

## **Rare Occurrence of Classical Hodgkin's Disease as a T Cell Lymphoma**

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### **Abstract**

Recent work identified Hodgkin and Reed-Sternberg (H/RS) cells in classical Hodgkin's disease (cHD) as clonal progeny of mature B cells. Therefore, it is generally assumed that cHD homogeneously represents a B cell lymphoma. In a subset of cHD, however, H/RS cells expressing T cell-associated proteins may be candidates for alternative lineage derivation. Single H/RS cells with cytotoxic T cell phenotype were micromanipulated from three cases of cHD and analyzed by single cell polymerase chain reaction for immunoglobulin heavy (IgH) and light chain (IgL) gene rearrangements, T cell receptor (TCR)- $\beta$  gene rearrangements, and germline configuration of the IgH and TCR- $\beta$  loci. H/RS cells from two cases of cHD harbored clonal, somatically mutated Ig gene rearrangements, whereas TCR- $\beta$  loci were in germline configuration. In contrast, H/RS cells from an additional case harbored clonal TCR- $\beta$  variable/diversity/joining (VDJ) and DJ gene rearrangements, whereas the IgH locus was in germline configuration on both alleles. Thus, in two cases of cHD with H/RS cells expressing cytotoxic T cell molecules, the tumor cells are derived from mature B cells that aberrantly express T cell markers. In a third case, however, H/RS cells were derived from a T cell, demonstrating that cHD can also occur as a T cell lymphoma.

**Key words:** Hodgkin's disease • T cell receptor genes • immunoglobulin genes • somatic hypermutation • Epstein-Barr virus

### **Introduction**

In classical Hodgkin's disease (cHD), the malignant Hodgkin and Reed-Sternberg (H/RS) cells typically account for <1% of cells within a complex admixture of lymphocytes, plasma cells, histiocytes, and eosinophils (1). The origin of H/RS cells in cHD was enigmatic and a matter of debate for more than a decade. Although H/RS cells usually lack expression of B lineage markers, there is now strong evidence that H/RS cells represent the outgrowth of a dominant tumor clone derived from mature B cells (2, 3). This conclusion is based on the amplification of clonally related Ig gene rearrangements from single micromanipulated

H/RS cells (4, 5). The presence and pattern of somatic mutations in the rearranged V genes identified germinal center B cells as the precursors of the tumor cells (5).

In a minority of cHD cases (~5–15%), however, H/RS cells express cytotoxic T cell markers (granzyme B, perforin, and T cell intracellular antigen 1 [TIA-1]), raising the possibility that the tumor cells in these cases might originate from T lymphocytes (6–9). To clarify whether H/RS cells in such cases are indeed derived from T lymphocytes, H/RS cells were micromanipulated and subjected to single cell PCR analysis for rearranged Ig heavy chain (IgH), Ig $\kappa$ , and Ig $\lambda$  light chain genes, TCR- $\beta$  VDJ and DJ gene rearrangements, as well as IgH and TCR- $\beta$  germline configuration (i.e., absence of rearrangements). Whereas IgH VDJ gene rearrangements are specific for and restricted to B lineage cells, the presence of TCR- $\beta$  VDJ gene rearrangements identifies a T cell. Studying H/RS cells from three such cases, a B cell

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genotype was found in two, a genotype revealing T cell origin in one case.

## Materials and Methods

Clinical data on the three cases of cHD are summarized in Table I.

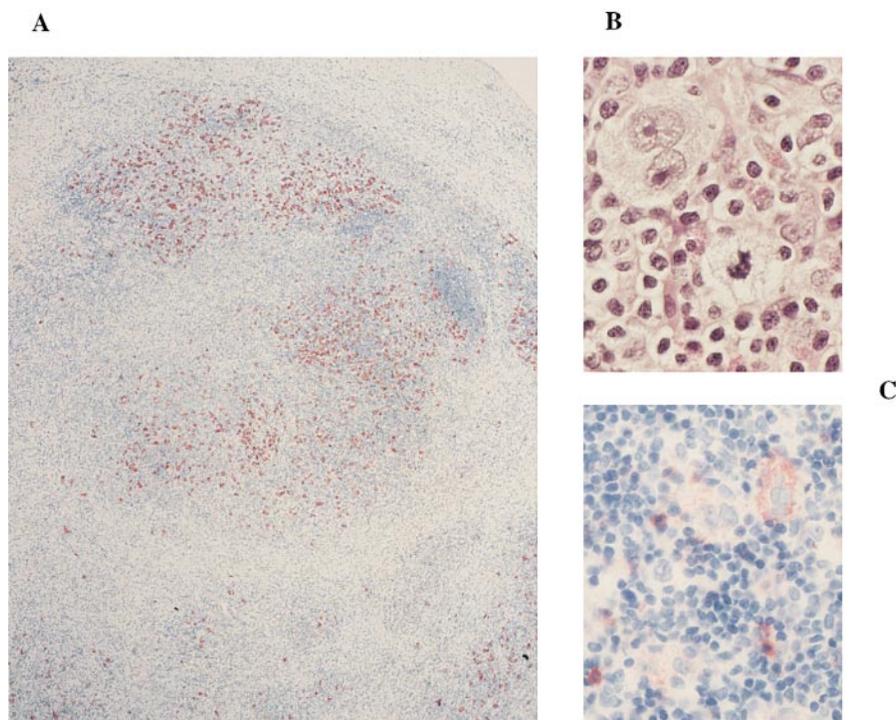
**Immunostaining and Micromanipulation.** For immunostaining, 6–7- $\mu$ m frozen tissue sections were stained using antibodies against CD30 (Fig. 1 A), CD20, LMP1, anaplastic lymphoma kinase (ALK)-1 (Dako), CD15 (Fig. 1 C; Becton Dickinson), CD3 (Ortho), TCR- $\alpha/\beta$  (T Cell Diagnostics), perforin (Neo Markers), granzyme B (Monosan), and TIA-1 (Immunotech). Stained cells were mobilized and aspirated with the help of a micropipette fixed to a hydraulic micromanipulator. Multiple cells were picked from each section. Buffer covering the sections was aspirated as negative controls for PCR analysis. For positive control of PCR, single B and T cells were either micromanipulated or sorted by flow cytometry.

**Single Cell PCR.** To analyze individual micromanipulated cells for IgH, Ig $\kappa$ , Ig $\lambda$ , as well as TCR- $\beta$  VDJ and DJ gene rearrangements or germline configuration of the IgH and TCR- $\beta$  loci, whole genome preamplification (10) was performed. Aliquots from these reactions were then subjected to two rounds of seminested PCR amplification as described previously. For analysis of the IgH and TCR- $\beta$  loci, three PCR strategies were applied (Fig. 2, A and B), one of which targets IgH (Fig. 2 A, iii) or TCR- $\beta$  (Fig. 2 B, iii) VDJ rearrangements, a second IgH (Fig. 2 A, ii) or TCR- $\beta$  (Fig. 2 B, ii) DJ rearrangements, and a third detects germline configuration of either the IgH (Fig. 2 A, i) or the TCR- $\beta$  (Fig. 2 B, i) locus. Rearranged V<sub>H</sub>, V $\kappa$ , and V $\lambda$  genes were amplified using family-specific leader or framework region V gene primers and two sets of J<sub>H</sub>, J $\kappa$ , and J $\lambda$  primers in a seminested approach (5, 11, 12). D<sub>H</sub>J<sub>H</sub> rearrangements and germline configuration within the IgH locus were detected using seven D<sub>H</sub> family-specific primers and two sets of J<sub>H</sub> gene-specific primers

in a seminested approach (5). In the case of germline configuration of the IgH locus, a 340-bp fragment was obtained with the D<sub>H</sub>7 primer, due to the close vicinity of the D<sub>H</sub>7-27 gene segment and J<sub>H</sub>1 (Fig. 2 A, i). D<sub>H</sub> family-specific primers were as follows: 5'-GTGTGCAGGCCTCRGTCTCTGTG-3' for the D<sub>H</sub>1 gene family; 5'-GCACTGGGCTCAGAGTCCTCTC-3' for the D<sub>H</sub>2 family; 5'-CCTCAGGTCAGCCCTGGACATC-3' for the D<sub>H</sub>3 family; 5'-TGAGATCCCCAGGACGCAGCAC-3' for the D<sub>H</sub>4 family; 5'-TCCCTGGGAAGCTCCTCCTGAC-3' for the D<sub>H</sub>5 family; 5'-GACACCAGACAGAGGGGCAGGC-3' for the D<sub>H</sub>6 family; and 5'-AGAGTGAAGTGGCAGGGTTG-AGG-3' for the D<sub>H</sub>7-27 gene. Amplification of TCR- $\beta$  VDJ gene rearrangements was carried out as described previously using a panel of 24 V $\beta$  family-specific primers and two sets of J $\beta$  gene-specific primers in a seminested approach (13). Germline configuration was detected separately for both C $\beta$  loci (Fig. 2 B, i) using primers binding to intronic sequences flanking the D $\beta$ 1 (5'-CCCCTTCGCCAAACAGCCTTA-3' as forward, 5'-GAG-TGAGGCAGAGGCATTCTGAAC-3' as external reverse, and 5'-GCAGAGGCATTCTGAACAAATTG-3' as internal reverse primer) or the D $\beta$ 2 gene (5'-TCAGGGTGATGCATGTTTCAAGGA-3' as forward, 5'-GGGACCCTGCAAGACCACAGCT-3' as external reverse, and 5'-ACTCTTCCCACCTGGTAGCTGCAT-3' as internal reverse primer). D $\beta$ J $\beta$  rearrangements were amplified using the primers specific for intronic sequences in the upstream regions of the D $\beta$ 1 and the D $\beta$ 2 genes, together with primers specific for the J $\beta$ 1 or J $\beta$ 2 gene clusters, respectively (Fig. 2 B, ii).

EBV infection of single H/RS cells was examined by amplification of a fragment of the EBV nuclear antigen 1 (EBNA1) gene by seminested PCR (5'-GGTCGCCGGTGTGTTTCGTATATGG-3' as forward, 5'-GCCGCAGCCCTTCCACCATAG-3' as external reverse, and 5'-AGGGAGGCAAATCTACTCCATCGTC-3' as internal reverse primer).

PCR products were gel-purified and directly sequenced.



**Figure 1.** Immunostaining of cHD, case III. Histological stainings were as follows: (A) the tissue is stained for CD30 with the use of alkaline phosphatase (4-fold magnification); (B) hemalaun-eosin staining of some multinucleated Reed-Sternberg cells at 60-fold magnification; (C) staining for CD15 at 40-fold magnification. Staining of this case for expression of perforin is shown on the cover illustration of this issue.



**Table I.** Case Description of Patients with cHD

Case	I	II	III
Age	31	26	51
Sex	Male	Male	Male
Presentation	First	First	First
LN biopsy site	Abdominal	Cervical	Inguinal
Stage	IIB	I	II
Hodgkin subtype	Mixed cellularity	Mixed cellularity	Nodular sclerosis
Phenotype of H/RS cells			
CD30	+	+	+
CD15	+	+	+
CD20	–	–	–
CD3	–	–	–
TCR- $\alpha/\beta$	–	ND	–
Granzyme B	+ (90%)	+ (60%)	+ (40%)
Perforin	–	–	+ (100%)
TIA-1	+ (60%)	+ (>90%)	+ (40%)
EBV	+	+	–
ALK	–	ND	–

Numbers in parentheses indicate percentages of positive Reed-Sternberg cells. Presence or absence of EBV was determined by amplification of a fragment of the EBNA1 gene from single H/RS cells, and the results were further confirmed in case I by LMP1 staining and in case III by EBER in situ hybridization. ALK expression is indicative for the t(2;5) translocation resulting in the expression of a fusion molecule with NPM.

From multiple H/RS cells of both subsets, a clonal Ig V<sub>H</sub>2-5-D<sub>H</sub>3-10-J<sub>H</sub>5b and a clonal D<sub>H</sub>3-9-J<sub>H</sub>6c gene rearrangement were amplified. The V<sub>H</sub>2 gene was rearranged in-frame, all sequences were identical, and the rearrangement was rendered nonfunctional by a somatic mutation generating a translation stop in codon 91 of framework region III (Table III). Thus, case I represents another example in which the H/RS cells have lost their capacity to express antigen receptor due to deleterious somatic mutations (5). No IgL, TCR- $\beta$  VDJ, or DJ gene rearrangements were obtained analyzing multiple cells (Table II). Fragments corresponding to germline configuration of the IgH locus were not obtained, but fragments specific for germline configuration of the TCR C $\beta$ 1 and C $\beta$ 2 loci were repeatedly amplified (Table II). For the TCR C $\beta$ 2 locus, germline configuration could be assigned to both alleles because of the detection of two polymorphic forms of the D $\beta$ 2 gene (G and/or A at position 13 of the D $\beta$ 2 gene; see reference 15).

Case II, which we studied previously for Ig gene rearrangements (4), was retrospectively found to express granzyme B and TIA-1 in a fraction of H/RS cells (Table I). 20 CD30<sup>+</sup> H/RS cells and 10 TIA-1<sup>+</sup> H/RS cells were micromanipulated and analyzed for IgH and Ig $\kappa$  gene rearrangements and configuration of the TCR- $\beta$  loci. Two clonal IgH and one clonal Ig $\kappa$  gene rearrangement were amplified from

H/RS cells regardless of their phenotype (Tables II and III). V<sub>H</sub> and V $\kappa$  genes were somatically mutated. The V $\kappa$  region gene and one of the V<sub>H</sub> gene rearrangements, which had both likely been originally productive, were rendered nonfunctional by somatic mutations (Table III). For unknown reasons, a second clonal V $\kappa$  gene rearrangement amplified in the first analysis was not obtained in the present study. No TCR- $\beta$  gene rearrangements, but instead germline configuration for the TCR C $\beta$ 1 and C $\beta$ 2 loci, were detected (Table II).

Taking cases I and II together, amplification of clonal V<sub>H</sub> gene rearrangements rendered non-functional by deleterious somatic mutations together with the detection of germline configuration in both TCR- $\beta$  loci identify the H/RS cells in these cases as the progeny of germinal center B cells that have lost their capacity to express antigen receptor due to “crippling” somatic mutations.

From case III, in which virtually all H/RS cell express perforin (Table I), 30 CD30<sup>+</sup> H/RS cells were analyzed for IgH, Ig $\kappa$ , and Ig $\lambda$  gene rearrangements (half of the cells were in addition analyzed with V<sub>H</sub> leader, IgH DJ, and IgH germline primer collections). Only one IgH VDJ gene amplicon was obtained, likely representing cellular or other contamination (Table II). No IgH DJ gene rearrangement was obtained. However, a fragment specific for germline configuration was repeatedly amplified from H/RS cells. Within these IgH germline fragments, two distinct sequences of the J<sub>H</sub> $\psi$ 1 pseudogene (G and/or A at position 45 of the J<sub>H</sub> $\psi$ 1 pseudogene; see reference 16) were detected, suggesting that both alleles of the IgH locus are in germline configuration. In contrast, analysis of the TCR- $\beta$  loci yielded two clonal rearrangements involving the two alleles of the TCR C $\beta$ 1 locus in about half of the H/RS cells. One allele harbors a clonal V $\beta$ 7.1-D $\beta$ 1-J $\beta$ 1.6 rearrangement (Fig. 2 C), whereas the other carries a clonal D $\beta$ 1-J $\beta$ 1.4 gene rearrangement (Fig. 2 D). The V $\beta$ 7.1 gene rearrangement is potentially functional. Unexpectedly, it exhibits intraclonal diversity (Fig. 2 C). Three different sequences were obtained (Fig. 2 C, sequences A–C). The three sequence variants were confirmed by repeated reamplification and sequencing from distinct aliquots of the whole genome amplification. Taken together, in case III the single cell PCR results identify a T cell as the progenitor of the tumor clone, thus classifying this case of cHD as a T cell lymphoma.

## Discussion

Derivation of H/RS cells from mature B cells was previously demonstrated in 18 out of 18 informative unselected cases of cHD analyzed in Cologne and Frankfurt (3–5, 17–19). In addition, recent results obtained by others indicate B cell derivation of H/RS cells in 24 out of 25 cases of cHD (20). On the other hand, in a nonselected collection of 13 cases of primary cHD, Daus and colleagues did not detect clonal TCR- $\gamma$  gene rearrangements in micromanipulated H/RS cells from any of these cases (21). Therefore, these data collectively indicate that cHD represents a homogeneous entity as a B cell lymphoma.

**Table II.** Summary of Single Cell PCR Analysis of Three Cases of cHD

Case	Gene locus*	Staining for micromanipulation	H/RS cells positive	PCR products		Rearrangements or germline configuration		Buffer controls positive
				Total	Sequenced	Repeated	Unique <sup>‡</sup>	
I	IgH	CD30	21/50	19 V <sub>H</sub> 2	17	17 V <sub>H</sub> 2		0/15
				1 V <sub>H</sub> 3	1		1 V <sub>H</sub> 3	
				1 V <sub>H</sub> 4	1		1 V <sub>H</sub> 4	
			9/14	9 D <sub>H</sub> 3-J <sub>H</sub>	9	9 D <sub>H</sub> 3-9-J <sub>H</sub> 6c		0/4
		Granzyme B	3/15	3 V <sub>H</sub> 2	3	3 V <sub>H</sub> 2		
	Igκ	CD30	0/16					0/4
	Igλ	CD30	0/8					0/4
	TCR Cβ1	CD30	9/20	9 germline	9	9 germline		0/14
		Granzyme B	3/15	3 germline	3	3 germline		
	TCR Cβ2	CD30	9/20	9 germline	9	9 germline <sup>§</sup>		0/14
Granzyme B		3/15	3 germline	2	2 germline			
EBNA1	CD30	7/10	7 EBNA1				0/4	
II	IgH	CD30	8/20	6 V <sub>H</sub> 1	6	6 V <sub>H</sub> 1		0/6
				7 V <sub>H</sub> 3	6	6 V <sub>H</sub> 3		
		TIA-1	3/10	2 V <sub>H</sub> 1	2	2 V <sub>H</sub> 1		0/6
				3 V <sub>H</sub> 3	2	2 V <sub>H</sub> 3		
	Igκ	CD30	9/20	9 V <sub>κ</sub> 1	7	7 V <sub>κ</sub> 1		0/6
		TIA-1	2/10	3 V <sub>κ</sub> 1	2	2 V <sub>κ</sub> 1		
	TCR Cβ1	CD30	4/20	4 germline				0/4
		TIA-1	6/10	6 germline				
	TCR Cβ2	CD30	9/20	9 germline				0/4
TIA-1		4/10	4 germline					
EBNA1	CD30	8/20	8 EBNA1				0/6	
	TIA-1	5/10	5 EBNA1					
III	IgH	CD30	1/30 <sup>  </sup>	1 V <sub>H</sub> 3	1		1 V <sub>H</sub> 3	0/10
			6/15	6 germline	6	6 germline <sup>§</sup>		0/4
	Igκ	CD30	0/30					0/10
			0/30					0/10
	TCR Cβ1	CD30	20/30	14 Vβ7	14	14 Vβ7		1/10 <sup>¶</sup>
				14 Dβ1-Jβ1	14	14 Dβ1-Jβ1.4		
				1 germline	1		1 germline	
TCR Cβ2	CD30	17/30	17 germline	7	7 germline		0/10	
EBNA1	CD30	0/10					0/4	
Controls:			Micromanipulated		Sorted by flow cytometry			
	Gene locus		B cells	T cells	B cells	T cells		
	IgH	VDJ	5/22	0/12	8/15			
		DJ/germline			9/10			
	Igκ		3/14		4/7			
	Igλ		3/14		3/7			
	TCR-β	VDJ		6/22		7/9		
		DJ/germline		16/22		16/18		

\*For IgH and IgL loci, only V(D)J rearrangements; for TCR-β loci, VDJ and DJ rearrangements as well as fragments specific for germline configurations were amplified. Cases I and III were also analyzed for D<sub>H</sub>J<sub>H</sub> rearrangements and germline configuration of the IgH locus.

<sup>‡</sup>Representing most likely cellular or other contamination.

<sup>§</sup>These loci were found in germline configuration on both alleles due to a germline polymorphism of the Dβ2 gene (case I) and the JHψ1 gene (case III).

<sup>||</sup>15 of the H/RS cells from case III were in addition analyzed using V<sub>H</sub> leader primers.

<sup>¶</sup>The contamination amplified from one buffer control represents a TCR Cβ1 germline PCR product.

**Table III.** Sequence Analysis of Clonal Ig and TCR- $\beta$  Gene Rearrangements

Case	Gene locus	Rearrangement*	Potentially functional	Percent mutation	Remarks
I	IgH	V <sub>H</sub> 2-5-D <sub>H</sub> 3-10-J <sub>H</sub> 5b	No <sup>‡</sup>	4.3	Stop (codon 91)
		D <sub>H</sub> 3-9-J <sub>H</sub> 6c	n.a.	0.9	TCR- $\beta$ loci in germline
II	IgH	V <sub>H</sub> 1-8-D <sub>H</sub> 3-22-J <sub>H</sub> 6b <sup>§</sup>	No <sup>  </sup>	3.5	47-bp deletion;
	IgH	V <sub>H</sub> 3-53-D <sub>H</sub> 2-2-J <sub>H</sub> 6b <sup>§</sup>	No <sup>‡</sup>	1.9	Stop in CDR3
	Ig $\kappa$ <sup>§</sup>	V $\kappa$ 1 (L12)-J $\kappa$ 1 <sup>§</sup>	No <sup>‡</sup>	1.3	Stop in CDR3 TCR- $\beta$ loci in germline
III	TCR- $\beta$	V $\beta$ 7.1-D $\beta$ 1-J $\beta$ 1.6	Yes	0.3-0.7	Sequence variations involving codons 98, 105, and 108; three variants
	TCR- $\beta$	D $\beta$ 1-J $\beta$ 1.4	n.a.	0	IgH locus in germline

n.a., not applicable.

\*Sequences of cases I and III are available from GenBank/EMBL/DDBJ under accession nos. AJ243643-AJ243648 and AJ251000-AJ251002.

<sup>‡</sup>These rearrangements were potentially functional before introduction of a stop codon.

<sup>§</sup>Sequences of this case have been published previously (reference 4).

<sup>||</sup>This rearrangement was potentially functional before the 47-bp deletion.

<sup>‡</sup>This rearrangement was likely originally nonfunctional due to a stop codon generated during VDJ recombination.

However, there are some observations raising the possibility that—in a subset of cHD—the tumor cells might stem from T lymphocytes. Some putative H/RS cell lines, for example, are derived from T cells (2). For these cell lines, proof of derivation from H/RS cells in the patients is missing. Furthermore, a TCR- $\alpha$  gene rearrangement was amplified from whole tissue DNA of lymphomatoid papulosis, cHD, and anaplastic large cell lymphoma occurring sequentially in one individual patient, suggesting a common T cell derivation of the three diseases (22). However, assignment of T cell genotype to H/RS cells of the Hodgkin's lymphoma was not conclusive: anaplastic large cell lymphoma versus classic cHD is often a difficult differential diagnosis (1, 14). In this case, a clear discrimination between the two entities was particularly complicated because H/RS cells coexpressing CD30 and CD15 were found in both lymphomas. Furthermore, the TCR- $\alpha$  gene rearrangement identified in the Hodgkin's disease biopsy with the help of clone-specific primers represented a faint band, which might have arisen from a few contaminating cells originating from either the lymphomatoid papulosis or the anaplastic large cell lymphoma instead of the cHD. Finally, H/RS cells in a minority of cHD cases express cytotoxic granular molecules, in particular granzyme B, perforin, and TIA-1, which are otherwise typically found in cytotoxic T lymphocytes (6-9). Three such cases were analyzed here.

In cases I and II, molecular analysis of Ig and TCR- $\beta$  loci revealed that the H/RS cells, despite expression of granzyme B and TIA-1 (Table I), were derived from germinal center B cells. Thus, expression of granzyme B and TIA-1 does not necessarily reflect a T cell origin of H/RS cells and shows that granzyme B and TIA-1 are aberrantly expressed by B lineage-derived H/RS cells. Similarly, expression of mole-

cules thought to be specific for cells of the dendritic/myeloid lineage (23) by H/RS cells does not apparently reflect a derivation of these cells from dendritic or myeloid cells. Thus, H/RS cells can mimic cells of various hematopoietic lineages in terms of cell surface marker expression.

In case III, the H/RS cells harbor a clonal TCR- $\beta$  VDJ and a clonal DJ gene rearrangement but no clonal Ig gene rearrangement. Furthermore, the IgH locus was found in germline configuration biallelically, directly demonstrating the absence of clonal IgH gene rearrangements. Given that the presence of a TCR- $\beta$  VDJ gene rearrangement defines a T cell, the H/RS cells in this case are derived from a T lymphocyte. The V $\beta$ 7.1 gene rearrangement amplified from the T cell tumor clone exhibits significant intraclonal diversity. This was not expected, since TCR genes in T cells are usually not subject to somatic hypermutation, although there are some reports claiming the rare occurrence of somatically mutated TCR genes (24-26). Notably, somatic mutations were not observed in the D $\beta$ 1-J $\beta$ 1.4 rearrangement or in intronic sequences flanking the germline D $\beta$ 2 gene (830-bp sequences; see Table II). The distribution of somatic mutations among the distinct gene fragments of the TCR- $\beta$  loci argues in favor of somatic hypermutation rather than some other type of somatic mutation (e.g., "genomic instability") as the cause for the mutations in the TCR- $\beta$  VDJ genes: in analogy to the Ig loci, somatic hypermutation would be expected to preferentially target VDJ joints rather than DJ rearrangements or germline genes, whereas genomic instability should not specifically target rearranged VDJ genes. Whether these mutations indeed reflect somatic hypermutation outside Ig loci, however, remains unclear.

In ~5-15% of all cases of cHD, the H/RS cells exhibit cytotoxic T cell phenotype. Studying three of these cases, we

found one to be T cell derived. Apart from this, 18 out of 18 nonselected cases of cHD in our collection are B lineage derived. On this basis, a rough estimate would be that cHD occurs as a T cell lymphoma at a low frequency (i.e., <5%).

This study establishes that rare cases of cHD derived from T cells indeed exist, indicating that cHD as defined by histopathology is not a uniform disease. It is remarkable that the transformation of both T and B cells can lead to the H/RS cell phenotype. Whether this reflects an initial transforming event inside the germinal center microenvironment in both cases and whether T and B cell derived H/RS cells can be distinguished in terms of gene expression patterns remain to be established.

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