

IDENTIFICATION OF COMMON GERMINAL-CENTER B-CELL PRECURSORS IN TWO PATIENTS WITH BOTH HODGKIN'S DISEASE AND NON-HODGKIN'S LYMPHOMA

ANDREAS BRÄUNINGER, PH.D., MARTIN-LEO HANSMANN, M.D., JOHN G. STRICKLER, M.D., REINHARD DUMMER, M.D., GÜNTHER BURG, M.D., KLAUS RAJEWSKY, M.D., AND RALF KÜPPERS, PH.D.

ABSTRACT

Background Hodgkin's disease and non-Hodgkin's B-cell lymphoma occasionally occur in the same patient. The identification of a common precursor of the two types of lymphoma would show definitively that Reed–Sternberg cells originate from B cells.

Methods We studied lymphomas from two patients, one with a composite lymphoma (classic Hodgkin's disease and a follicular lymphoma in the same lymph node) and the other with a T-cell-rich B-cell lymphoma that was followed by classic Hodgkin's disease. Single Reed–Sternberg cells and non-Hodgkin's lymphoma cells from frozen sections were micromanipulated. The rearranged immunoglobulin variable-region genes (*V* genes) of the heavy and light chains were amplified by the polymerase chain reaction from genomic DNA and sequenced.

Results In both patients, the Reed–Sternberg cells were related clonally to the non-Hodgkin's lymphoma B cells. The *V* genes carried somatic mutations (a hallmark of germinal-center B cells and their descendants). In both patients, some somatic mutations were shared by the Reed–Sternberg and non-Hodgkin's lymphoma cells, whereas other somatic mutations were found exclusively in one or the other cell type.

Conclusions In two patients with classic Hodgkin's disease and non-Hodgkin's B-cell lymphoma, we identified a common B-cell precursor, probably a germinal-center B cell, for both lymphomas. This finding suggests that the two types of lymphoma underwent both shared and distinct transforming events and provides proof of the B-cell derivation of Reed–Sternberg cells in classic Hodgkin's disease. (N Engl J Med 1999;340:1239–47.)

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MOLECULAR studies of single Hodgkin and Reed–Sternberg cells (hereafter referred to as Reed–Sternberg cells) in classic Hodgkin's disease (i.e., nodular sclerosis, mixed cellularity, and lymphocyte depletion) and lymphocyte-predominant Hodgkin's disease have shown that these cells are clonal populations of transformed B lymphocytes with somatically mutated immunoglobulin variable-region genes (*V* genes).^{1–8} Somatic mutations are introduced into rearranged *V* genes of B cells that are participating in an im-

mune response within a germinal center; these mutations are the distinctive feature of germinal-center B cells and their descendants.⁹ In classic Hodgkin's disease, the origin of Reed–Sternberg cells from germinal-center B cells was indicated by the finding that in some cases the *V* genes were rendered non-functional by crippling mutations (such as mutations creating stop codons).² Because such mutations usually cause the death of B cells by apoptosis within the germinal center, we surmised that the precursors of the Reed–Sternberg cells must have resided within the germinal center and been rescued from apoptosis by a transforming event.^{2,10} However, because Reed–Sternberg cells express several genes that are thought to be specific to dendritic or monocytic cells, or both, it has also been argued that Reed–Sternberg cells represent an unusual type of dendritic or monocytic cell.^{11–14}

In rare cases, Hodgkin's disease and a non-Hodgkin's B-cell lymphoma occur in the same patient, either simultaneously or sequentially.^{15–17} Definitive proof of the origin of Reed–Sternberg cells from B cells could be obtained by demonstrating that in such cases the B-cell lymphoma and the Reed–Sternberg cells are members of the same B-cell clone. In the current study, we used micromanipulation of single Reed–Sternberg cells and non-Hodgkin's B-cell lymphoma cells from two patients with such cases, and we amplified rearranged immunoglobulin *V* genes from genomic DNA of the single cells. Because rearrangements of immunoglobulin genes are highly diverse among B cells, they represent ideal clonal markers for these cells. In both patients in our study, the Reed–Sternberg cells and the non-Hodgkin's B-cell lymphoma cells shared a common germinal-center B-cell precursor.

CASE REPORTS

Patient 1 was a 75-year-old woman who presented with retroperitoneal lymphadenopathy in June 1990. A biopsy of retroperitoneal nodes showed a composite follicular small-cleaved-cell

From the Department of Pathology, University of Frankfurt, Frankfurt am Main, Germany (A.B., M.-L.H.); the Department of Pathology, Mayo Clinic, Rochester, Minn. (J.G.S.); the Department of Dermatology, Universitätsspital Zürich, Zurich, Switzerland (R.D., G.B.); and the Institute for Genetics, University of Cologne, Cologne, Germany (K.R., R.K.). Address reprint requests to Dr. Küppers at the University of Cologne, University Hospital, LFI E4 R706, Joseph-Stelzmannstr. 9, 50931 Cologne, Germany, or at rkuppers@mac.genetik.uni-koeln.de.

lymphoma and, in the same lymph node, mixed-cellularity Hodgkin's disease with abundant Reed–Sternberg cells. The Reed–Sternberg cells were CD30- and CD15-positive, whereas the tumor cells of the follicular lymphoma were negative for both markers. On a section of paraffin-embedded material, some Reed–Sternberg cells were CD20-positive. The patient was considered to have stage IVB disease on the basis of the presence of follicular lymphoma in the bone marrow and liver. She was treated with eight cycles of cyclophosphamide, vinblastine, procarbazine, and prednisone but the disease recurred (recurrence was not confirmed by biopsy) in December 1991.

Patient 2 was a 50-year-old man who presented with a T-cell-rich B-cell lymphoma of the skin in January 1994. The lymphoma was surgically removed, and no additional therapy was given. In January 1997, classic Hodgkin's disease was diagnosed in an axillary lymph node. The Reed–Sternberg cells were CD30- and CD15-positive. The patient also had Gardner's syndrome, and he underwent colectomy in 1992.

METHODS

Immunostaining and Micromanipulation

We stained frozen tissue sections, 5 to 10 μm thick, with anti-CD20 (L26; Dako, Hamburg, Germany) or anti-CD30 (BerH2; Dako) monoclonal antibodies.^{18,19} We visualized bound alkaline phosphatase with fast red. We overlaid stained sections with TRIS-buffered saline, and we isolated single cells using two hydraulic micromanipulators.^{18,19} Isolated cells were stored at -20°C in 20- μl Expand high-fidelity polymerase-chain-reaction (PCR) buffer (Boehringer Mannheim, Mannheim, Germany). From each section used for micromanipulation, we aspirated aliquots of the buffer covering the section and used them as negative controls in parallel with the analysis of the cell samples.

Single-Cell PCR Analysis

We amplified rearranged immunoglobulin genes from genomic DNA of single cells in a seminested PCR analysis using either family-specific V_H (variable-region heavy-chain gene) leader or framework region I primers and V_{κ} or V_{λ} (variable-region κ or λ light-chain) framework region I family-specific primers together with two sets of the respective joining-region (J gene)-segment primers.^{1,2,19} We carried out the first round of amplification using a collection of primers for V_H (and V_{κ} or V_{λ}) genes together with the respective J -segment primers. In the second round of amplification, we further amplified 1.5- μl aliquots (3 percent) from the first round with the same V gene primers in separate reactions and internal J -segment primer mixes. The PCR products were gel-purified and directly sequenced on an automatic sequencer (ABI377, Applied Biosystems, Wieterstadt, Germany). We analyzed the sequences using the data bases of Immunogenetics and GenBank. Reed–Sternberg cells and non-Hodgkin's B-cell lymphoma cells were analyzed in parallel with buffer controls.

RESULTS

PCR Analysis of Immunoglobulin Gene Rearrangements in Single Reed–Sternberg and Non-Hodgkin's Lymphoma B Cells

In both cases — the first a composite lymphoma consisting of classic Hodgkin's disease and a follicular lymphoma in the same lymph node, and the second a T-cell-rich B-cell lymphoma of the skin followed by classic Hodgkin's disease in a lymph node three years later — the Reed–Sternberg cells were CD30-positive and CD20-negative, whereas the non-Hodgkin's lymphoma cells were CD30-negative and CD20-positive (Fig. 1); in the composite lymphoma,

some Reed–Sternberg cells were CD20-positive. Consequently, for the micromanipulation of single cells, Reed–Sternberg cells in frozen sections were stained with the anti-CD30 antibody, and the non-Hodgkin's lymphoma cells in frozen sections were stained with the anti-CD20 antibody.

In the case of Patient 1, we analyzed 22 Reed–Sternberg cells for immunoglobulin gene rearrangements (Table 1) (not all cells generated a PCR product). The same V_H gene rearrangement was amplified from 12 of the 22 Reed–Sternberg cells, and the same V_{κ} (light chain) gene was amplified from 4 of the 12 cells analyzed for V_{κ} gene rearrangements. We obtained a clonal V_{λ} rearrangement from four of five cells analyzed for V_{λ} (light chain) gene rearrangements. The same three clonal V_H , V_{κ} , and V_{λ} gene rearrangements were also obtained from cells of the follicular lymphoma: the V_H gene from 26 of 35 samples (each containing two cells), the V_{κ} gene from 5 of 12 samples, and the V_{λ} gene from 4 of 5 samples (Table 1). From 1 of 11 buffer controls, we amplified a V_H gene rearrangement, which was most likely due to cellular contamination. One V_H gene that differed from the clonal rearrangement was amplified from a CD20-positive cell; it may have represented a rare normal B cell present in the follicular lymphoma.

In the case of Patient 2, we amplified clonal V_H and clonal V_{κ} gene rearrangements from 18 and 21 of 60 Reed–Sternberg cells, respectively (Table 1). The same rearrangements were also amplified from T-cell-rich B-cell lymphoma cells, the V_H gene from 23 of 60 cells and the V_{κ} gene from 9 of 60 cells. We amplified three different V gene rearrangements from 1 Reed–Sternberg cell and 2 of 48 buffer controls. We amplified one clonal V_H gene from another buffer control (Table 1). We assumed that these PCR products represented cellular contamination.

The presence of the same clonal V gene rearrangements in the Reed–Sternberg cells of Hodgkin's disease and the associated non-Hodgkin's lymphoma cells indicates that a single B cell is the common precursor of both lymphomas.

Analysis of Somatic-Mutation Patterns

The V_H and V_{λ} gene rearrangements amplified from the Reed–Sternberg cells of Patient 1 were potentially functional (translatable into protein RNA) and somatically mutated with mutation frequencies of 9.6 and 5.8 percent, respectively (Table 2). No intraclonal diversity was observed. Most somatic mutations in the V_H and V_{λ} genes of the Reed–Sternberg cells were shared by the corresponding rearrangements of the follicular lymphoma. However, in the heavy-chain rearrangement, the Reed–Sternberg cells harbored two point mutations that were absent in the V_H gene of the follicular lymphoma, and the cells lacked one point mutation present in the V_H gene of

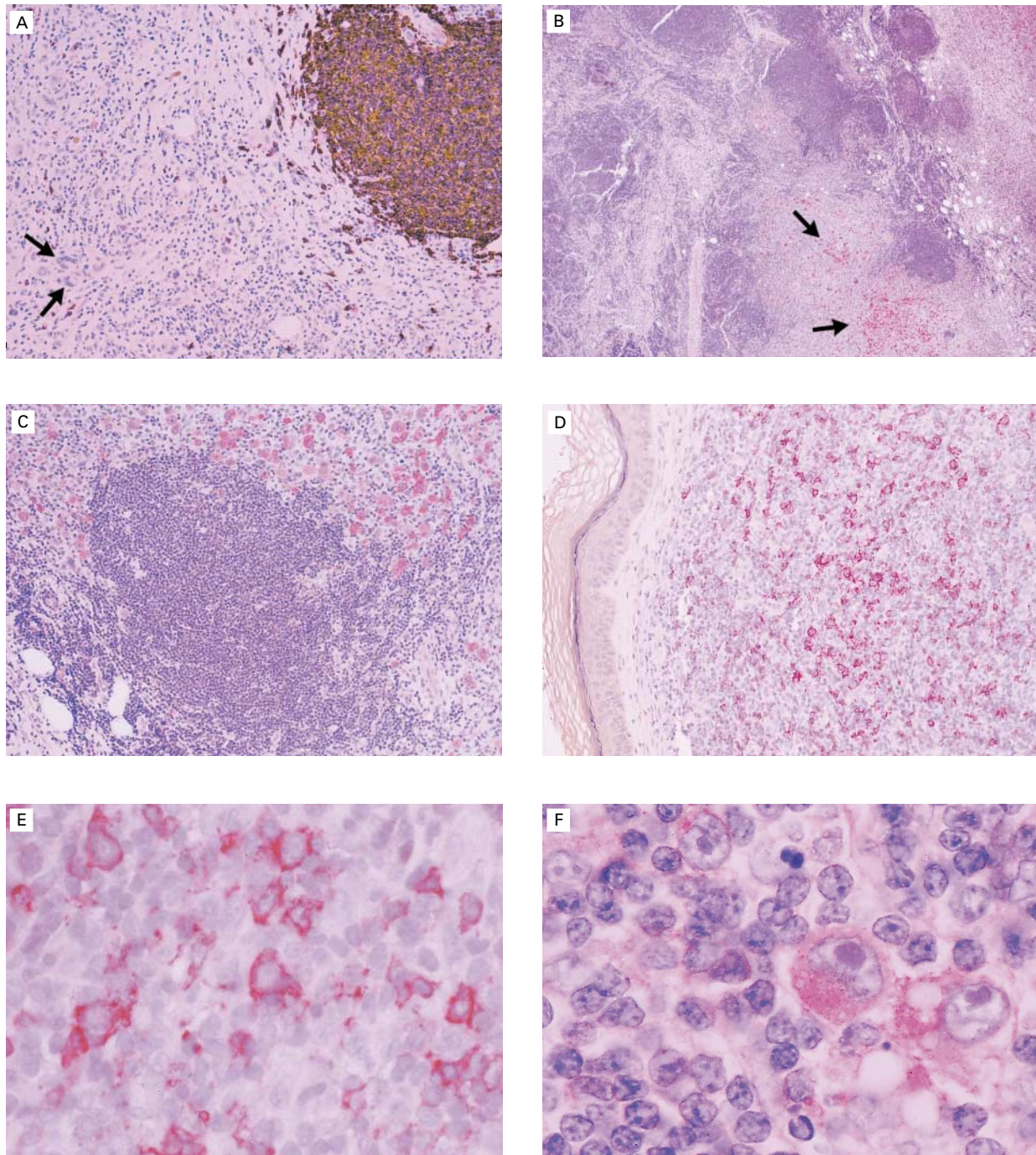


Figure 1. Immunostaining of the Composite Lymphoma from Patient 1 and the T-Cell-Rich B-Cell Lymphoma and Hodgkin's Disease Infiltrate from Patient 2.

Panel A shows the composite lymphoma from Patient 1 stained for CD20 with use of peroxidase. Positive immunostaining of the follicular lymphoma is shown at the upper right. Reed-Sternberg cells (arrows) were CD20-negative. Panel B shows the composite lymphoma stained for CD30 with use of alkaline phosphatase. Cells of the follicular lymphoma were negative, whereas Reed-Sternberg cells (arrows) were CD30-positive. Panel C shows anti-CD30 staining of a frozen section from the composite lymphoma. Panel D shows a skin-biopsy specimen of the T-cell-rich B-cell lymphoma from Patient 2. It shows numerous large CD20-positive blast cells in the corium (alkaline phosphatase). Panel E shows the T-cell-rich B-cell lymphoma with large CD20-positive B blasts surrounded by T lymphocytes. CD20 staining was visualized with alkaline phosphatase and fast red. Panel F shows Hodgkin's disease infiltrate in a lymph-node-biopsy specimen from Patient 2. Three CD30-positive Reed-Sternberg cells can be seen. CD30 staining was visualized with alkaline phosphatase and fast red.

TABLE 1. PCR AND SEQUENCE ANALYSIS OF *V* GENE REARRANGEMENTS OF SINGLE REED–STERNBERG AND NON-HODGKIN’S LYMPHOMA CELLS.

PATIENT NO.	SOURCE OF SAMPLES	V GENE*	No. OF POSITIVE SAMPLES	PCR PRODUCTS	No. OF REARRANGEMENTS		
					REPEATED	UNIQUE	
1	Hodgkin’s disease	V_H	12/22	12 V_H4	12		
		V_κ	4/12	4 $V_\kappa1$		4	
		V_λ	4/5	4 $V_\lambda1$		4	
	Buffer controls†	V_H	1/7	1 V_H4		1	
		V_κ	0/3				
		V_λ	0/2				
	Follicular lymphoma	V_H	27/35‡§	27 V_H4	26	1	
		V_κ	5/12‡	5 $V_\kappa1$		5	
		V_λ	4/5‡	4 $V_\lambda1$		4	
		Buffer controls†	V_H	0/4			
	2	Hodgkin’s disease	V_H	18/60	18 V_H3	17	1
			V_κ	21/60	21 $V_\kappa2$		21
Buffer controls†		V_H ¶	2/24	1 V_H1 , 1 V_H3		2	
		V_κ ¶	0/24				
T-cell–rich B-cell lymphoma		V_H ¶	23/60	23 V_H3	20		
		V_κ ¶	9/60	9 $V_\kappa2$		9	
Buffer controls†		V_H ¶	1/24	1 V_H3		1	
		V_κ ¶	0/24				

*Several primer collections were used for the analysis of the two cases: V_H framework region I family-specific primers together with V_κ framework region I family-specific primers; V_H leader region family-specific primers alone (Patient 1) or in combination with V_κ framework region I family-specific primers (Patient 2); and V_H framework region I family-specific primers together with V_λ framework region I family-specific primers. In both cases, V_H and V_L genes were coamplified repeatedly from the same cells.

†Buffer controls were aliquots of the buffer covering the tissue sections during micromanipulation. Additional controls with tubes lacking cells or aspirated buffer were also included but are not shown here. They were consistently negative.

‡Because the efficiency of PCR for the amplification of a *V* gene from a single cell is usually less than 50 percent, two neighboring CD20 cells were put into one reaction tube to shorten micromanipulation and PCR analysis.

§Eighteen of the samples were obtained by micromanipulating a group of 30 to 40 B cells and dividing the DNA of these cells into 18 samples after proteinase K digestion, so that each tube contained on average the DNA of 2 cells.

¶Ten cells of each lymphoma and four buffer controls each were analyzed with a V_H leader region family-specific primer mix and primers specific for A17 V_κ gene rearrangements. (The sequence of the A17-specific primer was 5’CTACCCTTGCCCTTGACTGATCAGAC3’.)

||Three V_H3 PCR products were not sequenced.

the follicular lymphoma (Fig. 2). Likewise, 15 point mutations in the V_λ gene were shared by the Reed–Sternberg and follicular-lymphoma cells, but the former carried 2 additional point mutations. In the V_H and V_λ genes of the follicular lymphoma, ongoing somatic mutation was observed, as is typical of follicular lymphoma.^{21,22} For the heavy-chain rearrangement, we observed 23 sequence variants among the 26 V_H gene sequences (Fig. 2 and Table 2). Most variants differed by one to four mutations from the sequence with the smallest number of mutations.

The V_κ gene amplified from Reed–Sternberg and

follicular-lymphoma cells of the composite lymphoma was nonfunctional and unmutated. In B cells expressing immunoglobulin λ light chains, the nonfunctional V_κ gene rearrangements that are often present in such cells are usually inactivated by deletion of the C_κ gene and the κ enhancers; this deletion also abolishes somatic hypermutation in persisting $V_\kappa J_\kappa$ joints of the rearranged variable-region genes.⁹

In Patient 2, the V_H and V_κ gene rearrangements of the Reed–Sternberg cells were both potentially functional and somatically mutated, with mutation frequencies of 11.7 and 8.3 percent, respectively (Ta-

TABLE 2. V GENE SEQUENCE AND MUTATION ANALYSIS OF IMMUNOGLOBULIN GENE REARRANGEMENTS FROM REED–STERNBERG AND NON-HODGKIN'S LYMPHOMA CELLS.

PATIENT NO.	SOURCE OF CELLS	V GENE (V FAMILY)	POTENTIALLY FUNCTIONAL	MUTATION FREQUENCY	INTRACLONAL DIVERSITY	SAMPLES WITH SEQUENCE VARIATION
						no. with variations/ total no.*
1	Hodgkin's disease	$V_H4-59 (V_H4)$	Yes	9.6	No	—/12
		L24a ($V_\lambda1$)	No	0	No	—/4
		V1-1g ($V_\lambda1$)	Yes	5.8	No	—/4
	Follicular lymphoma	$V_H4-59 (V_H4)$	Yes	9.3	Yes	23/26
		L24a ($V_\lambda1$)	No	0	No	—/5
		V1-1g ($V_\lambda1$)	Yes	5.1	Yes	2/4
2	Hodgkin's disease	$V_H3-30 (V_H3)$	Yes	11.7	Yes	2/17
		A17 ($V_\kappa2$)	Yes	8.3	No†	—/21
	T-cell-rich B-cell lymphoma	$V_H3-30 (V_H3)$	Yes	15.8	Yes	16/20
		A17 ($V_\kappa2$)	Yes	14.6	Yes	3/9

*The dash indicates that all sequences were identical.

†Two mixed sequences with two nucleotides at one or two positions on the sequencing gels (representing variants of the same clonal rearrangement) were amplified, indicating the presence of two copies of the rearranged immunoglobulin genes in the cell of origin, *Taq* DNA polymerase errors in one of the first PCR cycles, or both. The V gene sequences reported in this article have been submitted to the European Molecular Biology Laboratory data library under accession numbers AJ011130 through AJ011139. For the non-Hodgkin's lymphoma, the V gene sequences with the lowest numbers of mutations were submitted.

ble 2). One V_H sequence differed from the 17 others by a single nucleotide. The V gene rearrangements of the T-cell-rich B-cell lymphoma carried a higher load of somatic mutation than did the corresponding rearrangements of the Reed–Sternberg cells: 15.8 percent for the V_H gene and 14.6 percent for the V_κ gene. In this patient, most of the mutations were unique to either the Reed–Sternberg cells or the T-cell-rich B-cell lymphoma cells. For the heavy-chain gene rearrangement, only 7 mutations were shared between the two lymphomas; 28 of the mutations in the V_H gene of the Reed–Sternberg cells were not present in the T-cell-rich B-cell lymphoma cells, whereas the latter carried 39 mutations that were not present in the Reed–Sternberg cells (data not shown). The situation was similar for the V_κ light-chain genes (data not shown). As in Patient 1, the non-Hodgkin's lymphoma cells showed considerable intraclonal diversity (Table 2).

The pattern of somatic mutation in the rearranged V genes of the clonally related lymphomas is evidence of the reliability of our analysis. In both patients, the patterns of somatic mutations found in the clonally related immunoglobulin gene rearrangements of the Reed–Sternberg cells differed from those of the corresponding non-Hodgkin's lymphoma cells (Fig. 2). We found these distinct patterns of somatic mutation in different populations of tumor cells that were isolated and analyzed in parallel, ruling out the possibility of contamination.

Because crippling mutations rendering previously functional rearrangements nonfunctional were detected in some cases of classic Hodgkin's disease in our earlier studies,² we amplified and sequenced additional upstream and downstream fragments of the V genes of the lymphomas analyzed here (data not shown). However, no obviously crippling mutations (such as stop codons) were detected. This finding does not argue against the possibility that Reed–Sternberg cells in these cases derive from crippled germinal-center B cells, because most types of crippling mutations (such as those that disturb heavy- or light-chain folding or reduce affinity to the selecting antigen) cannot be identified easily.

DISCUSSION

The occurrence of a non-Hodgkin's B-cell lymphoma and Hodgkin's disease in the same patient is rare.^{15,16} The most frequent combination of the two is a diffuse large-cell lymphoma that develops after lymphocyte-predominant Hodgkin's disease.¹⁵⁻¹⁷ Because the B-cell origin of the Reed–Sternberg cells in the lymphocyte-predominant subtype of Hodgkin's disease has long been suspected,²³ a transformation to a high-grade lymphoma (as is well known to occur in several types of low-grade non-Hodgkin's B-cell lymphoma) would not be surprising. However, studies of the clonal relation between lymphocyte-predominant Hodgkin's disease and subsequent or concurrent non-Hodgkin's B-cell lym-

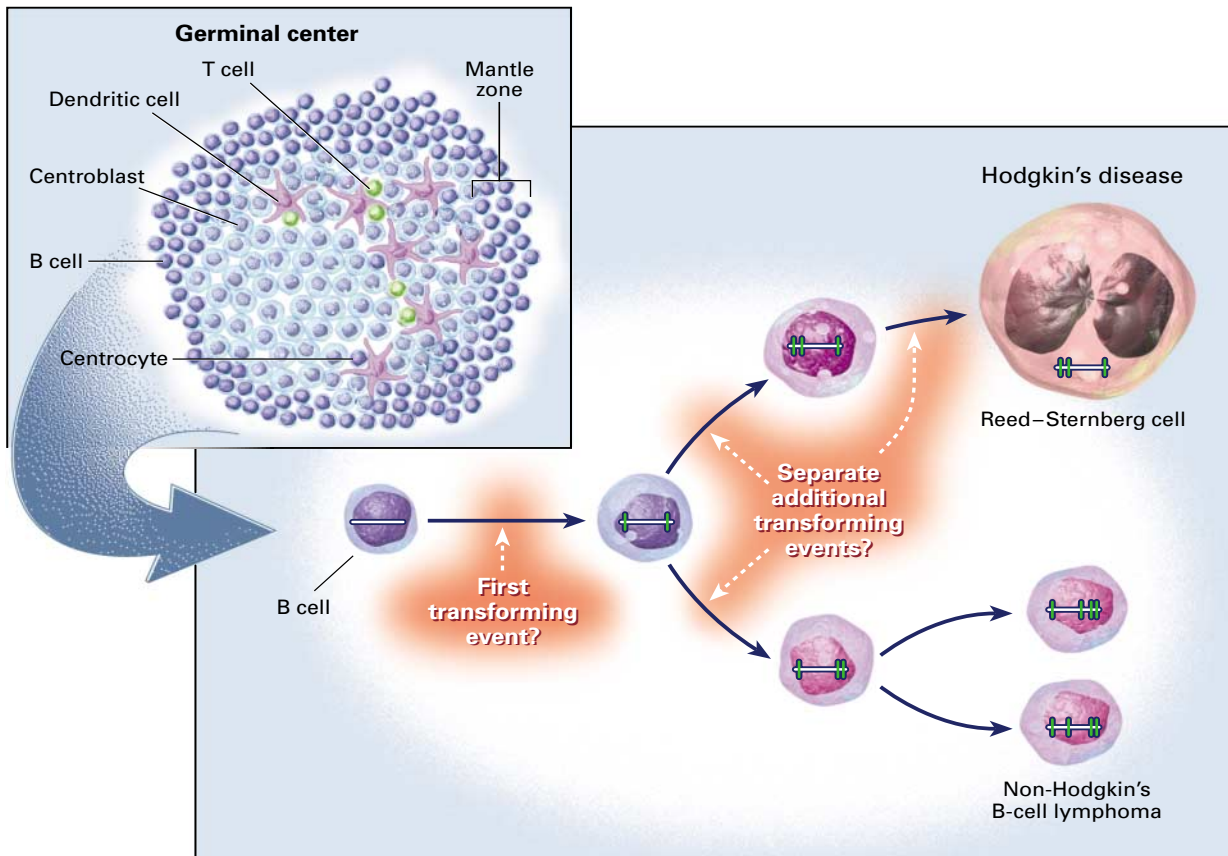


Figure 3. Scenario for the Generation of a Composite Lymphoma.

The horizontal lines within the circles indicate a V gene rearrangement; vertical lines within the circles indicate somatic mutations.

phoma found a clonal relation between the two lymphomas in only some cases.²⁴⁻²⁷ In the example of classic Hodgkin's disease in which the patient also had a non-Hodgkin's lymphoma, only a single case has been analyzed with molecular techniques.²⁸ The two lymphomas were clonally unrelated. In this instance, the non-Hodgkin's lymphoma may have been related to the therapy received, because the patient underwent radiotherapy and chemotherapy before the non-Hodgkin's lymphoma developed.

Both lymphomas in each of the two patients we studied were derived from a common B-cell precursor. In Patient 2, in whom Hodgkin's disease was diagnosed three years after the T-cell-rich B-cell lymphoma, the Reed-Sternberg cells or their precursor must have been present during those three years.

In both patients, there was considerable ongoing mutation within the tumor cells of the non-Hodgkin's lymphoma, as indicated by numerous variations in the clonal sequences. This phenomenon is well known in follicular lymphoma^{21,22} and also typical of T-cell-rich B-cell lymphoma.²⁹ Because the process of somatic hypermutation is thought to be restricted

to germinal-center B cells, these findings — together with other morphologic, histologic, and immunohistochemical features of the two types of lymphomas^{30,31} — indicate that the non-Hodgkin's lymphomas in both patients were cancers derived from germinal-center B cells.

In the composite lymphoma, most of the somatic mutations were the same in the Reed-Sternberg cells as in the follicular-lymphoma cells. This finding indicates that a germinal-center B cell that had already acquired a high load of somatic mutation gave rise to two daughter cells, one of which was the origin of the Reed-Sternberg cell clone, and the other of the follicular lymphoma. In the other patient, the number of shared mutations was lower (only 7 of a total of 74 mutations in the heavy chain). Nevertheless, in this case the pattern of V gene mutations also indicates that the tumor clones originated from a common germinal-center B-cell progenitor.

In our previous work, the interpretation that the Reed-Sternberg cells in classic Hodgkin's disease derive from germinal-center B cells was based on the argument that transformed B cells with crippled

V genes reside within the germinal center and must have been rescued from apoptosis in this microenvironment by some transforming event.^{2,10} The two cases presented here address the issue of the cellular origin of Reed–Sternberg cells in classic Hodgkin’s disease independently and more directly. The finding of a clonal relation and the pattern of *V* gene mutations of the Reed–Sternberg and non-Hodgkin’s lymphoma cells in the same patient unequivocally demonstrate the derivation of Reed–Sternberg cells from mature B cells and are further evidence of their germinal-center B-cell derivation.

Because Reed–Sternberg cells express a number of genes typical of dendritic cells, monocytic cells, or both,^{11–14} it has been argued that Reed–Sternberg cells derive from dendritic cells in which immunoglobulin gene rearrangements accidentally occurred. The results presented here rule out this possibility. Rather, the Reed–Sternberg cells are B cells that in the course of malignant transformation can acquire features of dendritic cells, an event that may be of key importance for the pathogenesis of Hodgkin’s disease.

The finding of a shared precursor of the lymphomas in each patient has implications for the pathogenesis of lymphomas. Because it is unlikely that two B cells belonging to the same germinal-center B-cell clone give rise to two lymphomas independently, we assume that both lymphomas in each patient shared one or more transforming events. These transforming events could have taken place before the tumor precursor entered the germinal center or in the course of the germinal-center reaction. Additional transforming events that are specific for the Reed–Sternberg or the non-Hodgkin’s lymphoma precursor cell presumably occurred later, probably within the germinal center, thus explaining the development of two distinct diseases from the same B-cell precursor (Fig. 3).

Because the Epstein–Barr virus is thought to participate in the pathogenesis of classic Hodgkin’s disease in some cases,³² the presence of Epstein–Barr virus was sought in the Reed–Sternberg and non-Hodgkin’s lymphoma cells in both cases, but the results were negative (data not shown). Moreover, although most follicular lymphomas carry translocations of the *bcl-2* oncogene into the immunoglobulin heavy-chain locus,³³ we failed to detect such a translocation in the Reed–Sternberg and follicular lymphoma cells of the composite lymphoma using a PCR-based method (data not shown). For these reasons, the shared and unique transforming events involved in the development of these lymphomas remain to be identified.

In summary, the two combinations of Hodgkin’s disease and non-Hodgkin’s lymphoma that we studied exemplify the close relation between these diseases and provide definitive evidence of the origin of Reed–Sternberg cells from B cells in classic Hodg-

kin’s disease. Moreover, the development of two lymphomas from members of a single germinal-center B-cell clone supports the emerging concept that molecular processes in germinal-center B cells play a decisive part in the malignant transformation of B lymphocytes and further supports the concept that Reed–Sternberg cells derive from B cells residing within the germinal center.⁹

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