

CLONALITY IN NODULAR LYMPHOCYTE-PREDOMINANT HODGKIN'S DISEASE

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ABSTRACT

Background There is general agreement that lymphocytic and histiocytic (L&H) cells, the variants of Reed–Sternberg cells in nodular lymphocyte-predominant Hodgkin's disease, belong to the B-cell lineage. However, the clonality of L&H cells remains controversial.

Methods We used complementarity-determining region 3 (CDR3) of the immunoglobulin heavy-chain gene as a clonal marker to study individual L&H cells isolated by micromanipulation from tissue sections of five patients with nodular lymphocyte-predominant Hodgkin's disease. The heavy-chain CDR3 of each cell was amplified by the polymerase chain reaction. The products were analyzed by gel electrophoresis, and representative amplification products from each patient were sequenced.

Results L&H cells whose heavy-chain CDR3 was related, indicating the presence of a clonal population, were detected in all five patients and were the dominant population in three. In four of the five patients, members of the clone were found in different nodules in the tissue section, different tissue blocks from the same tumor, or different lymph nodes from the same patient. The CDR3 sequences in each clone frequently contained nucleotide substitutions indicative of intraclonal mutation.

Conclusions Clonal populations of L&H cells occur in nodular lymphocyte-predominant Hodgkin's disease. Intraclonal variation in nucleotide sequences suggests that hypermutation of the heavy-chain CDR3 continues to occur among the clonal progeny. (N Engl J Med 1997;337:459-65.)

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NODULAR lymphocyte-predominant Hodgkin's disease, a subtype of Hodgkin's disease, is an indolent disorder in which tumors contain a variant of Reed–Sternberg cells known as lymphocytic and histiocytic (L&H) cells, which are admixed with small lymphocytes and histiocytes in nodular aggregates.¹⁻⁴ Immunohistochemical studies have provided strong evidence that these L&H cells originate from B cells,⁵⁻⁹ but whether they represent monoclonal or polyclonal populations remains unclear.

Studies of immunoglobulin-gene rearrangement as a clonal marker in nodular lymphocyte-predominant Hodgkin's disease have yielded conflicting results.¹⁰⁻¹⁸ To resolve the controversy, investigators have isolated single L&H cells and studied them by the polymerase chain reaction (PCR) for rearrange-

ments of the genes for the immunoglobulin heavy chain, the light chain, or both.^{19,20} Küppers et al.¹⁹ reported a clonal population of L&H cells in a patient with nodular lymphocyte-predominant Hodgkin's disease, whereas Delabie et al.²⁰ did not detect such populations in two patients they studied. It is unclear whether case selection or technical factors, such as sampling bias, account for these discordant findings. We performed single-cell analysis of five patients with nodular lymphocyte-predominant Hodgkin's disease; different nodules in the tumor and, when available, different tissue blocks and lymph nodes were sampled. We found a clonal population in each of the five patients, and in four the population appeared to be present throughout the tumor.

METHODS

Case Selection

We studied seven formalin-fixed, paraffin-embedded specimens of tissue from five patients with nodular lymphocyte-predominant Hodgkin's disease who were selected from the data base of the lymphoma registry at the University of Nebraska Medical Center. All the specimens had the characteristic histopathological features and immunophenotype of the disease. The specimens from Patients 1, 2, 3, and 4 had moderately abundant L&H cells and relatively few small B lymphocytes in the nodules; in the specimen from Patient 5 the nodules contained few L&H cells and numerous small B lymphocytes.

Immunohistochemical Analysis

Tissue sections 4 to 6 μm thick were stained with anti-CD20 or anti-CD3 (Dako, Carpinteria, Calif.) by a three-step immunoperoxidase method.²¹ Before the staining with anti-CD20, the sections were heated in citrate buffer (pH 6.0) for five minutes in a microwave oven; before the staining with anti-CD3, the sections were digested with pepsin.

Isolation of Single Cells from Tissue Sections

With the use of an inverted microscope (Nikon, New York), the L&H cells stained with anti-CD20 were isolated with a microknife and a micropipette that were fixed to hydraulic micromanipulators (Narishige, New York) and expelled into a tube containing PCR buffer as described by d'Amore et al.²² In addition to the L&H cells, CD20+ small B cells and CD3+ small T cells were isolated by micromanipulation for use as positive and negative controls. The phosphate-buffered saline overlying the section was sampled periodically to serve as the negative control for the supernatant.

PCR Amplification of Single Cells

The heavy-chain CDR3 of each single cell was amplified after digestion with proteinase K by a seminested PCR using consensus

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TABLE 1. RESULTS OF PCR AMPLIFICATION OF SINGLE CELLS FOR HEAVY-CHAIN CDR3.*

SOURCE OF CELLS	L&H CELLS	B CELLS
	no. of cells amplified/ no. studied	
Patient 1		
1st tissue block	17/100	4/16
2nd tissue block	17/60	
Patient 2	21/90	7/16
Patient 3		
1st lymph node	6/40	
2nd lymph node	2/39	4/16
Patient 4	27/72	6/20
Patient 5	20/100	5/16

*Sixteen T cells from each patient were also studied, none of which could be amplified by PCR. For Patient 1, two separate blocks of tissue from the same tumor were studied. For Patient 3, two separate inguinal lymph nodes were studied. The B and T cells studied were small lymphocytes all present in the same biopsy specimen.

VH and JH primers under hot-start conditions.^{20,23} The details of this procedure and of its use with formalin-fixed archival tissue have been described previously.²² The amplified products were subjected to electrophoresis on 8 percent polyacrylamide gels and visualized with ultraviolet light after staining with ethidium bromide.

DNA Sequencing

For each patient, representative heavy-chain CDR3 PCR products from cells that could be amplified were sequenced to determine their clonal relation. For this purpose, the original CDR3 PCR product was reamplified in a volume of 50 μ l with VH and JH nested primers with M13 sequences attached to their 5' ends (sequences, M13+VH, TGAAAACGACGGCCAGTCTGTCGACACGGCCGTTACTG; M13R+JH, GGAAACAGCTATGACCATGACCAGGTCCTTGGCCCCA). The products were gel-purified and directly sequenced with primers to M13 in forward orientation and M13R in reverse orientation and a cycle-sequencing procedure that used fluorescent dideoxynucleotide chain terminators (Applied Biosystems, Foster City, Calif.).²⁴ The sequences were analyzed with a model 373A sequencer (Applied Biosystems).

RESULTS

PCR was performed to amplify the CDR3 of the rearranged heavy-chain genes of the cells isolated from tissues affected by nodular lymphocyte-predominant Hodgkin's disease. The procedure yielded products for 5 to 38 percent of the L&H cells, 25 to 44 percent of the small B cells, and none of 80 T cells (Table 1).

The results of the analyses of the single cells from each patient are shown in Tables 2 and 3. For Patient 1, CDR3 PCR products were obtained from 17 of 100 L&H cells in the first tissue block and 13 of 40 L&H cells in the second tissue block (Table 2). PCR products of identical lengths were found in 22 cells from different nodules in the two tissue blocks. Sev-

en of these 22 products, from nodules 1, 2, 3, 5, and 6, were sequenced and found to be identical, indicating that clonal L&H cells were present in different nodules in both tissue blocks from the same tumor. The second tissue block from this patient was studied again after a 12-month interval (Table 2). The same clonal population was again identified, and sequence analysis showed that the sequences were identical to those determined previously.

For Patient 2, CDR3 PCR products were obtained from 21 of 90 L&H cells. Two sets of products, each with identical lengths, were detected (Fig. 1): 2 cells from nodule 2 (lanes 3 and 4) and 13 cells from nodules 1, 2, and 4. Sequence analysis of the two products (lanes 3 and 4, Fig. 1) from nodule 2 showed that they were unrelated. Eight of the 13 products from nodules 1, 2, and 4 were sequenced, and all were found to be related; 4 had identical nucleotides, and 4 contained nucleotide substitutions (Table 3). These findings indicated that there was a clonal population of L&H cells with intraclonal mutations in three nodules from the same tumor.

For Patient 3, in whom two inguinal lymph nodes were studied, CDR3 PCR products were obtained from 6 of 40 L&H cells from the first lymph node and from 2 of 39 L&H cells from the second. PCR products of identical lengths were found in two cells, one from each lymph node. Sequence analysis showed that the two cells were related, but there were multiple nucleotide substitutions, indicating that a clonal population of L&H cells with intraclonal mutations was present in the two inguinal lymph nodes.

For Patient 4, CDR3 PCR products were obtained from 27 of 72 single L&H cells. PCR products of identical lengths were found in 23 cells from four different nodules. Sequence analysis of eight of the products (two from each nodule) (Fig. 2) confirmed the presence of a clonal population with frequent intraclonal mutations in all the nodules. One additional, smaller clone was found in nodule 3 (Fig. 2). The sequence of this minor clone (lanes 5 and 6, Fig. 2) was related to that of the major clone (typified by cell 8, Table 3). Two additional PCR products from nodule 3 were also identical in size (Table 2), but the sequences were unrelated to each other and to the two related clones found in the same specimen.

For Patient 5, CDR3 PCR products were obtained from 20 of 100 L&H cells. Fourteen of the PCR products were classified into three groups in which the products had almost identical lengths (Fig. 3), and all 14 products were sequenced. Only 2 of the 14 PCR products (lanes 1 and 4, Fig. 3) had identical sequences (Table 3). All the other sequences appeared unrelated. This patient differed from Patients 1 through 4 in having fewer L&H cells and more abundant small B lymphocytes.

TABLE 2. RESULTS OF PCR AND SEQUENCE ANALYSIS OF SINGLE L&H CELLS.*

SOURCE OF CELLS	HEAVY-CHAIN CDR3 AMPLIFICATION BY PCR			SEQUENCE ANALYSIS	
	NO. OF CELLS STUDIED	NO. WITH PRODUCTS	NO. WITH PRODUCTS OF IDENTICAL LENGTH	NO. OF PRODUCTS STUDIED	NO. IDENTICAL OR RELATED
Patient 1, 1st tissue block					
Nodule 1	30	5	2	1	1
Nodule 2	30	8	7	3	3
Nodule 3	30	3	3	1	1
Nodule 4	10	1	—		
Total	100	17	12	5	5
Patient 1, 2nd tissue block					
Nodule 5	30	8	5	1	1
Nodule 6	10	5	5	1	1
Total	40	13	10	2	2
Entire section 1 yr later	20	4	3	2	2
Patient 2					
Nodule 1	20	2	2	2	2
Nodule 2	30	7	2,3	2,2	0,2
Nodule 3	10	1	—		
Nodule 4	30	11	8	4	4
Total	90	21	2,13	2,8	0,8
Patient 3, 1st lymph node					
Nodule 1	10	2	1	1	1
Nodule 2	10	1	—		
Nodule 3	10	1	—		
Nodule 4	10	2	0		
Total	40	6	1	1	1
Patient 3, 2nd lymph node					
Nodule 1	9	0			
Nodule 2	10	1	—		
Nodule 3	10	0			
Nodule 4	10	1	1	1	1
Total	39	2	1	1	1
Patient 4					
Nodule 1	10	4	4	2	2
Nodule 2	10	4	4	2	2
Nodule 3	42	17	2,2,13	2,2,2	2,0,2
Nodule 4	10	2	2	2	2
Total	72	27	2,2,23	2,2,8	2,0,8
Patient 5					
Nodule 1	20	3	1,1,1	1,1,1	1,0,0
Nodule 2	20	9	2,2,3	2,2,3	0,0,0
Nodule 3	10	1	—		
Nodule 4	10	1	1	1	1
Entire section	40	6	1,1,1	1,1,1	0,0,0
Total	100	20	5,4,5	5,4,5	2,0,0

*In some cases there are two or more groups of PCR products that appear to have identical molecular weights, and these groups are separated by commas. The corresponding sequence data are shown similarly.

DISCUSSION

The detection of B-cell antigens, such as the J-chain, CD19, CD20, and CD79a, was the first indication of the B-cell origin of L&H cells.⁵⁻⁹ However, whether nodular lymphocyte-predominant Hodgkin's disease is a monoclonal or a polyclonal B-cell disorder has been controversial. Southern blot and PCR analyses for immunoglobulin-gene rearrangements have detected only occasional cases of mono-

clonal immunoglobulin-gene rearrangements in all studies,^{10-15,17,18} with a single exception.¹⁶ These studies are difficult to interpret because of the admixture of numerous reactive cells in addition to the L&H cells. Some investigations using in situ hybridization have found messenger RNA with a single light-chain isotype in a predominance of L&H cells, as is consistent with a clonal population.^{25,26} The detection of clonality by the analysis of single L&H cells in one

TABLE 3. NUCLEOTIDE SEQUENCES OF THE HEAVY-CHAIN CDR3 OF SINGLE L&H CELLS.*

PATIENT AND CELL No.	NODULE No.	V _H FAMILY	nDn SEGMENT		J _H SEGMENT
Patient 1, 1st tissue block					
		VH4	<u>DN4(R)</u>	<u>DNQ52</u>	JH4
1	1	TGCGA	caacaaccgaCTATACTgacggcaatgTgACTGGGGggtcaggggcgcgatttgcg		TrC
2	2	-----	-----		---
3	2	-----	-----		---
4	2	-----	-----		---
5	3	-----	-----		---
Patient 1, 2nd tissue block					
6	5	-----	-----		---
7	6	-----	-----		---
8	Section†	-----	-----		---
9	Section†	-----	-----		---
Patient 2					
		VH3	<u>DIR2</u>	<u>DXP4</u>	JH6
1	1	TGCGAAA	atttettctcaCCgCgGCCTCCGGeaaGaATTACGgTTTTGGAGTGaTTATcgagagg		AaTACcACTACTACgACATGGACGTC
2	1	-----	-----		-----
6	2	-----	-----		-----
8	2	-----	-----		-----
10	4	-----	-----		-----
13	4	-----	-----		-----
15	4	-----	-----		-----
17	4	-----	-----		-----
Patient 3, 1st lymph node					
		VH6	<u>DM2(R)</u>	<u>DXP4</u>	JH6
1	1	TGCAcG	gattcttcttcaatccatactTGGTTCCGtcaaaTATTACGATTTTGGAGTGGTcATTATggagtcg		AgTACaAgTACTACgTATGGACGTC
Patient 3, 2nd lymph node					
2	4	-----	-----g-----t-----tc-----g-----a---g---t-----a-----		-----
Patient 4					
		VH4	<u>DK1(R)</u>		JH4
3	2	TG	tcgtaagCcCTATATratctegggcgcttaeact		ACcAC
4	2	--	-----		-----
1	1	--	-----c-----		-----
2	1	--	-t-----c---c-----		-----
7	3	--	--c-----a---c-a---a-tc--		-----
8	3	--	--c-----c---c-a---a-tc--		-----
9	4	--	-----		-----
10	4	--	-----c-----		-----
		VH4	<u>DK1(R)</u>		JH4
5	3	TG	tcctaagCcCTATATtaegtgtgcaagtgcagtcgacgtcaatect		ACcAC
6	3	--	-----t-----a-----		-----
<i>Comparison of the major and minor clonal sequences</i>					
8	3	TG	tcctaagCcCTATATtacc	tcggcgacgtcaatect	ACcAC
5	3	--	-----gtcgtgcaagtgcagtcg-----		-----
Patient 5					
		VH2	<u>DIR2</u>		JH5
1	1	TGCGCG	aggAGCCTaCGGtGaCtaaggcgcg		AACTGGTTCGACCCC
4	4	-----	-----		-----

*V, D, and J sequences that differ from the germ-line sequence are indicated by lowercase letters. (R) denotes reverse orientation.
 †These L&H cells were isolated from the entire tissue section in additional experiments performed 12 months after the first experiments.

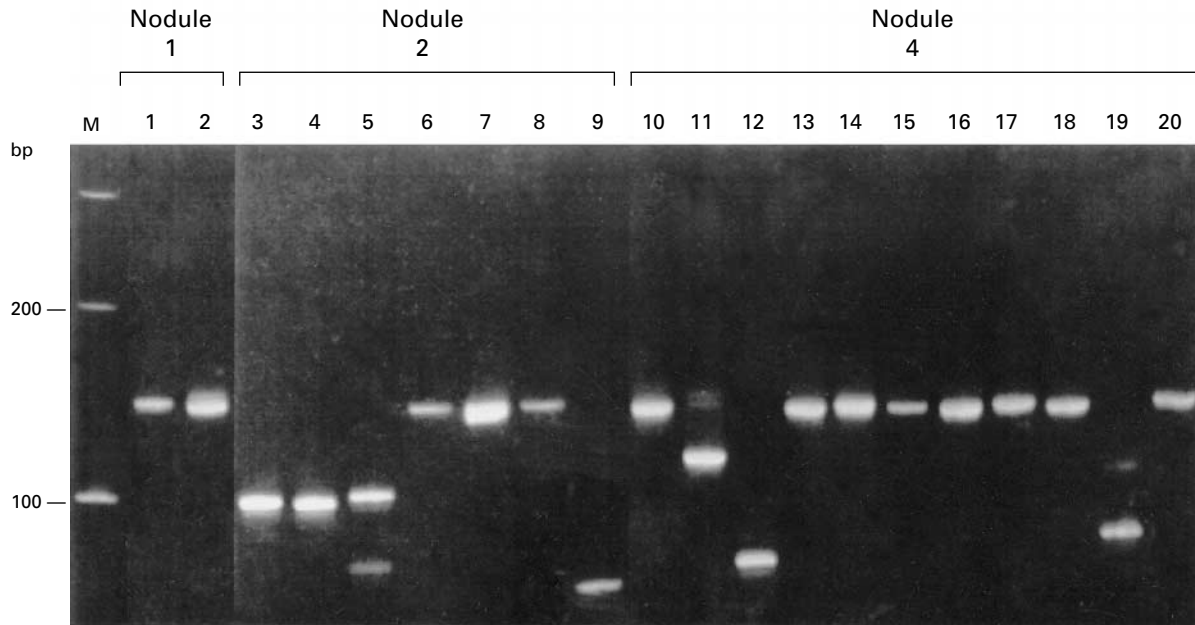


Figure 1. Results of Polyacrylamide-Gel Electrophoresis of the Heavy-Chain CDR3 PCR Products from Each of the Single L&H Cells from Patient 2.

Products with sizes identical to that in lane 1 were found in three nodules, and all the amplification products sequenced were identical or related. In addition, the products in lanes 3 and 4 (from nodule 2) appeared to be identical to each other, but sequencing showed them to be unrelated. M denotes a molecular-size ladder.

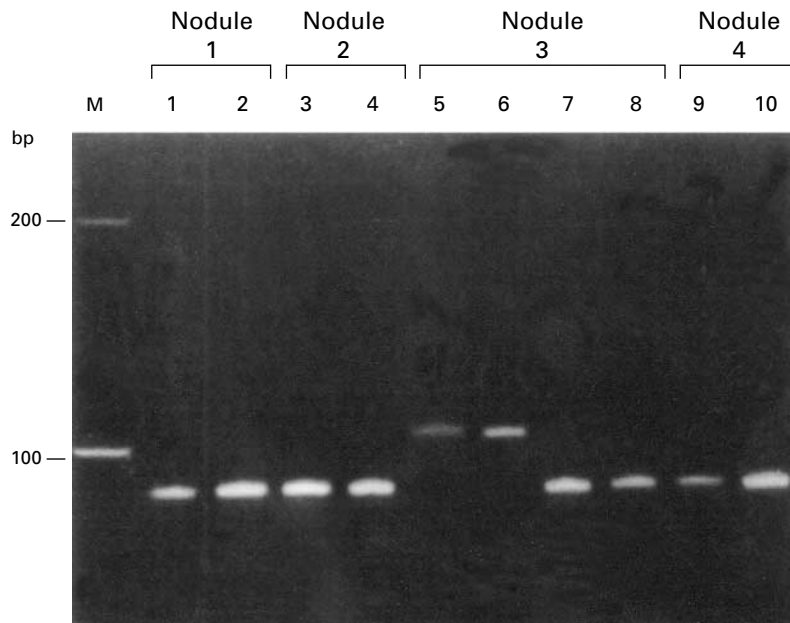


Figure 2. Results of Polyacrylamide-Gel Electrophoresis of the Heavy-Chain CDR3 PCR Products from the Single L&H Cells from Patient 4 That Had Related Sequences.

Products of identical size (less than 100 bp) were identified in all four nodules. In addition, two larger amplification products of identical size and related sequence (lanes 5 and 6) were seen in nodule 3. M denotes a molecular-size ladder.

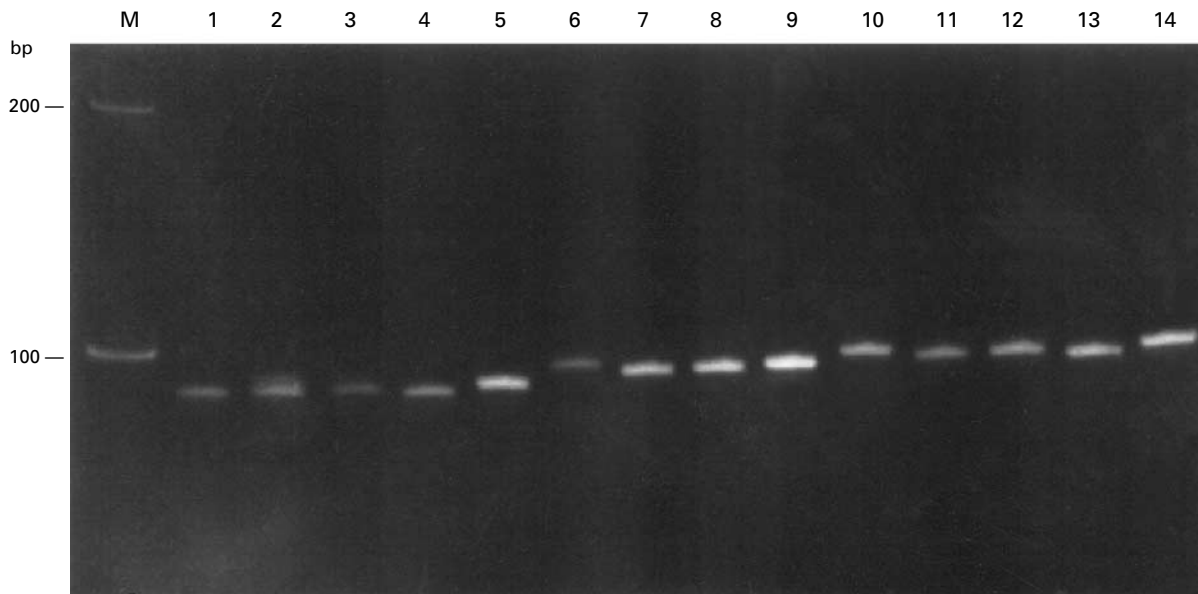


Figure 3. Results of Polyacrylamide-Gel Electrophoresis of the Heavy-Chain CDR3 PCR Products from 14 Single L&H Cells from Patient 5.

These products were classified into three groups, each of which contained amplification products almost identical in size, but only two products (lanes 1 and 4) had identical sequences. M denotes a molecular-size ladder.

patient with nodular lymphocyte-predominant Hodgkin's disease has been reported recently.¹⁹ In a previous study, we did not find clonal heavy-chain-gene rearrangements in L&H cells from two patients with nodular lymphocyte-predominant Hodgkin's disease.²⁰ In that study, single L&H cells were obtained from cell suspensions and a relatively small number of cells were analyzed, which may have hampered the detection of a clonal population of L&H cells. In this study, however, using PCR analysis of single cells obtained by micromanipulation, we have demonstrated clonal populations of L&H cells in all five patients with nodular lymphocyte-predominant Hodgkin's disease. These populations appeared to be present throughout the tumor in four of the five patients, since L&H cells with identical or related heavy-chain CDR3 sequences were found in different nodules in the same tissue section, in different tissue blocks from the same tumor, and in two separate lymph nodes in one patient. Patient 1 was unusual in that all the CDR3 sequences from the clonal population were identical. The sample from this patient was studied again after a 12-month interval. The presence of the initial clonal population of L&H cells was confirmed, and the sequences were identical to those obtained previously.

It has been proposed that L&H cells originate from B cells in germinal centers, because of the presence of follicular dendritic cells and CD57+ T-helper cells in the tumor nodules.²⁷⁻²⁹ The clonal sequenc-

es in three of the specimens we studied and in the case reported by Küppers et al.¹⁹ showed nucleotide substitutions in individual cells belonging to the clone, indicating intraclonal mutation. In Patient 4, an apparent second clonal population was detected, but sequence analysis (Table 3) suggested that one of the clones was derived from the other, either by deletion or by the insertion of the central portion of the sequence. The frequent mutation of heavy-chain genes we found in L&H cells is also characteristic of normal B cells in the germinal center³⁰⁻³² and suggests that the L&H cells are altered centroblasts in which hypermutation of heavy-chain genes takes place in a germinal-center-like environment.

In each of the five patients we also found L&H cells with heavy-chain-gene rearrangements that were unrelated to the clonal population we identified. This may indicate the concurrent presence of polyclonal L&H cells, but the possibility that the polyclonal heavy-chain-gene rearrangements were due to contamination by normal B cells should also be considered. No heavy-chain CDR3 products were amplified in any of the T cells or supernatant controls we studied, but this does not rule out an occasional contamination by B cells or the misidentification of L&H cells. The specimens from Patients 1, 2, 3, and 4 were selected because there were moderately abundant L&H cells and relatively few small B lymphocytes in the nodules, which would minimize contamination of the L&H cells by normal

B lymphocytes, but the selection criteria may have led to the inclusion of specimens in which a dominant clonal population was more likely. The specimen from Patient 5, on the other end of the spectrum, had few L&H cells and numerous small B lymphocytes. Only a small clonal population was detected in this patient, a fact that seems to support the existence of an early polyclonal phase of the disease. This hypothesis would be much strengthened if multiple unrelated clones were detected.³³

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