Nodular Lymphocyte Predominance Hodgkin’s Disease: A Monoclonal or Polyclonal B-Cell Disorder?


Nodular lymphocyte predominance Hodgkin’s disease (NLPHD) is characterized by the presence of atypical putatively neoplastic cells (L & H cells) with a B-cell phenotype. A proportion of patients with NLPHD develop a simultaneous or subsequent large cell B lymphoma (LCL) that is thought to evolve directly from the L & H cells of NLPHD. However, the clonal nature of L & H cells remains controversial, and the relationship between NLPHD and complicating LCL has not been fully established. In an attempt to determine the clonality of L & H cells and to clarify the link between NLPHD and complicating LCL, we used polymerase chain reaction (PCR) to analyze 33 cases of NLPHD, including 15 cases with simultaneous or subsequent LCL, for clonal immunoglobulin (Ig) heavy chain variable region (VH) gene rearrangements. PCR amplifications with consensus primers covering framework 2 or framework 3 to joining region were performed on paraffin-embedded tissue sections and, in 12 cases, on microdissection-enriched L & H cells. No clonal Ig rearrangements were detected. In eight of the 15 LCL, monoclonal IgVH regions were amplified, four of which were cloned and sequenced. Clone specific primers were designed based on the unique N region sequences. These allowed detection of LCL clones at a sensitivity up to 1,000 times greater than the consensus primers, as determined by dilution assays. However, no LCL clones were detected in the preceding NLPHD, including microdissection-enriched L & H cells. Our results suggest that populations of L & H cells do not carry monoclonal Ig rearrangements and provide no evidence for a clonal link between NLPHD and complicating LCL.

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MATERIALS AND METHODS

Tissue samples. Paraffin-embedded blocks or unstained tissue sections from 33 cases of NLPHD were retrieved from the surgical files of the Histopathology Department, University College London Medical School. Fifteen of the 33 NLPHD cases had complicating high grade B-cell lymphomas (LCL), five of which developed simultaneously, five developed 1 to 5 years after diagnosis of NLPHD (including cases 3 and 4) and 5 after more than 10 years (including cases one and two). Hematoxylin and eosin stained sections of all cases were available for histological review.

Immunostaining. Four-micron tissue sections were cut. After de-waxing, the sections were heated in a pressure cooker or a microwave oven to retrieve antigenicity and immunostaining was performed as previously described.25-27 Whenever possible, depending on availability of adequate tissue in the blocks, sections were stained with antibodies to Ig kappa/lambda, CD20, CD79a, CD3, and CD21 (Dako, High Wycombe, UK).

Microdissection and DNA preparation. Microdissection was performed as previously described. Briefly, L & H cells were highlighted by immunostaining with antibodies to CD20 or CD79a. Clusters of L & H cells from a single nodule or from different nodules

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of the same section were microdissected by a drawn-out glass pipette in the presence of 20% ethanol under a microscope (Fig 1). The microdissected fragments were placed into a solution of 10% ethanol on a clean slide where L & H cells were further separated from small immunostained B cells under the microscope with similar drawn-out glass pipettes or 'knives'. At least 50 L & H cells with no visible small immunostained B cells were collected from each nodule or pooled from several nodules in cases with relatively sparse L & H cells. CD3 positive cells (about 100 cells per case with an estimated purity over 90%) in the T-cell areas were also isolated with the same double microdissection technique. These cells together with washings were collected as negative controls. Cells were transferred into a microtube, dried, and digested with 20 μL of proteinase K digestion buffer. Extraction of DNA from whole tissue sections was performed as previously described.28 Digests were heated to 95°C to destroy proteinase K before PCR.

**Consensus PCR amplification of Ig heavy chain gene.** Amplification of Ig heavy chain gene from the framework 3 (FR3) to the joining (JH) region was performed using the seminested method of Ramasamy et al13 with the modifications described by Tamaru et al.22 All LCL samples and extracts from 25 NLPHD cases (including all the LEL associated cases) were analyzed using both sets of primers. The remaining eight NLPHD cases and micro-dissected samples were analyzed using FR3/JH primers alone due to limited amounts of DNA. All samples were analyzed in duplicate and run in parallel with positive (DNA from a monoclonal follicular lymphoma) and negative (no DNA) controls. Products were run on 10% (FR3/JH) or 6% (FR2/JH) polyacrylamide gels, stained with ethidium bromide and viewed under ultraviolet (UV) light.

**Cloning and sequencing.** Twenty to forty microliters of PCR products were end-filled by adding 5 U (1 μL) of DNA polymerase klenow fragment (Pharmacia Ltd, Milton Keynes, UK) and incubated...
were sequenced using Sequenase PCR Product Sequencing kit (Stratagene Ltd, Cambridge, UK). Clones containing fragments of the appropriate size were screened with PCR. Positive clones were sequenced using Sequenase PCR Product Sequencing kit (USB, Cleveland, Ohio) following the manufacturer’s instructions.

Clone specific primer design and PCR amplification. Clone specific primers (CSP) were designed using the unique complementarity determining region (CDR) III sequences centered on N regions of rearranged Ig VH genes amplified from LCL. Sequences between the FR3 and VLJH primer regions of rearranged Ig VH genes amplified from LCL are shown. Oligonucleotide sequences taken from the deduced unique N regions that were selected as targets for CSP are shown in bold capitals. The entire sequences of all the selected clones are available on request from the corresponding author.

Clones were characterized by cells bearing a close resemblance to typical L & H cells, four by large centroblast-like cells and one by large cells with typical features of Burkitt’s lymphoma. In one case the morphology was obscured by necrosis.

Immunostaining. L & H cells in each case of NLPHD were clearly defined by immunostaining with antibodies to CD20 or CD79a. These cells were usually present within nodules composed of small B cells and variable numbers of T cells. Characteristically the L & H cells were surrounded by rosettes or large clusters of T cells. In all cases there were larger concentrations of L & H cells, sometimes outside the B-cell nodules, usually separated by intervening T cells. These concentrations of L & H cells were preferentially selected for microdissection (Fig 1). In eighteen of the 25 cases, there was sufficient material for immunostaining of Ig light chains, and kappa light chain restriction was demonstrated in the L & H cells of 11 cases. Staining in six cases was not satisfactory. One case was negative for both kappa and lambda light chains. All 15 LCLs showed staining with CD20 and CD79a. Four showed kappa light chain restriction, and staining of three cases was unsatisfactory. Eight cases were not stained for light chains due to shortage of material.

PCR amplification with consensus primers. Using FR3/JH primers, all extracts from 33 complete NLPHD sections and microdissected L & H cells from 12 cases of NLPHD gave rise to a smear or variable ladder of PCR products. With FR2/JH primers a smear or ladder of products was seen in 16 (including cases 1 through 4) of the 25 NLPHD cases, whereas nine were not amplifiable. Thus, no monoclonal populations of B cells were detected in the NLPHD samples. Of 15 cases of LCL, eight gave rise to a reproduc-

<table>
<thead>
<tr>
<th>Case</th>
<th>CSP Sequence</th>
<th>2nd Primer</th>
<th>Product Size (bp)</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-CAAAGCATGCTATCTGTCT-3'</td>
<td>FR2\textsuperscript{31}; FR3\textsuperscript{30}</td>
<td>214; 51</td>
<td>5×; 10×</td>
</tr>
<tr>
<td>2</td>
<td>5'-TCGAGACCACCTTTTACC-3'</td>
<td>FR3\textsuperscript{30}</td>
<td>78</td>
<td>10×</td>
</tr>
<tr>
<td>3</td>
<td>5'-CCGATTATTTGTTGGACG-3'</td>
<td>FR3\textsuperscript{30}</td>
<td>93</td>
<td>1000×</td>
</tr>
<tr>
<td>4</td>
<td>5'-TACCTTGTCACCAGTA-3'</td>
<td>FR2\textsuperscript{31}; FR3\textsuperscript{30}</td>
<td>216; 55</td>
<td>100×; 1×</td>
</tr>
</tbody>
</table>

CSP-PCR: Reverse sequences complementary to the clone specific sequences shown in Table 1 were used in conjunction with FR2 or FR3 primers for PCR amplification. Sensitivity of the CSP-PCR is compared with that of consensus primer PCR.
CLONALITY OF LP HODGKIN’S DISEASE

Fig 2. PCR amplification of LCL and microdissected L & H cells using consensus primers (FR3/JH). Products were run on a 10% polyacrylamide gel. Lane M, PhiX/Hinfl molecular weight markers (the 100-bp fragment is indicated); P, positive control follicular lymphoma; N, negative control without DNA; A, LCL extract from case 2; B, microdissected L & H cells from case 2; C, LCL extract from case 1; D, microdissected L & H cells from case 1.

ible dominant band when amplified using FR3/JH primers, four (cases 1 through 4) of which showed a dominant band using FR2/JH. The remaining seven cases showed a smear of PCR products. Thus, monoclonality was confirmed in 53% of LCL cases. Comparison of PCR products from the LCL cases with those from the corresponding cases of NLPHD, including microdissected L & H cells showed no matching dominant bands to the clone in the LCLs (Fig 2). PCR reactions with water (no template) and samples from microdissected T-cell populations and washings showed no products, whereas the positive control gave rise to the expected dominant band in each reaction.

Cloning and sequencing. IgVH PCR products (cases 2 through 4 from FR2 to JH region; case 1 from FR3 to JH region) from four cases of LCL were cloned. At least six clones from each case were sequenced. Sequence analysis showed that all clones contained combinations of variable (V), diversity (D), and joining (J) fragments and had a variable number of N nucleotides at the V-D (N1) and D-J (N2) junctions (Table 1). None of the N region sequences from any one case was similar to those from other cases or showed high homology to any sequence in the GCG gene database. The three cases amplified from FR2/JH were analyzed for VH usage and all found to belong to the VH3-7 family. When compared with germ line sequences, several silent and replacement mutations were identified in CDR2 and FR3 in all cases. In case 4, some mutations were common to all the clones, but others varied. These intrainival variations may indicate ongoing mutations in the tumor.

Clone specific PCR. All four clone specific primers, in conjunction with FR2 or FR3 primer, gave rise to products of the predicted size when applied to the specific LCL extract, but no products were seen from other lymphoid tissue samples. Efficiency of clone specific detection varied between cases and different framework primers (Table 2). Using FR2 with the clone specific primers, sensitivities of specific PCR increased by five (case 1) to 100 (case 4) times, when compared with consensus PCR (Fig 3). Using FR3 as the second primer, the sensitivities ranged from identical (case 4) to 1,000 times more sensitive. No products were seen using CSP on extracts of complete sections or microdissected L & H cells from NLPHD, which preceded specific LCL (Fig 4).

DISCUSSION

Southern blot and PCR analyses for Ig gene rearrangements have failed to demonstrate monoclonal Ig gene rearrangements
in NLPHD in the majority of studies. This failure has largely been thought to result from the paucity of the L & H cells in tissue samples and the relative insensitivity of the techniques used. However, in a recent study of Tamaru et al, using an improved PCR strategy, monoclonality was demonstrated in over 60% of NLPHD cases without prior microdissection. Monoclonality was also shown in single L & H cells microdissected from a single case of NLPHD. In the present study, we did not detect clonal Ig gene rearrangement in any of our cases, even with an optimized strategy using two seminested PCR reactions (FR2 to JH and FR3 to JH), one of which (FR2 to JH) was identical to that used by Tamaru et al. In contrast, we consistently demonstrated polyclonal patterns of PCR products in microdissection-enriched L & H cells. The reasons for this disparity are unclear, although it is likely that some cases of NLPHD are monoclonal. It is also possible that the polyclonal PCR products were amplified from contaminating small B cells rather than from L & H cells in the microdissected samples. However, this is unlikely as no visible CD20/CD79a positive small cells were seen in the selected populations.

Polyclonal Ig gene rearrangements have also been demonstrated in the L & H cells from two cases of NLPHD by Delabie et al using PCR and single cell isolation techniques. In our study, clusters of L & H cells were clearly defined by immunostaining of tissue sections. This allowed reliable isolation of the target cells using a well established microdissection technique. Our polyclonal PCR results further support the findings of Delabie et al. Polyclonal rearrangements of Ig genes may occur in tumors of precursor B cells, such as acute lymphoblastic leukemia, due to ongoing VH gene replacements within the tumor clone. It is unlikely that such secondary Ig gene rearrangements are occurring in NLPHD, as sequence analysis in previous studies showed no VH replacements among the clones obtained from the L & H cells. In addition, the L & H cells have a mature B-cell phenotype. False negatives due to failure of primer binding are unlikely in such a large series of cases.

Attempts have also been made to determine monoclonality of the L & H cells by analysis of Ig light chain protein or mRNA expression. Polyclonal patterns of immunostaining have been observed in L & H cells., but this is generally considered a result of passive uptake by the cells. Using an improved immunohistochemical method, Schimid et al were able to demonstrate kappa light chain restriction in L & H cells in 18 of 19 NLPHD cases. This finding was supported by two in situ hybridization studies in which monotypic Ig kappa mRNA was detected in the L & H cells in 50% to 80% of NLPHD cases. These results conflict with our PCR findings for reasons, as yet, unclear. It is possible that preferential synthesis of kappa light chain within L & H cells is associated with microenvironmental influences, such as viral infections, and does not reflect monoclonality. Further studies are needed to verify this possibility.

A proportion (3% to 5%) of patients with NLPHD develop simultaneous or subsequent large cell lymphomas (LCL) irrespective of treatment. In many cases, the tumor cells of LCL bear morphological and phenotypic similarities to L & H cells. For these reasons, the complicating LCLs are considered to have evolved directly from the L & H cells. However, no direct evidence for a clonal link between the two entities has been reported. In our series of 15 cases of LCL complicating NLPHD, monoclonal rearrangements of IgVH genes were detected in 53% of cases, a similar detection rate to other high grade B-cell lymphomas., confirming the B-cell lineage and monoclonality of the tumors. However, we could not prove a clonal link with associated NLPHD, even with highly sensitive and LCL clone specific PCR analysis on microdissection-enriched L & H cells, the same strategy widely used for detection of minimal residual disease in lymphoid malignancies. The failure to trace the LCL progenitors in NLPHD could be explained by derivation of the LCLs from transformed B-cell progenitors that have the ability to differentiate into polyclonal L & H cells, but which require further genetic alterations before emergence of a monoclonal high-grade lymphoma. Identification of IgVH somatic mutations in the L & H cells of a NLPHD case by Kuppers et al and in the majority of LPHD cases by Tamaru et al suggests germinal-center B cells as the progenitor of the tumor. Our sequence analysis also shows IgVH somatic mutations in the NLPHD associated LCLs with evident intrachlonal variation in one case indicating a similar derivation of the disease from germinal-center B cells. It is possible that the clones responsible for the LCLs were present in the NLPHD tissue samples, but were undetectable by the clone specific primers due to somatic point mutations within the N regions or to scarcity of target cells. However, previous studies have shown that the accumulation of somatic point mutations during tumor progression usually involves only a few nucleotides in the IgVH region. Such a rate should not be sufficient to totally abolish PCR amplification of the VH region of four cases. Furthermore, the inclusion of thymidine at the 3' end of the clone specific primers makes them more tolerant to mismatch at the 3' end. Therefore, the most probable explanation of our negative clone specific PCR results in NLPHD is that the tumor clones were not present or were extremely rare in that phase of the disease.

In conclusion, by analyzing Ig gene rearrangements with PCR, we have failed to demonstrate monoclonality in whole tissue sections and microdissected L & H cells of NLPHD and found no evidence that L & H cells evolve directly into subsequent LCL. Further studies using markers of clonality other than Ig genes may show whether the L & H cells of NLPHD are part of the same disease process as later LCL. The previous observation of Ig light chain restriction in some nodules in many cases of NLPHD and demonstration of monoclonal Ig gene rearrangement in microdissected single L & H cells may reflect development from polyclonal to oligoclonal and, finally, monoclonal disease. Although our present results provide no support for this hypothesis, they do not rule out the possibility that the LCLs are derived from further transformed L & H cells. The mechanisms driving proliferation of polyclonal L & H cells in NLPHD and the factors that cause development of LCL remain to be elucidated.

NOTE ADDED IN PROOF

Since acceptance of this manuscript, Wickert et al have published the results of a similar study in which nine cases of NLPHD with complicating LCL were analyzed. As in our
investigation, no dominant clones were detected in NLPHD using consensus primers, whereas five of the LCL showed a dominant band. In contrast to our results, clone-specific primers derived from LCL CDR III regions confirmed the presence of the LCL clone in the NLPHD in two cases. We have now used clone-specific PCR to analyze an additional two cases, one of which shows no evidence of the LCL tumor sequence in the NLPHD and in the other we have amplified fragments of the appropriate size from 2 of 73 aliquots (each containing an estimated 10 to 100 ng of DNA) of the NLPHD extract. We are currently evaluating this result by sequencing of products. If the specificity of the products is confirmed, in the light of the results of Wickert et al., these findings would suggest that LCL clones may be present during the NLPHD phase of the disease as minor subsets of polyclonal L & H cell populations.

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