in the same block but could be readily separated from each other. It is noteworthy that the LCL clone was not detected in the LPHD portion in this latter case, suggesting that cross-contamination is preventable even when the two components occur in the same tissue block.

We chose to amplify the LCL components with consensus primers to the FR3 (V\textsubscript{6\textalpha}) and FR4 (J\textsubscript{\textgreek{n}}) of the V\textsubscript{\textgreek{n}} genes. The small product amplified with these primers was ideal when working with DNA extracted from routinely fixed, paraffin-embedded biopsy specimens. Indeed, two of our LCL cases showed no amplification at 280 bp with the \beta-tubulin primers but gave clonal bands of approximately 100 bp with the consensus V and J primers. In contrast, two other LCL cases were successfully amplified for the \beta-tubulin target but exhibited no CDR III amplification products. PCR methods for the detection of B-cell clonality using the consensus primers to amplify the CDR III may be falsely negative for a variety of reasons, and about 30% of Southern blot positive cases do not generate clonal CDR III bands.  

Our current data and data from our previous study do not demonstrate a dominant clonal B-cell population in LPHD. Multiple-sized CDR III products with no clearly dominant band were identified in all seven of our cases with amplifiable DNA. In contrast, analysis of the corresponding LCL component showed clonal B-cell populations in six cases (one case by Southern analysis only), whereas a pattern more consistent with an oligoclonal proliferation was observed in one other case. Of the six clonal cases, five were suitable for analysis with CS methods.

The application of DGGE to purify clonal products from a polyclonal background before sequencing was useful. A discrete clonal band without the heavy polyclonal background smear was obtained in case 8 after DGGE. Two presumably clonal CDR III bands were detected by routine polyacrylamide gel electrophoresis in case 9. When they
Fig 3. Sensitivity of CS amplification. The top panel shows an ethidium bromide-stained 2% agarose gel electrophoresis of CS products from case 8, and the bottom panel shows the resulting autoradiograph after Southern transfer and hybridization with labeled CS probe. Lane A, molecular size marker (bottom band is 100 bp, and others are consecutive multiples of 100); lane B, CDRIII products amplified from LCL DNA (120,000 targets); lanes C through G, products obtained from serial 10-fold dilutions of the LCL DNA into HL-60 DNA; lane H, products from HL-60 DNA alone; lane I, water control. Specific CDRIII products are detectable down to the 10⁻⁴ dilution (lane F).

Fig 4. Detection of the LCL clone in LPHD (case 3). The top panel shows a 2% agarose gel of CDRIII products, and the bottom panel shows the resulting autoradiograph after Southern transfer and hybridization with labeled CS probe. Lane A, molecular size marker (bottom band is 100 bp, and others are consecutive multiples of 100); lane B, 1:1,000 dilution (120 targets) of LCL DNA in HL-60 DNA; lane C, 1:10,000 LCL dilution (12 targets); lanes D and E, LPHD DNA amplified with CS primer; lanes F through K, LPHD DNA amplified with consensus primers only; lane L, 1:100 dilution of Namalwa cell line into K562; lane M, CDRIII amplified from PBMC isolated from a normal donor; lane N, water control. CS products are identified in the LPHD tumor when either the CS primar or consensus primers are used for amplification.
were independently excised, purified, and sequenced, the resulting data were not interpretable. We then analyzed each band using DGGE, and only the lower band proved to be clonal and was subsequently sequenced successfully. The failure to produce good sequence data in the first experiments was interpreted as contamination by irrelevant background CDRIII sequences. The upper band in case 9, as visualized in the standard gel, may have been a heterogenous mixture of similar-sized CDRIII products.

The CS assays did not detect an occult clone in three of the five LPHD tumors studied. Before concluding that the clone is absent in the corresponding LPHD, several issues need to be considered: (1) The IgH V region genes can undergo somatic mutation, which takes place in germinal center B cells. The nodules in LPHD have the structure of altered germinal centers, and it is possible that L&H cells have a high rate of V\textsubscript{H} mutation, as do normal germinal center cells. Thus, if enough significant mutations have occurred in the LCL clone, the specific oligonucleotides based on its sequence may not be efficient in detecting the original sequence present in the LPHD and may result in a false negative. (2) Our sensitivity studies with serial dilutions of tumor DNA revealed a consistent level of detection at one tumor cell per 10,000 normal cells when both a CS primer and a CS probe were used. Alteration or degradation of cellular DNA in the archival tissue might have prevented us from detecting tumor DNA at the single-copy level. To increase the chance of detecting the neoplastic clone, a total of about 200,000 cells were sampled in six assays per case. (3) If the clonal population in LPHD was present only focally, it might not have been included in the tissue sample studied. (4) The possibility of Taq polymerase-induced er-

### Table 5. Detection of the LCL Clone in the LPHD Tumor

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Amplification Method</th>
<th>Specific Primer</th>
<th>Consensus Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

All results were confirmed by hybridization to a radiolabeled CS probe: +, detection; –, no detection. Abbreviation: ND, not done.

### Table 6. CDRIII Sequence Comparison

<table>
<thead>
<tr>
<th>Case No. and Source</th>
<th>Sequence Between Primers</th>
<th>% Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>TGGGAAAGAT TTGGGACTAAAGACTCTA</td>
<td>97 (28/29)</td>
</tr>
<tr>
<td>LPHD</td>
<td>TGGGAAAGAT TTGGGACTAAAGACTCTA</td>
<td>100 (34/34)</td>
</tr>
<tr>
<td>LCL</td>
<td>TGGGAAAGAT TTGGGACTAAAGACTCTA</td>
<td>100 (34/34)</td>
</tr>
<tr>
<td>8</td>
<td>TGCGAGAGTC TGCGAAAGTTGACACTGGTACAAAA</td>
<td>100 (34/34)</td>
</tr>
</tbody>
</table>

Vertical lines indicate homology; underscore, probe sequence. Calculation of the percentage of homology excluded the primer sequences (not included in the figure). N indicates base assignment not possible.
HISTOLOGICAL PROGRESSION:

PTGC → LPHD → LCL.

CLONAL PROGRESSION:

Polyclonal → Oligoclonal → Monoclonal

Fig 6. Hypothesized relationship of PTGC, LPHD, and LCL with the postulated changes in the clonality of the L&H cells.

errors in the primer/probe sequences is unlikely as direct product sequencing greatly reduces errors due to imperfect enzyme fidelity. From the above considerations, our investigation tends to underestimate the frequency of a clonal relationship between LPHD and B-LCL.

In cases I and II, we were able to confirm that the B-cell clone comprising the LCL was also present in the LPHD, not only from the CS assay but also from direct sequencing of the PCR products from the LPHD samples. The CDR3 of each B cell is unique, and it is highly unlikely for such homology (Table 6) to be obtained from two unrelated B cells. These data indicate that at least some LCLs represent a clonal progression of the LPHD. This study and the findings of other investigators support the following hypothesis on the evolution of LPHD: Progressively transformed germinal centers (PTGC) share many structural and immunologic similarities with the nodules of LPHD and may represent a precursor lesion to LPHD in some cases. Most cases with PTGC resolve without further consequence. However, in a small percentage of patients, events still unknown act to perpetuate this process and transform some of the B cells into L&H cells, the presence of which confirms a diagnosis of LPHD. At an early stage of the disease, these cells are likely to be polyclonal, as demonstrated in our previous study of single L&H cells. As the L&H cells continue to slowly proliferate, the cellular composition of the lesion becomes progressively more oligoclonal, which would account for the reported detection of clonal IgH rearrangements in some cases. In most patients, this indolent state exists for years, but in a minority, additional genetic events act on one of the B-cell subclones, resulting in its preferential expansion into a monoclonal proliferation that is morphologically recognized as a B-LCL. This hypothetical relationship of PTGC, LPHD, and B-LCL is shown in Fig 6. The hypothesis could be tested by combining the CS assay with single-cell studies and/or in situ PCR in suitable cases. Although the progression to LCL occurs in only a subset of individuals, it will be important to further study the molecular evolution of this process. Knowledge regarding this phenomenon may eventually lead to a better understanding of the pathogenesis of this enigmatic condition.

ACKNOWLEDGMENT

We thank Dr G. Zatarri and the members of the Nebraska Lymphoma Study Group for their referral of the cases, Martin Bast for clinical information, Karen Hansen for typing the manuscript, and George Pallas for photography.

REFERENCES


From www.bloodjournal.org by guest on November 16, 2017. For personal use only.


Clonal relationship between lymphocytic predominance Hodgkin's disease and concurrent or subsequent large-cell lymphoma of B lineage

RS Wickert, DD Weisenburger, A Tierens, TC Greiner and WC Chan