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.

NODULAR LYMPHOCYTE predominance Hodgkin’s disease (NLPHD) exhibits many features that distinguish it from other subtypes of Hodgkin’s disease. Clinically, NLPHD is very indolent and can remain stable for years, even without treatment. Histologically, the disease is characterized by the presence of the putative tumor cells, L & H (lymphocytic and histiocytic) cells, which have been shown to have a B-cell phenotype. Although NLPHD is now generally accepted as a B-cell disorder, whether it represents a true neoplasm or merely a reactive or premalignant condition remains unresolved. A proportion of patients with NLPHD develop large cell B lymphoma (LCL), and it is often assumed, although not proved, that the lymphoma is clonally linked to the NLPHD.

Numerous attempts have been made to verify the neoplastic nature of NLPHD by demonstration of monoclonality using immunohistochemical or molecular-genetic techniques. Analyses for Ig light chain protein and mRNA expression in L & H cells by immunostaining and in situ hybridization have yielded heterogeneous results, ranging from absence of expression to polyclonal or monoclonal expression of light chain protein or mRNA. Investigations for monoclonal rearrangements of Ig genes using Southern blot or polymerase chain reaction (PCR) analysis have not proved fruitful in most studies. However, in two recent investigations using an improved PCR method and a single cell isolation technique, monoclonal rearrangements of Ig VH genes were demonstrated in whole tissue sections and in single L & H cells of NLPHD cases. In another molecular study based on single cell PCR techniques, polyclonal rearrangements of the Ig genes were demonstrated in the L & H cells. Therefore, the clonality of NLPHD remains controversial.

We have been able to collect 33 cases of NLPHD, 15 of which were complicated by LCL, providing a unique opportunity for us to study the clonal nature of NLPHD and its relationship with LCL. We have used an optimized PCR strategy to analyze each case for clonal IgVH rearrangement. To correlate the PCR results with the atypical cells, we have performed the analysis on L & H cells microdissected from tissue sections. To study the clonal relationship between NLPHD and complicating LCL, we have cloned and sequenced monoclonally rearranged IgVH regions obtained from LCL. Tumor cell specific IgVH sequences have been identified and used to design primers for clone specific PCR, which has been used to trace LCL progenitors in associated NLPHD.

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. 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
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 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. All LCL samples and extracts from 25 NLPHD cases (including all the L&L associated cases) were analyzed using both sets of primers. The remaining eight NLPHD cases and microdissected 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.

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 Wan et al. Amplification from framework 2 (FR2) to JH was performed using the seminested method of Ramasamy et al. with the modifications described by Tamaru et al. All LCL samples and extracts from 25 NLPHD cases (including all the L&L associated cases) were analyzed using both sets of primers. The remaining eight NLPHD cases and microdissected 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 prekit (Promega Ltd, Southampton, UK). DNA diluted with polyclonal DNA extracted from hyperplastic tonsil

Four LCL cases (sequences of rearranged IgVH regions from LCL applied to at least one case) 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 33 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-

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<tr>
<th>Table 1. Sequence Analysis</th>
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<tr>
<td>N1</td>
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<td>Case 1: gccagagact gAGACGGAT GCCGCTTCTT Gtatctggg gccaaggac c</td>
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<tr>
<td>N1</td>
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<tr>
<td>Case 2: gccagacata agagctcaaat tattttttctg gaggagttt AG GOTA AAGGGT GCTGCTGAA ac g</td>
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<tr>
<td>N1</td>
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<tr>
<td>Case 3: tcgagagata agtggtttat tatttggg ggcctcaatta tattagggg gta ACTGCC ACCAATAA CGU tatggac gt</td>
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<td>N1</td>
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<tr>
<td>Case 4: gccagagggg ggcagTTAC TGGAGACCAA GGIAT ctga tga tctgggtgct ctattgct ctatttgact a</td>
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Table 2. Efficiency of Clone-Specific Detection

<table>
<thead>
<tr>
<th>Case</th>
<th>CSP Sequence</th>
<th>2nd Primer</th>
<th>Product Size (bp)</th>
<th>Sensitivity</th>
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<tbody>
<tr>
<td>1</td>
<td>5'-CAAAAAGCATGATCCTGGCTC-3'</td>
<td>FR2&lt;sup&gt;37&lt;/sup&gt;; FR3&lt;sup&gt;38&lt;/sup&gt;</td>
<td>214; 51</td>
<td>5×; 10×</td>
</tr>
<tr>
<td>2</td>
<td>5'-TCGAGACCACCCTTACCTTGGAC-3'</td>
<td>FR3&lt;sup&gt;38&lt;/sup&gt;</td>
<td>78</td>
<td>10×</td>
</tr>
<tr>
<td>3</td>
<td>5'-CCGATATTATCTGGGGGAG-3'</td>
<td>FR3&lt;sup&gt;38&lt;/sup&gt;</td>
<td>93</td>
<td>1000×</td>
</tr>
<tr>
<td>4</td>
<td>5'-TATCCCTGATGTACCAGAAG-3'</td>
<td>FR2&lt;sup&gt;37&lt;/sup&gt;; FR3&lt;sup&gt;38&lt;/sup&gt;</td>
<td>218; 55</td>
<td>100×; 1×</td>
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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.
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.

Fig 3. Sensitivity of detection of tumor clone from case 4 diluted with polyclonal tonsillar DNA using consensus primers (FR3/JH 10% gel; top) and clone specific amplification (FR2/CSP 6% gel; bottom). 1, 50% tumor extract; 2, 5%; 3, 0.5%; 4, 0.05%; and 5, 0.005%.

Fig 4. PCR amplification using clone specific primers. Products were run on 10% gels. Lane M, PhiX/Hinfl molecular weight markers (the 100 bp fragment is indicated); case 3: 1, LCL extract; 2, NLPHD; 3, microdissected L & H cells; case 2: A, LCL extract; B, NLPHD; and C, microdissected L & H cells.
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, Schmid 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 monoclonic 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. 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 intraclonal 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 CDR3 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,40 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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LX Pan, TC Diss, HZ Peng, AJ Norton and PG Isaacson