

ORIGIN OF NODULAR LYMPHOCYTE-PREDOMINANT HODGKIN'S DISEASE FROM A CLONAL EXPANSION OF HIGHLY MUTATED GERMINAL-CENTER B CELLS

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ABSTRACT

Background The atypical cells of nodular lymphocyte-predominant Hodgkin's disease, designated lymphocytic and histiocytic (L&H) cells, have a B-cell phenotype. To clarify the clonality of these cells, we studied rearranged immunoglobulin genes for the variable region of the heavy chain (V_H genes) in individual L&H cells from 11 patients with nodular lymphocyte-predominant Hodgkin's disease. We also studied the expression of immunoglobulin light chains by those cells in six of the same patients.

Methods Single CD20+ L&H cells were isolated from frozen sections by a technique of micromanipulation. The rearranged V_H genes of these cells were amplified by the polymerase chain reaction (PCR), sequenced, and compared with germ-line V_H genes. Immunoglobulin light-chain messenger RNA (mRNA) was detected by in situ hybridization.

Results Of 615 L&H cells isolated from all the frozen sections, 160 yielded PCR products. In each of the 11 patients, the L&H cells that could be evaluated had identically rearranged V_H genes, whether they were isolated from the same nodule, different nodules, or different blocks of tissue. All the V_H sequences derived from the L&H cells were highly mutated (7.5 to 27.2 percent). In two cases the coding capacity of the V_H genes was completely or partially disrupted by mutations. Intracлонаl diversity was found in six cases, and monotypic immunoglobulin light-chain mRNA was found in six.

Conclusions The L&H cells of nodular lymphocyte-predominant Hodgkin's disease represent a monoclonal expansion of B cells. The high load of V_H gene mutations and signs of intracлонаl diversity suggest a relation between L&H cells and germinal-center B cells at the centroblastic stage of differentiation. (N Engl J Med 1997;337:453-8.)

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LYMPHOCYTE-PREDOMINANT Hodgkin's disease,¹⁻³ an indolent disorder associated with a long survival,⁴ represents approximately 5 percent of all cases of Hodgkin's disease.⁵ Its histopathological features include an obliterated lymph-node structure that usually contains small numbers of large, dysplastic cells, designated lymphocytic and histiocytic (L&H) cells, and numerous small lymphocytes. There is a nodular subtype of the disease that occurs frequently and a diffuse sub-

type that occurs rarely.³ L&H cells have consistently been found to express B-cell markers,⁶⁻⁸ but it is unclear whether lymphocyte-predominant Hodgkin's disease is a monoclonal neoplasm or a premalignant or reactive condition with polyclonal L&H cells. Most studies have not found clonal populations of B cells by the analysis of Southern blots or polymerase-chain-reaction (PCR) products of DNA extracts from tissue or single L&H cells that have been isolated,⁹⁻¹⁶ but some investigators have demonstrated the expression of monotypic immunoglobulin light chains.¹⁷⁻¹⁹ These conflicting data probably result from technical difficulties. We used an improved method of isolating cells from frozen sections of tissue from 11 patients with typical cases of nodular lymphocyte-predominant Hodgkin's disease and analyzed their V_H gene rearrangements. Six of these patients were also studied for immunoglobulin light-chain transcripts by in situ hybridization. The two approaches yielded congruent results: the pattern of rearranged V_H genes in the L&H cells indicated a monoclonal population, and the cells expressed only one type of light chain.

METHODS

Tissue Samples

We collected frozen specimens of tissue from patients with typical cases of nodular lymphocyte-predominant Hodgkin's disease that met the criteria of the revised European-American lymphoma²⁰ classification. The samples were obtained from files at the institutes of pathology in London; Toulouse, France; Perugia and Bologna, Italy; and Berlin, Germany. Hyperplastic tonsils were used as controls.

Isolation of Single Cells

Seven-micrometer-thick frozen sections were immunostained for CD20 (L26) or the beta chain of the T-cell receptor (β F1). From 25 to 89 CD20+ L&H cells (615 cells in all) and 7 normal

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T cells (84 in all) were isolated from the frozen tissue from the 11 patients; for 1 patient, two samples obtained nine months apart were studied. In addition, 260 B cells were extracted from two hyperplastic tonsils (158 cells) and seven patients with nodular lymphocyte-predominant Hodgkin's disease (102 cells). In contrast to previous studies of single cells,^{12,16,21,22} our method involved suspending the isolated cells in a minimal volume of buffer (less than 250 nl). To check for the presence of contaminating V_H sequences in the buffer, aliquots of at least 1 μ l were drawn after the isolation of each cell (959 aliquots in all). Cells were isolated from each section two to six times, depending on the number of L&H cells that could be amplified.

PCR and Analysis of DNA Sequences

To detect single copies of rearranged V_H genes, we used a fully nested PCR with family-specific FW1 primers²³ and a consensus JH primer (LJH)²⁴ in the first amplification and modified family-specific FW2 primers in conjunction with a nested JH primer (VLJH) in the second amplification.^{15,24,25} The isolated PCR products were sequenced, and the number of somatic mutations in the amplified V_H region was determined by comparison with corresponding germ-line V_H segments. To establish the coding capacity of the L&H cells, the sequences of the amplification products were translated into the amino acids and studied as described elsewhere.^{25,26}

In Situ Hybridization

After the linearization of plasmids containing the immunoglobulin kappa and immunoglobulin lambda light-chain constant regions, antisense and sense transcripts labeled with sulfur-35 were generated with T7 or SP6 RNA polymerases (Promega-Biotech, Madison, Wis.). In situ hybridization was performed as described previously.²⁷

RESULTS

Immunoglobulin V_H Rearrangements

Individual CD20+ L&H cells (from 25 to 89 per patient) were isolated from frozen sections of tissue from 11 patients with nodular lymphocyte-predominant Hodgkin's disease and studied by single-copy PCR for rearrangements of the V_H genes (Fig. 1A and Table 1). From a total of 615 L&H cells and 260 B cells from hyperplastic tonsils as well as patients with nodular lymphocyte-predominant Hodgkin's disease, 160 (26 percent) and 102 (39 percent) amplification products of appropriate size for rearranged V_H genes were obtained, respectively. Eighty-four T cells isolated from frozen sections of tissue from the 11 patients (with two samples from Patient 1) did not generate V_H amplification products, with one exception (Table 1). Aliquots from the buffer overlying the frozen sections during the process of cell isolation were tested after the selection of each cell, and none of these 959 samples of buffer gave rise to PCR products.

By gel electrophoresis and sequence analysis, all but 1 of the 160 V_H gene amplification products derived from the L&H cells from the same patient proved to be identical, whether the cells were isolated from the same nodule, different nodules, or different sections of tissue that were analyzed independently. In addition, the two biopsy specimens obtained from Patient 1 nine months apart had iden-

tical V_H sequences. In contrast, the V_H gene rearrangements in all the single B cells from hyperplastic tonsils and patients with nodular lymphocyte-predominant Hodgkin's disease were unrelated to each other, to the rearrangements in the L&H cells, and to sequences from a data bank (GenBank release 98).

Somatic Mutations

The rearranged V_H genes of the L&H cells from all 11 patients were found to contain from 11 to 40 nucleotide substitutions (7.5 to 27.2 percent) when they were compared with the corresponding germ-line segments. Most of the mutations were located in complementarity-determining region (CDR) 2 (average mutation rate in CDR2, 21.6 percent), whereas framework region 3 was affected less often (average mutation rate, 8 percent).

Among these clones with hypermutated L&H cells, six had intraclonal diversity (Table 2 and Fig. 1B). In the L&H cells from two patients, the coding capacity of the V_H genes was disrupted by the mutations. In Patient 4, the V_H genes of all 13 L&H cells were silenced by two stop codons and a shift in the reading frame. In Patient 7, the coding capacity was only partly lost, because in 11 of the 13 L&H cells there was a stop codon in framework region 3.

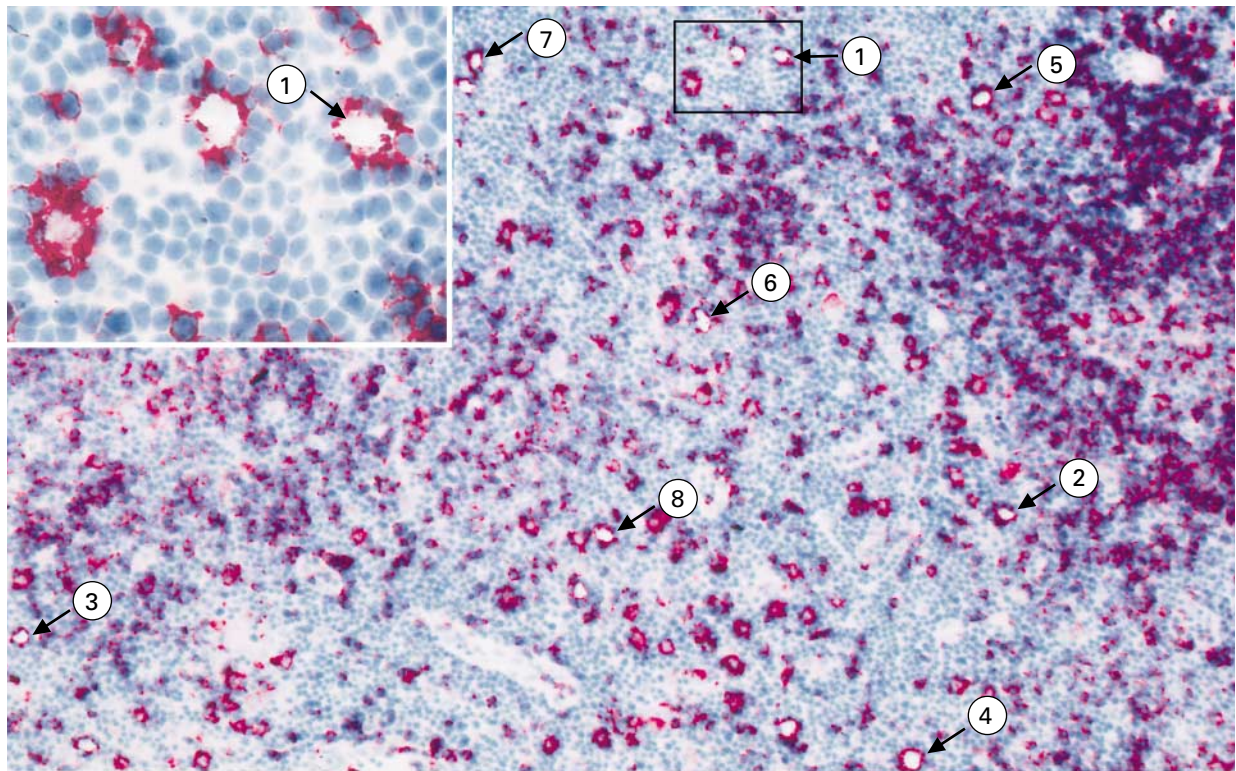
Immunoglobulin Light-Chain Transcripts

In 6 of the 11 patients we used in situ hybridization of frozen tissue to study immunoglobulin light-chain transcripts. In all six patients, the L&H cells had moderately strong labeling for immunoglobulin light-chain kappa messenger RNA, but they were not labeled by the immunoglobulin light-chain lambda antisense probes (Table 2). Both probes for immunoglobulin light chains marked plasma cells strongly and marked a proportion of reactive lymphoid cells weakly. Sense probes produced only background signals.

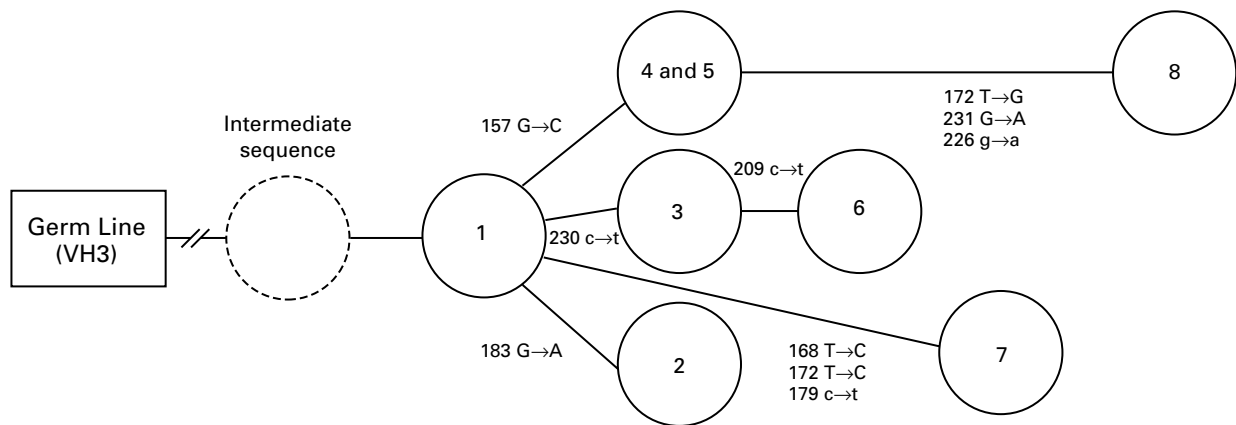
DISCUSSION

It is generally agreed that L&H cells have a B-cell phenotype, but their clonality is controversial. To clarify this issue, we studied the rearranged V_H genes in individual L&H cells isolated from frozen sections of tissue from 11 patients with nodular lymphocyte-predominant Hodgkin's disease. We identified the cells morphologically and with the B-cell marker CD20. The L&H cells in each patient had the same rearrangement of V_H genes. This finding confirmed that L&H cells were B cells (because only B cells contain rearranged V_H genes) and showed that L&H cells arise as a result of clonal expansion. The clonality of these cells was further evidenced by the monotypic expression of immunoglobulin light-chain transcripts, which is in accordance with earlier studies.¹⁷⁻¹⁹

Previous studies by single-cell PCR of both the



A



B

Figure 1. Studies of the Clonal Relation among Eight Lymphocytic and Histiocytic (L&H) Cells Isolated from Patient 11.

Panel A shows a frozen section of a lymph node from the patient after immunostaining for CD20 (alkaline phosphatase–anti-alkaline phosphatase method, $\times 100$), with the reaction products staining red. The white zones surrounded by red rims are the spaces formerly occupied by the L&H cells that were isolated from the section by a micropipette. Among these isolated L&H cells, those that gave rise to V_H amplification products are indicated (arrows; the numbering is arbitrary and does not correspond to the order in which the cells were isolated). The boxed portion of the section is shown at higher magnification (inset at upper left, $\times 400$).

Panel B shows a genealogic tree indicating the somatic mutations in the clonally related V_H sequences in the same eight L&H cells, numbered as in Panel A. The germ-line sequence (VH3) and the hypothetical intermediate sequence are also shown. The numbers and positions shown adjacent to the lines indicate the locations of the nucleotide substitutions and their types. The length of the lines corresponds to the number of mutations in the sequence. Uppercase letters denote replacement mutations, and lowercase letters silent mutations. The location numbers follow the system of Cook and Tomlinson.²⁶

TABLE 1. CLINICAL DATA AND REARRANGEMENTS OF IMMUNOGLOBULIN HEAVY-CHAIN GENES IN L&H CELLS FROM 11 PATIENTS WITH NODULAR LYMPHOCYTE-PREDOMINANT HODGKIN'S DISEASE.

PATIENT NO.	AGE (YR)/ SEX	DISEASE STAGE	V_H REARRANGEMENTS*		REARRANGEMENTS IN L&H CELLS	
			T CELLS	L&H CELLS	NO. IDENTICAL†	NO. UNRELATED‡
			no. PCR-positive/ no. studied§			
1¶						
Biopsy 1	60/M	II	0/7	18/35	18	0
Biopsy 2	61/M	IV	0/7	14/51	14	0
2	32/M	II	0/7	24/73	24	0
3	37/M	I	0/7	22/64	21	1
4	43/M	III	0/7	13/29	13	0
5	41/M	I	0/7	5/53	5	0
6	Not available		0/7	14/41	14	0
7	47/M	I	0/7	13/25	13	0
8	50/M	I	1/7	8/89	8	0
9	39/M	III	0/7	12/36	12	0
10	73/F	I	0/7	9/56	9	0
11	15/M	I	0/7	8/63	8	0

*L&H cells were studied together with T cells and B cells (not shown) as controls.

†Identical rearrangements do not differ in their nucleotide sequences, except for substitutions of nucleotides that represent somatic mutations among clonal variants.

‡Unrelated rearrangements differ in complementarity-determining region 3 from all other V_H rearrangements.

§The number of cells that gave rise to an amplification product by PCR is shown, followed by the total number of cells isolated from the tissue section.

¶Nine months elapsed between the two biopsies in Patient 1.

||This patient relapsed 10 years after the initial diagnosis.

nodular lymphocyte-predominant^{12,16,28} and classic types of Hodgkin's disease have had discordant results.^{16,22,28} These discrepancies were most likely due to difficulties with the technique of cell isolation, among which problems with the amplification of contaminating V_H sequences are the most serious. We overcame this difficulty by reducing the volume of buffer used in the suspension of single cells. With this technical modification, none of the 959 samples that were drawn from the buffer covering the frozen sections after the isolation of each cell contained V_H -specific amplification products, and only 1 of the 84 T cells isolated from the tissue sections from the patients with nodular lymphocyte-predominant Hodgkin's disease yielded a PCR product. Moreover, 260 B cells isolated from frozen sections of hyperplastic tonsils and samples of tissue affected by the disease gave rise to 102 PCR products with unrelated V_H genes.

The CDR3 sequence is the molecular signature of a B cell, because randomly selected nucleotides are inserted in the course of the V_H rearrangement. None of the V_H sequences derived from the clonal L&H cells that we studied were homologous with

sequences we studied previously or with CDR3 sequences in the data bank. The single V_H rearrangement in the sample from Patient 3 that was unrelated to the highly mutated clonal population of L&H cells probably originated from a neighboring B cell, as was evidenced by the absence of somatic mutations in the rearranged V_H gene. That two biopsy specimens from Patient 1 obtained nine months apart contained identical V_H rearrangements provides further support for the validity of our method.

Physiologically, somatic mutations are introduced into immunoglobulin V genes at the centroblastic stage of B-cell differentiation in germinal centers during the immune response.^{29,30} Through this process, B cells acquire immunoglobulin receptors with higher or lower affinity for the antigen. The high-affinity B cells are selected by antigen for further differentiation into memory B cells and plasma cells.³⁰ These cells have a low-to-moderate number of somatic mutations (up to 6 percent).²⁹ By contrast, receptors on B cells that have highly mutated V genes lose affinity for the antigen and undergo apoptosis.³⁰ In our study, the high load of somatic mutations (7.5 to 27.2 percent) in all 11 cases of nodular lym-

TABLE 2. MOLECULAR FEATURES OF THE IMMUNOGLOBULIN HEAVY- AND LIGHT-CHAIN GENES IN L&H CELLS OF 11 PATIENTS WITH NODULAR LYMPHOCYTE-PREDOMINANT HODGKIN'S DISEASE.

PATIENT NO.	NO. OF L&H CELLS STUDIED	V _H FAMILY	SEQUENCES WITH SOMATIC MUTATIONS (%)*			INTRACLONAL VARIANTS	CODING CAPACITY	LIGHT-CHAIN TRANSCRIPTS FOUND
			ALL SEQUENCES					
			CDR2	FR3				
1†								
Biopsy 1	18	VH3	17.0	17.6	16.6	Yes	Yes	No data
Biopsy 2	14	VH3	16.3	13.7	17.7	Yes	Yes	No data
2	24	VH3	27.2	43.1	18.7	Yes	Yes	κ
3	21	VH1	19.7	15.6	21.8	Yes	Yes	κ
4	13	VH3	7.5	0	11.4	No	No‡	κ
5	5	VH1	27.2	31.3	25.0	No	Yes	κ
6	14	VH3	8.2	15.6	4.1	No	Yes	No data
7	13	VH3	14.2	21.5	10.4	Yes	Partial§	No data
8	8	VH3	10.2	17.6	6.3	No	Yes	No data
9¶	12	VH3	11.5	21.5	6.3	Yes	Yes	κ
10	9	VH3	12.2	27.4	4.2	No	Yes	No data
11	8	VH3	9.5	13.7	7.3	Yes	Yes	κ

*CDR2 denotes complementarity-determining region 2, and FR3 framework region 3. Values shown for somatic mutations are percentages of the length of CDR2 (51 bp) and FR3 (96 bp).

†Nine months elapsed between the two biopsies in Patient 1.

‡All 13 cells studied in this patient had two stop codons and a frame-shift mutation.

§Eleven of the 13 cells studied in this patient had a stop codon in the FR3 region.

¶One amplification product for this patient contained an in-frame deletion of 3 bp.

phocyte-predominant Hodgkin's disease and the signs of ongoing mutation in 6 of these cases suggest that L&H cells are resistant to these physiologic mechanisms, perhaps because of a block in the apoptotic pathway. The presence in two patients of L&H cells with disrupted capacity for coding by immunoglobulin genes supports this theory, because normally such B cells cannot survive.

The evidence that L&H cells are related to B cells in germinal centers or their progeny includes the pattern of V_H gene mutation, the consistent association of L&H cells with progressively transformed germinal centers,³¹ the distribution of L&H cells within these structures, the expression of cell antigens characteristic of the germinal center,³²⁻³⁴ and the resemblance of the L&H cells to large centroblasts. Our findings show that L&H cells arise from a single clone of germinal-center B cells.

Although our data are limited and the light-chain V genes of L&H cells were not sequenced, it appears that the rearranged V genes in L&H cells and those in Reed-Sternberg cells have undergone somatic mutation to a similar extent²¹ (and this study). However, in L&H cells these mutated genes usually retain the potential for translation into protein, whereas in Reed-Sternberg cells they often lose their coding capacity.²¹ Moreover, intracлонаl variants seem to be more frequent among L&H cells than among Reed-Sternberg cells. If further investigation substantiates

these differences, they would constitute important molecular distinctions between nodular lymphocyte-predominant Hodgkin's disease and classic Hodgkin's disease.

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