Hodgkin and Reed-Sternberg Cells in Hodgkin's Disease Represent the Outgrowth of a Dominant Tumor Clone Derived from (Crippled) Germinal Center B Cells

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Summary

In Hodgkin's disease (HD), the Hodgkin and Reed-Sternberg (HRS) cells represent only a minute population in the diseased tissue. The investigation of lineage derivation and clonal origin of these cells has yielded conflicting results. We have analyzed HRS cells micromanipulated from infiltrated tissue sections of 10 primary HD patients for rearranged V genes, extending a previous study. Clonally related rearrangements were found in nine cases, indicating that HRS cells represent a dominant clone of B lineage–derived cells in at least a large fraction of cases of HD. Rearranged V_H genes from HRS cells carried a high load of somatic mutation, indicating that HRS cells are derived from germinal center (GC) cells or their progeny. Stop codons in some in-frame V gene rearrangements suggest that the HRS cell precursors reside inside GCs, have acquired crippling mutations that prevent antigenic selection, but escape apoptosis through some transforming event.

The Hodgkin and Reed-Sternberg (HRS)[1] cells, which are considered to represent the malignant tumor cell population in Hodgkin's disease (HD), still pose riddles as to their origin and clonality. In contrast to the abundance of tumor cells in other neoplasias, the HRS cells in HD (usually ~1% of cells) reside within a complex admixture of lymphocytes, plasma cells, histiocytes, eosinophils, and other cells. The composition of the cellular background and morphological criteria have led to the subdivision of HD into the three "classical HD" subtypes, namely, nodular sclerosing (NS), mixed cellularity (MC), and lymphocyte depleted (LD), besides the fourth subtype, lymphocyte predominant (LP) (1, 2). HRS cells of the NS, MC, and LD categories of HD express the activation markers CD30 and CD15 as detected by immunohistochemistry (3). In the LP subtype, HRS cell variants often lack these antigens but express several B cell–associated molecules, and therefore are commonly accepted to be of B lineage origin (4).

Despite considerable effort, lineage derivation and clonality of the HRS cells in classical HD are controversial (3, 5). This is mainly due to the sparseness of the HRS cells and difficulties encountered in attempts to isolate these cells (6, 7). Immunohistochemical studies yielded conflicting results, revealing B and/or T cell markers on HRS cells and occasionally also monocyte markers (3). In a recent study of HRS cells at the level of single cells, expression of genes characteristic of several hematopoietic lineages was found (8). Furthermore, Southern blot hybridization and PCR approaches for the detection of Ig or TCR gene rearrangements using whole tissue DNA from HD infiltrates (as successfully perfomed on other lymphomas) detected such rearrangements only in a small number of cases (5). These population-based approaches now are considered inappropriate because of low sensitivity and the inherent inability to specifically assign rearrangements detected to the sparse HRS cells (9). However, the demonstration of clonal numerical chromosome aberrations in HRS cells and EBV genome studies suggest a monoclonal origin of HRS cells (10–12).

Recently, we overcame the problems associated with the small number of HRS cells by isolating individual HRS cells from frozen sections by micromanipulation and analyzing them by amplification of gene rearrangements (13). Using this approach, the HRS cells in four cases of HD (2 of MC, 1 of NS and 1 of LP subtype) were found to represent a single clone and to originate from the B lineage, because Ig V gene rearrangements are restricted to B lineage cells and highly specific for a B cell clone (13, 14). Single-

[1] *Abbreviations used in this paper:* FR, framework region; GC, germinal center; HD, Hodgkin's disease; HRS, Hodgkin and Reed-Sternberg; LD, lymphocyte depleted; LMP, latent membrane protein; LP, lymphocyte predominant; MC, mixed cellularity; NS, nodular sclerosing.

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cell approaches have also been applied by other groups to the analysis of classical HD, but with differing results (15, 16; see Discussion). Roth et al. (15) isolated HRS cells from suspensions of LN from 13 HD patients (12 with classical HD) and failed to detect any V_H gene rearrangements. Hummel et al. (16) suggested that HRS cells carrying polyclonal Ig V gene rearrangements in a large fraction of cases might exist.

In our previous studies, only four HD cases (and only three of the "classical" category) were analyzed, leaving open whether clonality and B lineage derivation are common features of HD (13, 14). Therefore, we have now investigated a larger number of cases to determine whether HRS cells in general represent a clonal population and originate from B lineage cells. Clonal Ig gene rearrangements were identified in most cases, and their sequence analysis allowed us to determine the stage of maturation of the B cell from which the tumor clone arose.

Table 1. *Case Description of 10 Patients Suffering from Primary Hodgkin's Disease Whose HRS Cells Were Analyzed for Ig Gene Rearrangements*

Patient*	Age (yr)/sex	Disease subtype	Disease stage‡	HRS cells CD30§	HRS cells CD15§	HRS cells EBV‖
1	18/F	NS	II B	+	+	−
2	51/F	NS	II A	+	+	+
3	28/F	NS	III A	+	+	−
4	17/F	NS	II B	+	+	−
5	27/F	NS	II A	+	+	+
6	36/M	NS	I B	+	−	+
7	36/M	MC	II A	+	+	−
8	29/M	MC	III B	+	+	+
9	18/M	MC	II A	+	+	−
10	19/M	MC	I A	+	+	+

HRS cells in the cases analyzed were negative for CD20 and CD3 expression as determined by immunostaining.
*All biopsies were cervical LN, except for one axillary LN biopsy for patient 4.
‡According to the Ann Arbor classification system (17).
§Presence (+) or absence (−) of $CD30^+$ or $CD15^+$ cells determined by immunostaining.
‖Presence (+) or absence (−) of EBV detected by staining with an antibody against LMP 1.

Materials and Methods

Tissues and Clinical Data. Clinical features of the HD patients are summarized in Table 1. Biopsy specimens of 10 cases of classical HD were chosen from the files of the LN registery at the Pathology Department at the University of Cologne. All biopsies were LN taken out for primary diagnosis of HD. A tonsillar specimen from a child suffering form chronic tonsillites was used as control in some experiments.

Immunostaining and Micromanipulation. Immunohistochemical staining procedures using antibodies against CD30, CD20, Ki67, LMP1 (Dako, Hamburg, Germany), CD15 (Becton Dickinson & Co., Mountain View, CA), and CD3 (Ortho Diagnostic Systems, Inc., Raritan, NJ) were as described in our previous publications (18, 19). The stained sections were overlaid with Tris-buffered saline (TBS), and single cells were isolated under the microscope (Olympus, Hamburg, Germany; 600×) with the help of micropipettes fixed to hydraulic micromanipulators (see Fig. 1) (18, 19). Isolated cells were transfered into 20 μl PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) containing 1 ng/μl 5S rRNA (Boehringer Mannheim GmbH, Mannheim, Germany) and stored at −80°C. Single $CD30^+$ HRS cells and $CD3^+$ T cells were obtained from adjacent sections of the same specimen. In a few experiments (see Table 2, experiments *2a* and *8*), single mantle zone B cells were micromanipulated from a Ki67 stained tonsillar section of another individual. The isolated cells were coded and analyzed in a blinded fashion. In the repeat experiments, separate sections were analyzed following the protocol outlined above.

Sorting of IgD^+ and $Ig\lambda^+$ Peripheral Blood B Cells. PBMC were isolated by Ficoll gradient centrifugation (PAQUE R; Pharmacia/LKB, Uppsala, Sweden). B cells were enriched by magnetic cell sorting using the MACS system and anti-CD19 beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) (20). The $CD19^+$ fraction was stained by a combination of anti–CD20-PE (Becton Dickinson & Co.) and anti–IgD-FITC (Southern Biotechnology Associates, Birmingham, AL) or anti-Ig λ-PE (Becton Dickinson & Co.) and anti-Ig κ-FITC (Becton Dickinson & Co.) antibodies. After washing with PBA (PBS, 1% BSA, 0.02% sodium azide), single IgD^+CD20^+ and single $Ig\lambda^+$ cells were isolated by fluorescence-activated cell sorting using a FACS® 440 (Becton Dickinson & Co.). Dead cells were excluded from the analysis by staining with propidium iodide. Single cells were sorted into 0.5-ml microtubes containing 20 μl PCR buffer supplemented with 1 ng/μl 5S rRNA (Boehringer Mannheim GmbH) and stored at −80°C.

Single-Cell PCR. Rearranged Ig genes were amplified from single cells using the seminested PCR approach and V-gene family–specific primers together with J_H and J_κ primers as described previously (18, 19). Briefly, single cells in 20 μl PCR buffer were incubated with 0.25 mg/ml proteinase K for 1 h, subsequently inactivating the enzyme at 95°C for 10 min. In the same tube, the first amplification round was carried out with 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μM each dATP, dGTP, dCTP, and dTTP, 6.9 nM each V_H, V_κ, $3'J_H$, and $3'J_\kappa$ primer (19), and 1.25 U of Taq DNA polymerase (GICBO BRL, Gaithersburg, MD) in a total volume of 50 μl. The PCR consisted of one cycle of 95°C for 2 min, 65°C for 5 min, and 72°C for 1 min, followed by 34 cycles of 95°C for 1 min, 59°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. Enzyme was added during the 5-min incubation at 65°C. A 1-μl aliqout of the first round was reamplified in a second round in separate reactions for each of the six V_H and six V_κ family–specific primers. The 50-μl reaction mix of the second PCR contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$ (1.5 mM for V_H primers), 100 μM of each dATP, dGTP, dCTP, and dTTP, 0.125 μM of one of the V_H or V_κ primers together with 0.125 μM of the respective $5'J_H$ or $5'J_\kappa$ primer mixes, and 1.25 U Taq polymerase (GIBCO BRL). The cycle progam was the same as for the first round except that the annealing temperature in the first cycle was 68°C and the annealing temperature in the following 44 cycles was 61°C (65°C for V_H3 and V_H4). Amplification

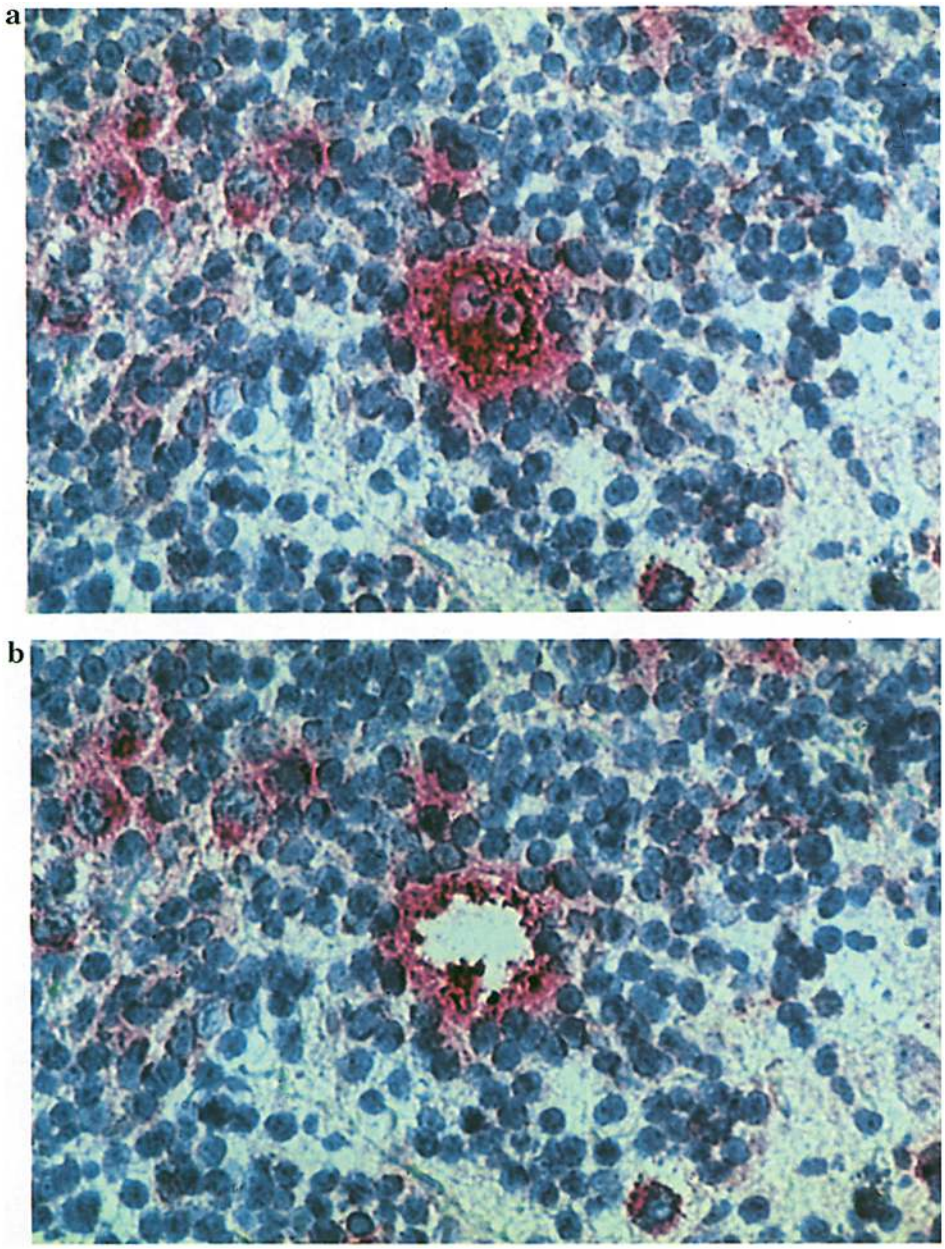

Figure 1. Micromanipulation of a single HRS cell. HD-infiltrated LN biopsy with a $CD30^+$ HRS in the middle of the picture (*a*) before and (*b*) after isolation. Note that the surrounding cells were not disturbed by the micromanipulation. Frozen section, anti-CD30 antibody staining, hematoxylin counterstain, ×600.

was performed in a thermal cycler (model 480 Perkin Elmer, Foster City, CA, or Trio-Thermoblock; Biometra, Göttingen, Germany). An 8-μl aliquot of the reaction mix was analyzed on a 2% agarose gel. The primers used have been published elsewhere (19). The following modifications were introduced: a new J_H3-specific primer (5′-GAC GGT GAC CAT TGT CCC TTG GCC-3′) was used in the second round of amplification and primer $5'J_H$ 1,3,4,5 was modified to 5′-GAC GGT GAC CAG GGT KCC CTG GCC-3′. In some experiments, V_H gene family–specific framework region (FR) I primers (19) in the first round of PCR were exchanged for a set of V_H FRII primers (Kanzler, H., R. Kippers, M.-L. Hansmann, and K. Rajewsky, unpublished results). The $V_\lambda1$ rearrangement in patient 2 was amplified with a $V_\lambda1$ family–specific primer and a set of nested J_λ primers (Kippers, R., M.-L. Hansmann, and K. Rajewsky, unpublished observation).

Sequence Analysis. PCR products were gel purified from 2.5% agarose gels using a gel extraction kit (Qiaex II; Qiagen, Hilden, Germany) and directly sequenced. Cycle sequencing was performed using the Ready Reaction DyeDeoxyTerminator cycle sequencing kit (Perkin Elmer, Foster City, CA) and automatic sequencing (Applied Biosystems/Perkin Elmer) or the ds cycle sequencing system (GIBCO BRL) following the instructions of the manufacturer. Sequencing primers were the same as in the second round of amplification. DNASIS software (Pharmacia/LKB) and the GenBank data library (release 92) were used to analyze the V gene sequences.

Results

PCR Analysis of Isolated HRS Cells. Single $CD30^+$ HRS cells were micromanipulated from HD-infiltrated frozen tis-

Table 2. *Summary of the Single-Cell Analysis of Micromanipulated HRS Cells for Ig Gene Rearrangements*

Patient	Experiment	Cells positive	PCR products		Rearrangements	
			Total	Sequenced*	Repeated	Unique
1	1a	3/9	4	3	2 V_H4	
					1 $V_\kappa3$	
	1b	10/20	14	14	10 V_H4	1 V_H3
					1 $V_\kappa3$	1 $V_\kappa1$
						1 $V_\kappa2$
2	2a	9/20	12	12	3 V_H1	
					9 $V_\kappa3$	
	2a‡	2/9	2	2	2 $V_\lambda1$	
	2b‡	4/18	4	4	4 $V_\lambda1$	
3‡	3a	8/20	8	7	7 $V_\kappa2$	
	3b§	2/9	2	2	2 $V_\kappa2$	
4	4	15/21	17	16	14 V_H3	1 V_H3
						1 $V_\kappa2$
5‡	5a	4/20	8‖	6	2 V_H3	1 V_H4
						1 V_H6‖
						1 $V_\kappa1$
						1 $V_\kappa4$‖
	5b	3/13	3	3	3 V_H3	
	5c	2/10	2	2	2 V_H3	
6‡	6a	8/20	10	10	7 $V_\kappa3$	2 V_H3
						1 $V_\kappa1$
	6b§	2/9	2	2	2 $V_\kappa3$	
7‡	7a	8/9	14	14	6 V_H1	
					8 $V_\kappa3$	
	7b	5/10	7	6	3 V_H1	
					3 $V_\kappa3$	
8	8	13/18	23	23	12 V_H3	
					5 V_H3	
					3 $V_\kappa1$	
					3 $V_\kappa3$	
9	9§	0/17				
10	10a	9/20	13	11	2 V_H3	1 V_H1
						3 V_H3
						2 V_H4
						2 $V_\kappa2$
						1 $V_\kappa3$
	10b	9/23	9	9	8 V_H3	1 $V_\kappa1$
	10c	2/9	2	2	2 V_H3	
Control¶	B cells	72/89	158	83		Multiple**
	T cells	6/132	7	7		Multiple**

HRS cells were analyzed together with B cells and T cells as controls. The number of cells analyzed and cells yielding at least one PCR product are indicated. *Repeated rearrangements* indicates clonally related rearrangements; *unique rearrangements* are rearrangements amplified only once and not related to any other sequence. The same repeated rearrangements were detected in the HRS cells in the repeat experiments for patients 1–3 and 5–7. For patient 10, the V_H3 gene rearrangement amplified twice in experiment 10a was different than the V_H3 rearrangement in experiments 10b and 10c, whereas identical V_H3 gene rearrangements were obtained in experiments 10b and 10c.

*Among 8 PCR products from HRS cells not sequenced were one $V_\kappa3$ gene rearrangement (experiment 1a), one $V_\kappa2$ gene rearrangement (experi-

sue sections of LN biopsies and analyzed by single cell PCR (Fig. 1). The panel of cases analyzed consisted of 10 primary HD patients, 6 of the NS and 4 of the MC subtype (Table 1). HRS cells did not detectably express the CD20 and CD3 markers. In 50% of the patients, the presence of EBV could be detected by latent membrane protein (LMP) 1 immunostaining (Table 1). In 5 of the 10 cases, a repeat experiment was carried out. Cases 5 and 10 were examined three times (Table 2). As negative control, single T cells micromanipulated on the same day as the HRS cells from a CD3-stained adjacent section of the same LN were analyzed together with the HRS cells in a blinded fashion. As positive control, single IgD^+ peripheral blood B cells were sorted by flow cytometry and analyzed in parallel with the HRS and T cells (Table 2, in experiments 2a and 8, B cells micromanipulated from a tonsillar section were used). From 132 T cells analyzed, 7 Ig gene rearrangements were obtained. These originated most likely from cellular or other contamination during the micromanipulation procedure. V_H and/or V_κ PCR products were obtained from 72 of 89 control B cells (including 74 FACS®-sorted B cells, of which 66 (89%) yielded at least one product), demonstrating the high efficiency of the PCR strategy. The Ig V gene sequences from control B cells and T cells were unique and clonally unrelated to V gene sequences from HRS cells. The finding of identical Ig gene rearrangements in HRS cells in repeated experiments (see below) further demonstrated the validity of the single-cell approach chosen.

HRS Cells Harbor Clonal Ig Gene Rearrangments. From each HD patient, between 17 and 52 individual HRS cells were analyzed for Ig gene rearrangements (Table 2). From a total of 304 HRS cells, 118 yielded amplification products. In the nine cases in which rearrangements were identified, the efficiency of amplification from the HRS cells varied from 20 to 89% positive cells in different experiments. Direct sequencing of the PCR products confirmed that the HRS cells indeed carried rearranged V_H and/or V_L gene rearrangements (Table 2). Furthermore, the vast majority of Ig V gene rearrangements gained from HRS cells of the same case were clonally related, with one possible exception (see below).

No Ig gene rearrangements were amplified from the HRS cells from patient 9, even when we exchanged the V_H gene family–specific FRI primers in the first round of PCR by a set of FRII primers (Table 2). The FRII primer set was used to detect potential V_H gene rearrangements that could not be amplified with V_H FRI primers (e.g., because of somatic mutations; see Discussion). The V_H FRII primer set or a group of V_λ gene–specific primers were also used to investigate HRS cells from other patients. Except for the amplification of a clonal $V_\lambda 1$ gene rearrangement in HRS cells from patient 2, this did not lead to the detection of Ig gene rearrangements beyond those already identified (Table 2 and data not shown).

In the case of patients 1–3, 5–7, and 10, repeat experiments were performed. Identical Ig V gene rearrangements were detected, with one exception (patient 10, see below). For patient 5 (the case with only 20% amplification efficiency), a third experiment was carried out yielding the same results as in experiments 5a and 5b. The results in repeat experiments 10b and 10c differed from those in experiment 10a. Whereas in experiment 10a a number of clonally unrelated Ig V gene rearrangements were obtained besides two HRS cells harboring a related $V_H 3$ gene rearrangement, HRS cells in experiments 10b and 10c showed a $V_H 3$ gene rearrangement differing from the one in experiment 10a but identical between the two repeat experiments.

Sporadically, HRS cells also gave rise to Ig V gene rearrangments that were not related to the clonal rearrangement(s) carried by the majority of HRS cells from the respective case (Table 2). These "unique" rearrangements were detected in 11 out of 304 (3.6%) of HRS cells analyzed (the unique Ig gene rearrangements obtained in experiment 10a are discussed below). T cells included as controls for cellular or other contamination gave rise to Ig gene rearrangements with about the same frequency (6 out of 132 [4.5%] of T cells analyzed; Table 2), suggesting that the unique Ig gene rearrangements amplified from HRS cells were also due to cellular or other contamination. Indeed, the unique Ig gene rearrangements from HRS and T cells were either unmutated or harbored between 0.5 and 6.3% mutation (on the average 2.5% for mutated rearranged V genes). Because naive B cells with unmutated V genes as well as memory B cells (harboring on the average 4% mutation) are present in human LNs (18, 21, 22), the mutation

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ment 3a), one $V_H 2$ gene rearrangement (experiment 4), one $V_H 4$ and one $V_\kappa 3$ rearrangement (experiment 5a), one $V_\kappa 3$ rearrangement (experiment 7b), and two $V_H 4$ gene rearrangements (experiment 10a).

‡HRS cells from patient 2 (27 HRS cells), patient 3 (11 HRS cells), patient 5 (7 HRS cells), patient 6 (10 HRS cells), and patient 7 (11 HRS cells) were analyzed for Ig V_λ gene rearrangements. Because rearranged V_λ genes from the HRS cells of patient 2 only were amplified, data from this case only are included.

§V_H gene primers annealing to the FRII were used instead of the V_H FRI primer set (in experiment 9, 8 HRS cells were analyzed using the V_H FRI primer set and 9 cells using the V_H FRII primer set).

‖Unique rearrangements (two sequenced, two not sequenced) originate from one cell.

¶Between 2 and 9 B cells and 3 and 10 T cells were analyzed in each experiment. The number of cells indicated represents a summary of all control experiments.

**Clonally unrelated V_H and V_κ gene rearrangements of various V gene families were detected. In B cells, members of all V_H and V_κ gene families were amplified except for $V_\kappa 6$. None of 90 sequenced Ig gene rearrangements obtained from B cells or T cells was clonally related to a repeated sequence from an HRS cell. For 4 PCR products, sequences could not be determined because we obtained double sequences, or PCR products were lost upon purification. 71 PCR products from B cells were not sequenced because the V genes belonged to other V gene families than those of the HRS cells of the respective patient.

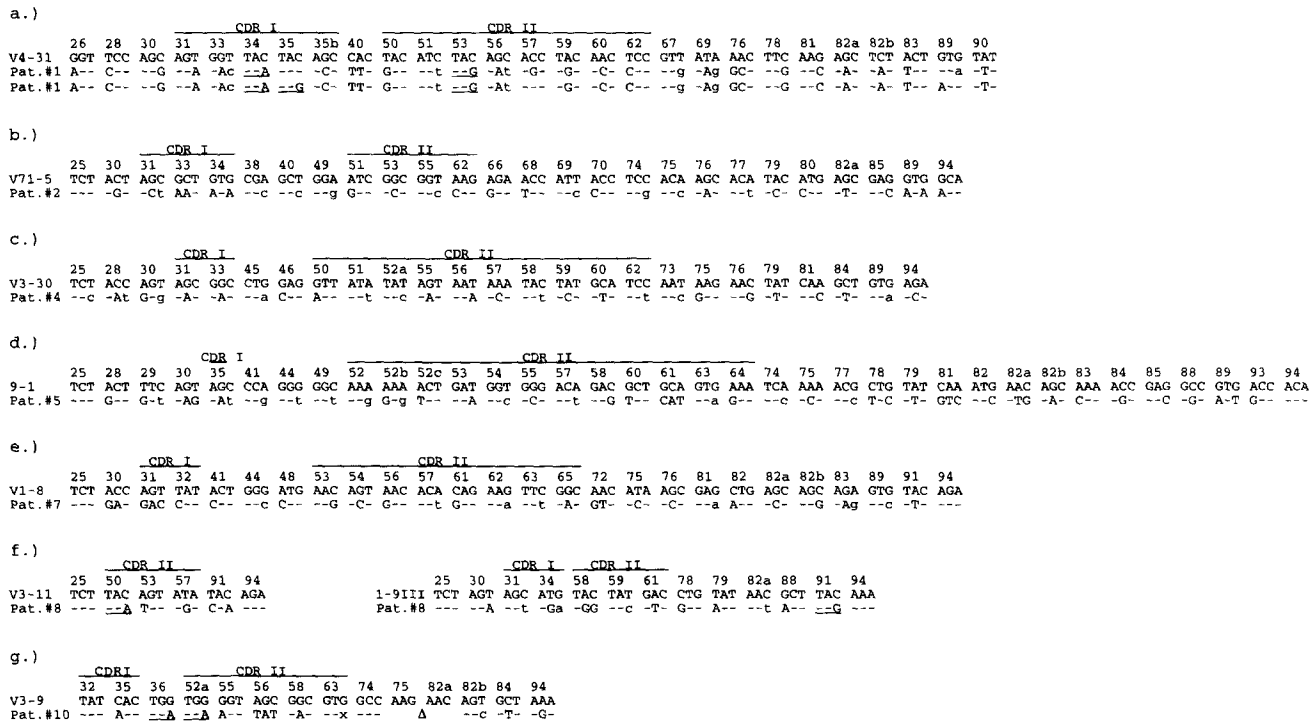

Figure 2. Comparison of V_H region gene sequences from HRS cells to the most homologous germline genes. Nucleotide sequences of V_H region genes amplified from individual HRS cells of (*a*) patient 1, (*b*) patient 2, (*c*) patient 4, (*d*) patient 5, (*e*) patient 7, (*f*) patient 8, and (*g*) patient 10 are compared with the most homologous germline genes. Only the first and the last codon and those codons of the V_H genes are shown that differ from the respective germline genes. Dashes indicate sequence identity. Replacement mutations are indicated by uppercase letters, silent mutations by lowercase letters. In cases in which two point mutations were present in a codon, it is assumed that the mutation that lead to a replacement (independent whether it occured first or second to the other mutation) occured first. Three mutations in a codon are not classified as replacement or silent mutation separately. Underlined codons mark stop codons originating from mutation. The upper sequence from patient 1 (*a*) was amplified from seven HRS cells, the lower sequence from five HRS cells, indicating intraclonal diversity in this patient. × marks a nucleotide not clearly readable on the sequence gels. A deletion of codons 75–82a in the V3-9 gene rearrangement in HRS cells from patient 10 (*g*) is indicated by Δ. Codons are numbererd according to Kabat et al. (23). For reference of V_H germline genes see Matsuda et al. (24) and Berman et al. (25). Sequence data of the clonally related Ig V gene rearrangements from HRS cells have been deposited in the GenBank data base under accession number Z77316-Z77331. All other sequence data are available from the authors upon request.

pattern found for the unique Ig gene rearrangements is compatible with a derivation of those products from polyclonal, surrounding B cells. They thus most likely represent cellular contamination during the micromanipulation procedure. This is strongly supported by the fact that the sequence characteristics of the clonal gene rearrangements carried by the HRS cells distinguish them from those of normal B cells (see Discussion).

Rearranged V Genes in HRS Cells Have Been Affected and Sometimes Silenced by Somatic Mutation. The sequences of the clonal V_H gene rearrangements amplified from HRS cells are shown in Fig. 2 and the results summarized in Table 3. The V_H gene sequences carried between 5 and 45 nucleotide differences (2.2–19.6%) compared with the most homologous germline genes. The vast majority of these nucleotide substitutions can be reliably assigned to somatic mutations because most human V_H genes are now known (24, 28). In this study, this is evidenced by the sequence analysis of V_H genes amplified from naive IgD^+ B cells (which are known to carry unmutated V genes) (21, 22, 29) for control. Among 55 V_H genes amplified from B cells from two donors, 48 were 100% homologous to published germline genes. Seven sequences harbored between one and eight nucleotide differences (on the average three to four differences) to the most homologous germline genes (R. Küppers, H. Kanzler, M.-L. Hansmann, and K. Rajewsky, unpublished observation). That the HRS cell–derived V gene rearrangements have indeed been subject to somatic hypermutation is further confirmed by the pattern of nucleotide substitutions. A striking similarity to nucleotide substitution preferences found for somatically mutated mouse Ig passenger transgenes was observed (Table 4) (for review see reference 30).

Some of the clonal $V_\kappa J_\kappa$ sequences from HRS cells also carried mutations (unambigious in these cases because all human V_κ genes have been sequenced) (26) while others did not. We tentatively attribute the absence of somatic mutation in the latter to the particular genomic structure of the human V_κ gene locus that allows the presence of unmutated rearranged V_κ genes in a B cell with a somatically mutated V_H gene rearrangement (see Discussion) (31).

In the HRS cells from patient 1, we found evidence for

Table 3. *Sequence analysis of Clonal Ig Gene Rearrangements Obtained from 10 Cases of Hodgkin's Disease*

	V_H				V_L			
Patient	V_H family	V_H gene	Percentage of mutation	In-frame*	V_L family‡	V_L gene	Percentage of mutation	In-frame*
1	4	V4-31	14.4	−	3	L6	7.1	+
2	1	V71-5	13.0	+§	3	A27	0	−
					1	LV122	9.3	+‖
3					2	A17	0	−
4	3	V3-30	11.8	+				
5	3	9-1	19.6	+				
6					3	L6	0	−
7	1	V1-8	13.0	+	3	A27	0	−
8	3	V3-11	2.2	−	1	L8	3.1	+
	3	1-9III	5.8	+‖	3	L2	1.6	−
10	3	V3-9	5.8	+‖¶				

Clonal sequences from HRS cells are compared with the most homologous germline genes (24–27).
In patient 1, evidence for intraclonal diversity of the V_H4 gene rearrangement was found.
*Out-of-frame rearrangement (−); in-frame rearrangement (+).
‡All V light chain gene rearrangements are V_κ gene rearrangements except for a $V_\lambda1$ gene rearrangement in patient 2 (LV122) (27).
§V71-5 is a pseudogene with a defective splice site.
‖In-frame rearrangements that are nonfunctional because a stop codon was introduced by somatic mutation.
¶V_H gene rearrangement with a 27-bp deletion in FRIII and stop codons in FRII and CDRII.

intraclonal diversification of the V_H gene rearrangement with identical substitutions at four positions of the V gene in 5 of the 12 HRS cells (Fig. 2). In all other patients the clonally related V gene rearrangements did not show sequence variation. In patient 4, 1 of 14 otherwise identical HRS cells carried a single base substitution. The absence of deletions or insertions in the clonal sequences except for a 27-bp deletion of codons 75–82a of the V_H gene rearrangement in HRS cells from patient 10 contrasts previous studies in which we detected aberrant V gene rearrangements in four of seven V_H region genes (13, 14). Three of the four V_H gene rearrangements with deletions and insertions were derived from two relapse patients who had undergone therapy before. This may indicate that deletions and insertions in rearranged V genes occur in the course of therapy (32, 33).

Among the eight clonally related V_H gene rearrangements in the collection, the rearrangements from patients 4, 5, and 7 could potentially be translated into protein (Table 3). The V_H1 gene in patient 2 was also rearranged in-frame, but because the most homologous V71-5 germline gene is a pseudogene (because of a mutated splice site outside the region amplified in our study), functionality of that rearrangement is unclear. Overall, the frequency of potentially functional Ig gene rearrangements was low in HRS cells. In patient 1, only an out-of-frame V_H gene rearrangement was detected. The 1-9III V_H3 gene rearrangement in patient 8 and the V3-9 V_H3 gene rearrangement in patient 10 were rearranged in-frame but rendered nonfunctional because of stop codons introduced by somatic mutation (Fig. 2 and Table 3). A stop codon most likely derived from somatic mutation was also found in the in-frame LV122 V_λ gene rearrangement in patient 2.

Table 4. *Nucleotide Substitution Preferences of Somatic Mutations in HRS Cells*

To	Frequency of nucleotide substitution			
From	T	C	A	G
T	−	0.074	0.033	0.041
		(0.068)	(0.040)	(0.022)
C	0.123	−	0.057	0.090
	(0.155)		(0.019)	(0.036)
A	0.086	0.086	−	0.115
	(0.084)	(0.055)		(0.151)
G	0.016	0.079	0.201	−
	(0.033)	(0.118)	(0.219)	

Nucleotide substitutions were calculated from 244 point mutations carried by the clonal Ig gene rearrangements amplified from HRS cells. Numbers in brackets represent the frequencies of nucleotide substitutions characteristic for somatic hypermutation reported by Neuberger and Milstein (30).

Discussion

HRS Cells in Classical HD Are B Lineage Derived and As a Rule Represent a Dominant Tumor Cell Clone. In 9 out of the 10 cases analyzed in this study, HRS cells carried rearranged V_H and/or V_L genes, indicating their B lineage origin. Furthermore, the V gene rearrangements of individual HRS cells from a given patient were clonally related. These findings are in agreement with our previous observation of an expanded HRS cell population with clonally related V gene rearrangements in two cases of MC HD and one of NS HD (13, 14). We conclude that classical HD in most if not all cases is characterized by the outgrowth of a dominant clone of HRS cells. Recent combined fluorescence in situ hybridization and immunophenotyping studies by Weber-Matthiesen et al. (10) and DNA content and interphase cytogenetics investigations by Inghirami et al. (34) also support the clonal nature of the HRS cells.

However, the finding of clonal, B lineage–derived HRS cell populations is in conflict with the work of other groups who also investigated HRS cells in classical HD on the single-cell level. The failure of Roth et al. (15) to detect V_H gene rerarrangements in HRS cells from any of 13 HD cases (including 12 classical HD cases) could result from a technical difference between their work and ours, namely, the more stringent hybridization conditions that were used in their PCR analysis. As most V_H gene rearrangements in HRS cells carry a high load of somatic mutations (Table 3), mutations at the sites of primer binding might have prevented successful amplification of such rearrangements in the work of Roth et al. (15). In another recent article, Hummel et al. (16) reported on HRS cells harboring polyclonal V_H gene rearrangements in 6 of 12 cases of HD. Both polyclonal and clonal populations of HRS cells were present in three further cases, and in only three cases, HRS cells represented a single tumor clone. However, these initial findings were later partly corrected (35). In a reanalysis of the cases previously classified as polyclonal, HRS cells in 2 cases now turned out to carry clonally related V gene rearrangements (we analyzed one of these cases in parallel with an identical result) (36). In two cases, polyclonality of HRS cells was again found; lack of material prevented reanalysis of the two remaining cases (35). Given this confused situation, the work of Hummel et al. (16, 35) neither supports nor argues against the present data. Furthermore, the relevance of the study by Hummel et al. for a characterization of clonality and lineage derivation of HRS cells in typical cases of classical HD is unclear, because it is restricted to a small subset of cases of classical HD (~10–15%) (3), namely, those in which the HRS cells express the B cell antigen CD20 (37–39). In our analysis, cases of classical HD lacking expression of CD20 were analyzed (Table 1).

The HRS cells from one patient in our collection (patient 10) carried a number of clonally unrelated V gene rearrangements in one of three experiments (Table 2, experiment *10a*). Reevaluating the histological section used in experiment 10a, we recognized that the $CD30^+$ cells were smaller than typical HRS cells. It is known that EBV-infected B cells in infectious mononucleosis can express CD30 and have an HRS cell-like morphology (40). Thus, a possible explanation for the clonally unrelated V gene rearrangements amplified from $CD30^+$ cells in experiment 10a is that we mistakenly isolated $CD30^+$ EBV-infected bystander B cells (two of which happened to be clonally related). Unfortunately, presence of EBV could not be determined in the region of the LN from which cells micromanipulated in experiment 10a were derived. However, as revealed by the repeat experiments 10b and 10c, this patient also harbored a dominant clone of HRS cells (Table 2).

In 1 of the 10 patients (patient 9), we could not detect any V_H or V_κ gene rearrangement in HRS cells using either the standard FRI V_H and V_κ primer sets or a set of FRII V_H and V_κ primers (Table 2, experiment *9*). This failure could be due to somatic mutations at the primer binding sites. Alternatively, the HRS cells of this patient were not derived from B lineage cells. The occasional expression of T cell antigens on HRS cells is compatible with a T cell origin of HRS cells in a minor fraction of HD (3).

HRS Cells Seem to Originate from Germinal Center (GC) B Cells That at Least in Part Acquired Crippling Somatic Mutations Preventing Antigenic Selection. All clonally related V_H genes amplified from HRS cells in this study showed multiple nucleotide substitutions when compared with the most homologous germline genes (Fig. 2 and Table 3) and, as discussed in the Results section, most of these must have been introduced by the process of somatic hypermutation. The finding of unmutated, out-of-frame V_κ gene rearrangements in patients 3 and 6 does not exclude that these cases also harbor somatically mutated rearranged V genes that we failed to amplify. Deletion of the κ enhancers in λ-expressing B cells likely prevents somatic hypermutation of retained $V_\kappa J_\kappa$ joints, so that unmutated V_κ gene rearrangements can be amplified from B cells carrying mutated V_H and V_λ genes (31). Indeed, mutated, rearranged V_H genes were identified in HRS cells from patients 2 and 7 that also harbored unmutated, out-of-frame V_κ gene rearrangements (Table 3).

The frequency of somatic mutations in V_H genes amplified from HRS cells varied from 2.2 to 19.6% (Table 3). On average, HRS cells showed 10.7% somatic mutation in rearranged V_H genes. This contrasts to an average of 4 and 2% in human class-switched IgG^+ and IgM^+ memory B cells (21, 22, 29). The significantly higher average mutation frequency of the clonal Ig gene rearrangements amplified from HRS cells as compared to the mutation frequency of normal human B cells is a further strong argument against a potential contamination of the isolated HRS cells by surrounding B cells.

The finding of V gene rearrangements and somatic mutations in HRS cells allows one not only to assign these cells to the B lineage but also to determine the developmental stage of the B cell that was the precursor of the tumor clone. Because the process of somatic hypermutation appears to be restricted to B cells undergoing proliferation

and antigen selection in the microenvironment of GCs (18), the finding of somatic mutations in the V_H genes amplified from HRS cells suggests that HRS cells originate from GC B cells or their progeny. In retrospect, this also holds true for two previously published cases of HD (NS and MC) (13). At that time, the limited knowledge of the human V_H gene locus did not allow us to identify with confidence somatic mutations in the V_H gene rearrangements carried by the corresponding HRS cells. Furthermore, the presence of two nonfunctional V_H gene rearrangements in the case of MC HD led us suspect that in this instance the HRS cells originated from an early B cell progenitor that had failed to carry out a functional V_H gene rearrangement on either of its two IgH alleles (13). However, when we reanalyzed those sequences with the present knowledge of the human IgH locus, it became clear that the rearranged V_H genes of both cases harbor somatic mutations (with a frequency of 2 and 3% in the two V_H genes from the case of MC HD and 16% in the case of NS HD). Thus, also in these cases the HRS cells appear to be derived from GC B cells or their progeny.

Strikingly, we observe in several instances in-frame V gene rearrangements that were apparently rendered nonfunctional by somatic mutation. In this study, stop codons generated by somatic mutation were seen in three of nine cases from which Ig gene rearrangements could be amplified (V_H genes in patients 8 and 10 and the V_λ gene from patient 2). The situation in patient 8 is particularly informative, because two V_H gene rearrangements were amplified from the HRS cells, excluding the presence of a potentially functional V_H gene on the second allele. Similary, the HRS cells from the MC HD patient in our previous study (13) carried a nonproductively rearranged $V_HD_HJ_H$ rearrangement in addition to an in-frame one, the latter rendered nonfunctional by a stop codon in CDRIII. In light of the other cases, it seems straightforward to assign the introduction of this stop codon to somatic hypermutation as well.

Under physiological conditions, GC B cells are strongly selected for the expression of high-affinity antibodies. Only cells fulfilling this criterion are allowed to persist in the GC microenvironment and to finally leave it in order to join the pool of memory B cells (41). Thus, B cells bearing V gene rearrangements rendered nonfunctional by somatic hypermutation can only occur *within* GC and cannot represent postgerminal center cells. This suggests that at least those HRS cells that carry such a rearrangement originate from precursor cells that underwent a transforming event either before their entry into or within a GC, such that they escaped apoptotic death in the absence of antigenic selection. This may also hold true for those cases of HD in which we failed to detect mutations in V gene rearrangements that would preclude Ig expression. With one exception (14), we did not detect potentially functional, in-frame rearrangements for both H and L chain V regions in any HRS cell. In addition, in cases of potentially functional rearrangements, we might have missed deleterious mutations outside the region that was amplified. And perhaps most importantly, even if still able to express Ig, a precursor cell might lose its ability to bind antigen through somatic mutation of its antibody-binding site. The high load of somatic mutations in a large fraction of HRS cells goes well along with these possibilities. Independence of selection for expression of functional surface Ig might also result in downregulation of Ig expression in cases in which V gene rearrangements were not rendered nonfunctional because of crippling mutations as well.

HRS Cell Progenitor Generation: A Scenario. In light of the molecular data presented above, a hypothetical unifying scenario of the pathogenesis of HD can be envisioned. In this scenario, the key feature of the progenitor cell from which the HRS cells derive is that it survives in the GC, although, because of somatic mutation, it has lost its ability to be selected by an appropriate antigen—a prerequisite for normal B cells to survive in the GC microenvironment. Thus, in contrast to most other B cell lymphomas (2), the HRS cells in HD are independent of surface Ig expression and indeed have usually been found not to express antibody (3, 5). In this respect, classical HD is the true counterpart of follicular lymphoma, which also originates from a GC cell, but in this case a cell that still depends on signals through its antigen receptor (42). Consistent with its GC origin, follicular lymphoma is characterized by ongoing somatic hypermutation (42), whereas the process of hypermutation is largely abrogated in HRS cells (Fig. 2 and Table 3). This latter finding may indicate that somatic hypermutation requires triggering of the cell through its Ig receptor. Alternatively, abrogation of somatic hypermutation in HRS cells might be due to the disruption of the GC microenvironment (considered to be necessary for the process of somatic hypermutation) during tumor expansion.

Which transforming event has hit the HRS cell progenitor such that it survives in the absence of antigenic selection? It seems reasonable to think that this event should result in the premature upregulation of the gene that is known to protect GC cells from apoptosis, namely, bcl-2 (43); indeed, in a large fraction of HD, HRS cells express bcl-2 protein (44, 45). It is an intriguing possibility that protection of the HRS cell progenitor from apoptosis through bcl-2 upregulation might be accomplished by EBV infection: EBV has been detected in more than half of the cases of classical HD, where it is found in virtually all HRS cells (3, 46). Significantly, EBV-harboring HRS cells commonly express the LMP-1 gene (46) (all five EBV-positive cases in Table 1), which is known to upregulate expression of bcl-2 (47).

In this picture of the early events in the pathogenesis of HD, many questions remain unanswered. Thus, it is not clear whether HRS cells develop from a progenitor that had been infected by EBV before entry into the GC (in EBV-positive individuals the frequency of infected B cells is in the order of 10^{-5}) (48) or EBV infection takes place in the GC itself. It also remains to be resolved whether in cases of EBV-negative HRS cells other mechanisms of transformation are involved or EBV has still played a role,

through a mechanism of "hit-and-run" (49). Furthermore, rescue of "failed" GC cells from apoptosis can only be a first step in the series of events that finally leads to the development of HD. GC cells rescued from apoptosis might continue to proliferate in the GC microenvironment and further accumulate somatic mutations, thus being at an increased risk to acquire further genetic changes leading to malignancy. Such events may be promoted in GC cells by the occasional failure to properly target somatic hypermutation to rearranged antibody V region genes.

Taken together, this analysis demonstrates that in at least the major fraction of cases of classical HD, HRS cells represent a clonal population of B lineage cells. The presence of a high load of somatic mutations, together with the detection of mutations rendering Ig genes nonfunctional, suggests that these cells originate from a precursor located within the GC, which because of crippling somatic mutations had lost its ability to be selected by antigen and was prevented from apoptosis by a transforming event.

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