

HODGKIN'S DISEASE WITH MONOCLONAL AND POLYCLONAL POPULATIONS OF REED-STERNBERG CELLS

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Abstract Background. There is strong evidence that Reed-Sternberg cells have a lymphoid phenotype, but clonally rearranged genes for B-cell and T-cell antigen receptors have not been demonstrable in tumor tissue from most patients with Hodgkin's disease. To elucidate this issue, we assayed single Reed-Sternberg cells from 12 patients with classic Hodgkin's disease of a B-cell immunophenotype to detect rearranged immunoglobulin variable-region heavy-chain (V_H) genes.

Methods. We isolated single Reed-Sternberg cells from frozen sections that had been immunostained for CD30. The rearranged V_H genes of these cells were amplified by the polymerase chain reaction and analyzed by gel electrophoresis and nucleotide sequencing.

Results. In all 12 patients, the Reed-Sternberg cells studied contained rearranged V_H genes. Three patterns were observed: in three patients the rearrangements in

each patient were identical, in six patients all the rearrangements were unrelated and unique, and in three patients both identical and unrelated rearrangements were detected. Apparently somatic mutations of V_H genes were present in some Reed-Sternberg cells but absent in others.

Conclusions. Reed-Sternberg cells with B-cell phenotypes have rearranged V_H genes; therefore, these cells arise from B cells. The pattern of V_H gene mutations suggests that Reed-Sternberg cells can correspond to either immunologically naive or memory B cells. In half our patients the population of Reed-Sternberg cells was polyclonal; in the other half, monoclonal or mixed cell populations were found. Correlation with the clinical stage suggests that polyclonal Hodgkin's disease can present as a widespread lymphoma. (N Engl J Med 1995; 333:901-6.)

HODGKIN'S disease is generally regarded as a distinct type of malignant lymphoma, because Reed-Sternberg cells are present in an admixture of various nonmalignant cells. Immunologic studies of Reed-Sternberg cells from the nodular-sclerosing, mixed-cellularity, and lymphocyte-depleted types of Hodgkin's disease have revealed the presence of the lymphoid-activation markers CD30 and CD70 in nearly every case,^{1,2} and that of B-cell or T-cell markers in a substantial proportion of cases.³⁻⁷ These findings suggest that Reed-Sternberg cells originate in activated lymphocytes of either the B-cell or T-cell type. Studies of cell lines derived from tissue affected by Hodgkin's disease give further evidence of the lymphoid nature of Reed-Sternberg cells and the existence of B-cell and T-cell types.⁸⁻¹⁰ However, studies of rearrangements of antigen-receptor genes carried out on whole-tissue DNA from biopsies of patients with Hodgkin's disease were inconclusive, because in most instances the clonal rearrangement expected in a typical lymphoma was not found.¹¹⁻¹³ This result may have been due to the scarcity of clonally rearranged Reed-Sternberg cells or to the actual absence of a clonal rearrangement. Studies of karyotypes of cells in metaphase and interphase, DNA content, mutation patterns in the p53 locus, and the terminal repeats of Epstein-Barr virus (EBV) genomes were similarly inconclusive because the results were heterogeneous, applicable in only some cases, or not attributable specifically to Reed-Sternberg cells.^{10,14-21}

A new approach to ascertaining the origin and clonality of Reed-Sternberg cells is the analysis of single

Reed-Sternberg cells isolated from tissues affected by Hodgkin's disease. This approach has been used by three groups, but with differing results.²²⁻²⁴ The discrepancies may be due to the small numbers of patients and subtypes of Hodgkin's disease investigated, to differences in isolation methods, or both. In this paper, we report our results with the single-cell assay in 12 patients with Hodgkin's disease whose Reed-Sternberg cells had a B-cell immunophenotype. Our method of isolating immunostained cells directly from frozen sections²⁵ allows a clear morphologic and immunophenotypic identification of Reed-Sternberg cells, permits the reliable collection of single Reed-Sternberg cells, and prevents their contamination by other cells.

METHODS

Tissues

Biopsy specimens from 12 patients with Hodgkin's disease (4 with the nodular-sclerosing type and 8 with the mixed-cellularity type) containing CD20-positive Reed-Sternberg cells were obtained from the files in our departments. One hyperplastic tonsil specimen, one specimen of mantle-cell lymphoma, the B-cell line Raji, and the T-cell line HUT102 were used as control tissues and cells.

Immunophenotyping, Tumor-Cell Fraction, Mitotic Index, and In Situ Hybridization

Sections embedded in paraffin, frozen sections, or both were stained with antibodies against CD30, CD20, CD15, CD10, CD3, CD1a, EBV-encoded latent membrane protein, and terminal deoxynucleotidyl transferase (TdT) from Dako (Glostrup, Denmark) and were stained for T-cell-receptor β -chain (TCR β) with β F1 (T-cell Sciences, Cambridge, Mass.). In situ hybridization with probes for EBV-encoded nuclear RNA 1 and 2 (EBER 1 and 2) was performed as described elsewhere.²⁶ The number of CD30-positive Reed-Sternberg cells among 2000 cells of other types was determined. The mitotic index of the Reed-Sternberg cells was investigated by counting the number of mitotic figures in 300 CD30-positive Reed-Sternberg cells.

Isolation of Single Cells

Frozen sections 7 μ m thick were immunostained for CD30, CD20, or TCR β . In addition to 5 CD20-positive and 5 TCR β -positive cells used as controls, at least 20 CD30-positive Reed-Sternberg cells were

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isolated from each specimen and collected as described by Küppers et al. (Fig. 1).²⁵ All isolations of cells were performed at least twice by different persons.

Polymerase Chain Reaction

A nested polymerase chain reaction (PCR) was performed with the consensus primers FR1²⁷ and LJH²⁸ in the first round of amplification and the consensus primers FR2A and VLJH for reamplification.²⁸

The first PCR was carried out in the tube to which the single cell had been transferred, with 300 ng of FR1 primers (50 ng each), 75 ng of LJH primer, and 2.5 mmol of magnesium chloride per liter of solution in a total volume of 100 μ l. The PCR consisted of 5 cycles at 63°C and 35 cycles at 57°C for the annealing of primers. A 1 percent aliquot of the first amplification product was used as a template for reamplification, with 200 ng of FR2A, 400 ng of VLJH, and 1.5 mmol of magnesium chloride per liter of solution. The second PCR consisted of 40 cycles at 63°C. All other PCR conditions were the same as previously described.^{29,30}

Six microliters of each amplification product was subjected to polyacrylamide-gel electrophoresis and subsequently stained with ethidium bromide.

Analysis of DNA Sequences

The isolation of amplified products and subsequent analysis of DNA sequences were performed as described elsewhere.³⁰ The se-

quences obtained were compared with each other and with published sequences of immunoglobulin variable-region heavy-chain (V_H) germ lines (GenBank, release 87) and translated into protein. Sequences with substitutions of more than three bases were regarded as somatically mutated, because there is very little polymorphism in the germ-line V_H sequences.³¹

RESULTS

Control Experiments

The PCR method was capable of detecting identical V_H gene rearrangements in single Raji cells, a culture of monoclonal B cells (Fig. 2A), whereas DNA from single cells of the T-cell line HUT102 could not be amplified with the V_H gene primers. Approximately 60 percent of single B cells isolated from frozen sections of hyperplastic tonsils and a mantle-cell lymphoma yielded PCR products. The PCR assay revealed unrelated (polyclonal) V_H gene rearrangements in the tonsillar B cells and identical (clonal) rearrangements in the mantle-cell lymphoma cells. Single T cells and buffers that covered the frozen sections during the cell-isolation procedure yielded no amplification products (Fig. 2A).

PCR and Sequence Analysis of Single Cells Isolated from Hodgkin's Disease Tissues

The material analyzed by PCR from biopsy specimens of tissues affected by Hodgkin's disease included single CD30-positive Reed–Sternberg cells and, as positive and negative controls, single B cells and T cells, respectively. Whereas T cells yielded no amplification products, V_H gene-specific PCR products were obtained from 60 to 70 percent of B cells and about 50 percent of single Reed–Sternberg cells. The failure to obtain such products from the rest of the cells was probably due to the use of tissue sections, which often contain only parts of nuclei, especially in the case of large Reed–Sternberg cells. It proved impossible to increase the yield by increasing the thickness of the sections, which only reduced the quality of immunostaining and made the identification and isolation of single cells less reliable.

PCR products of V_H genes were obtained from individual Reed–Sternberg cells isolated from all 12 patients with Hodgkin's disease. Gel electrophoresis and analysis of V_H gene sequences revealed three patterns (Table 1 and Fig. 2B). In three patients (Patients 10, 11, and 12), all the amplification products of Reed–Sternberg cells from a given biopsy specimen had the same length and sequence. In six patients (Patients 1 through 6), the lengths and V_H gene sequences of the amplification products from each biopsy specimen differed. In the remaining three patients (Patients 7, 8, and 9), some PCR products had the same lengths and sequences, whereas other products had different ones. Each experiment was performed at least twice by two persons, with identical results in each case.

Comparison of Sequences and Translation into Protein

We compared the V_H gene sequences we studied with germ-line sequences in the GenBank data bank to identify individual V_H genes and infer the presence of V_H mutations in Reed–Sternberg cells (Table 1, and material on deposit with the National Auxiliary Publications

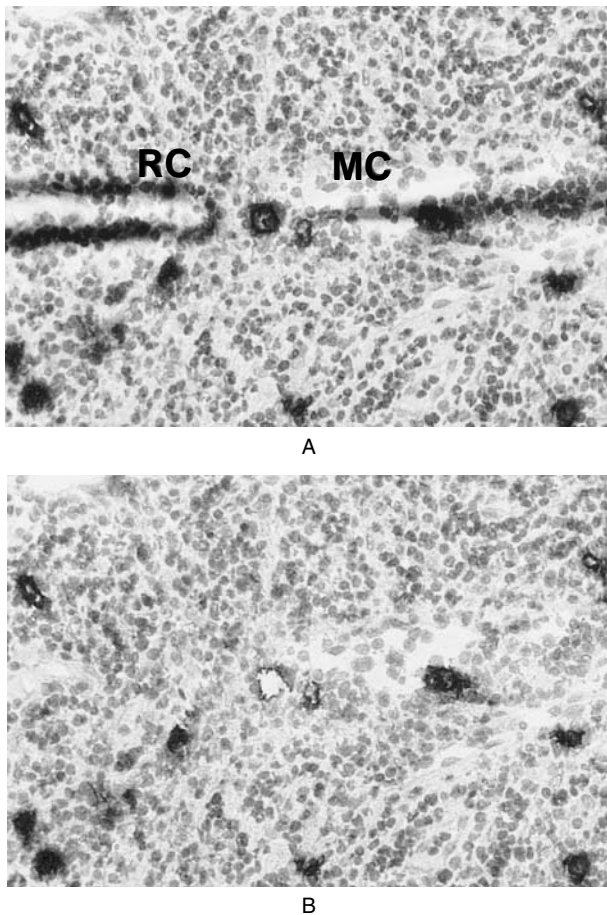


Figure 1. Isolation of a Single CD30-Positive Reed–Sternberg Cell from a Section of Tissue from a Patient with Hodgkin's Disease ($\times 600$).

The tissue section is shown before (Panel A) and after (Panel B) the isolation of the cell. The immunostained Reed–Sternberg cell was cut away from the surrounding cells with a manipulation capillary (MC) and transferred to the reception capillary (RC) without damage to surrounding cells and tissue, as described by Küppers et al.²⁵

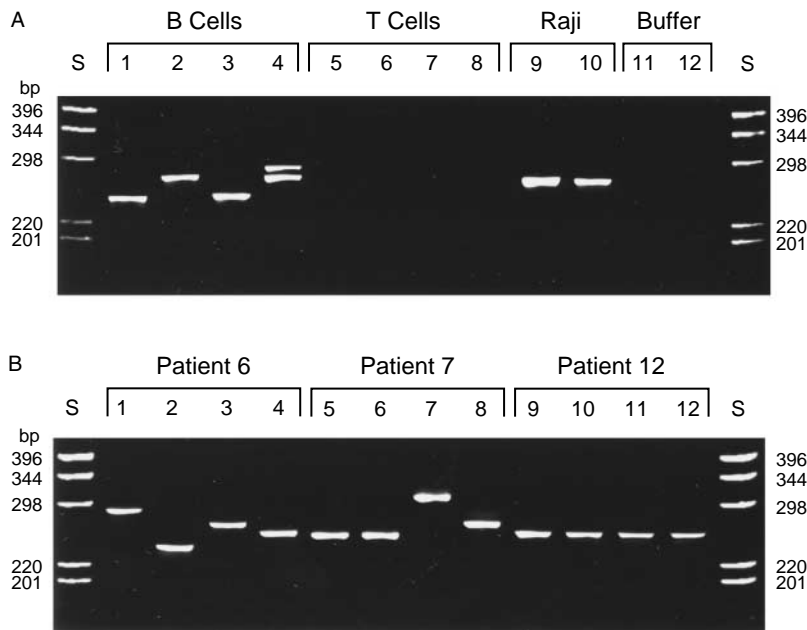


Figure 2. Amplification Products of the V_H Genes Generated by PCR with the Use of the FR2A and VLJH Primers.

Panel A shows amplification products after 6 percent polyacrylamide-gel electrophoresis and staining with ethidium bromide. The products were derived from single selected normal B cells (lanes 1 through 4) and cytospin preparations of single cells from the Raji cell line (lanes 9 and 10). No amplification products were obtained from single selected T cells (lanes 5 through 8) or overlying buffers (lanes 11 and 12). Panel B shows V_H -specific amplification products derived from single Reed-Sternberg cells isolated from tissue from three patients with Hodgkin's disease. In Patient 6 (lanes 1 through 4), all the PCR products differed in length; in Patient 7, products of both identical length (lanes 5 and 6) and different lengths (lanes 7 and 8) were found, whereas in Patient 12 (lanes 9 through 12) all the PCR products were the same length. S denotes a molecular-weight standard, and bp base pairs.

Service [NAPS]*). The population of V_H genes that the Reed-Sternberg cells had rearranged resembled the population used by normal B cells.³² Mutations of V_H genes varied greatly, from none to sequences that appeared to be highly mutated within the same patient and between different patients. One patient (Patient 6), with a polyclonal population of Reed-Sternberg cells, had only wild-type V_H sequences.

The DNA sequences of the V_H genes from the Reed-Sternberg cells were potentially translatable into protein, and were thus functional, with the exceptions of a deletion of 23 base pairs (in Patient 12) that resulted in a frame shift, and of three other sequences, each in a different patient, in which the translation broke off in the N region of the rearranged gene.

Correlation of Rearrangement Patterns with Other Features

The histologic, immunophenotypic, and other features shown in Tables 1 and 2 indicate that the Reed-Sternberg cells from all the patients expressed CD30

and, in variable quantity and density, the B-cell marker CD20. Early lymphoid-cell markers, such as CD1a, CD10, and TdT, were not detected (data not shown). The Reed-Sternberg cells of all four patients with nodular sclerosing Hodgkin's disease contained unrelated (polyclonal) V_H gene rearrangements and lacked detectable transcripts of EBER 1 and 2. The average mitotic index of the Reed-Sternberg cells was significantly lower (2.3 percent) in the polyclonal group, with late mitotic figures almost totally absent in three instances, as compared with the monoclonal group (mitotic index, 10.2 percent).

DISCUSSION

We conducted a PCR analysis of the rearranged V_H genes in single Reed-Sternberg cells with B-cell immunophenotypes that were isolated from 12 patients with classic Hodgkin's disease. We focused on this B-cell type of Reed-Sternberg cells, because with whole-tissue DNA we found a striking positive correlation between the expression of the B-cell marker CD20 on Reed-Sternberg cells and the presence of clonal V_H gene rearrangements.²⁹ However, analysis of whole-tissue DNA cannot determine whether the clonal rear-

rangements are derived from Reed-Sternberg cells or from other cells in the biopsy specimens. Therefore, we turned to the analysis of individual Reed-Sternberg cells. With this approach, we found rearranged V_H genes in Reed-Sternberg cells in all 12 patients studied. Our results, and the demonstration of immunoglobulin-gene rearrangements in single Reed-Sternberg cells in two other studies,^{22,24} suggest that technical factors may have contributed to the failure of Roth et al.²³ to obtain V_H PCR products from single Reed-Sternberg cells in any of their patients. By molecular means, our experiments show the B-cell origin of Reed-Sternberg cells that express the B-cell antigen CD20.

The pattern of V_H gene rearrangements in the 12 patients we studied was heterogeneous. In three patients, all the Reed-Sternberg cells isolated from the same biopsy specimen had identical V_H rearrangements. This result is consistent with the molecular findings in a monoclonal B-cell lymphoma. We were surprised, however, by the unrelated V_H rearrangements in other patients. The rearranged V_H genes of some Reed-Sternberg cells were identical, whereas those of the others in the same tissue sample were unrelated. These findings indicate a polyclonal proliferation of Reed-Sternberg cells in six patients and mixed populations of both monoclonal and polyclonal cells in the other three patients. Typically, the B cells in a reactive lymph node

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consist of a polyclonal population, whereas in a lymphoma the B cells are monoclonal. The heterogeneous pattern of V_H gene rearrangement can explain most of the differing results of previous studies using DNA that was extracted either from tissue samples or from enriched populations of Reed–Sternberg cells,³³ as well as the heterogeneous findings of analyses of the DNA content of Reed–Sternberg cells.^{20,21} The reproducibility of our results diminishes the possibility that they represent methodologic artifacts.

Nevertheless, our data seem to be at variance with the results obtained by several other groups. In the study of single cells by Delabie et al.,²⁴ only polyclonal populations of Reed–Sternberg cells were detected, whereas Küppers et al.²² found only monoclonal tumor cells. This discrepancy is probably due to the small numbers of patients — three and four, respectively — included in these two studies. A study of the DNA of Reed–Sternberg cells²⁰ has been interpreted to indicate that the cells have a monoclonal origin. However, it is possible that some of the patients in that study, especially those without aneuploidy, chromosomal aberrations, or p53 mutations, had Reed–Sternberg cells of polyclonal origin. Most studies of EBV genomes in Hodgkin’s disease³³ have found evidence of monoclonal EBV episomes with a molecular probe of the terminal-repeat region of the virus.³⁴ However, this probe has also found monoclonal EBV episomes in some samples of hyperplastic (polyclonal) lymphoid tissue³⁵ and in some cases of HIV-related immunoblastic lymphomas with polyclonal populations of rearranged immunoglobulin genes.³⁶

The rearranged V_H genes in the three patients with

Table 1. Analysis of Single Reed–Sternberg Cells from 12 Patients with Classic Hodgkin’s Disease with a B-Cell Immunophenotype, Performed to Detect V_H Gene Rearrangements.

REARRANGEMENT PAT- TERN AND PATIENT NO.	V_H AMPLIFICA- TION PRODUCTS		V_H SEQUENCES		V_H GENE FAMILY*				DEGREE OF DEVIATION FROM KNOWN SEQUENCES*		
	NO. WITH TOTAL IDENTICAL		TOTAL NO.	NO. IDENTICAL	1	2	3	4	LOW	MEDIUM	HIGH
	NO.	LENGTHS†									
<i>no. of sequences</i>											
Polyclonal											
1	11	0	10	0	—	1	4	5	7	1	2
2	23	0	11	0	—	—	8	3	7	3	1
3	9	2‡	9	0	—	—	7	2	4	2	3
4	6	0	6	0	1	—	5	—	2	3	1
5	9	0	8	0	3	1	3	1	2	5	1
6	22	4‡	6	0	1	—	4	1	6	—	—
Mixed polyclonal and monoclonal											
7	15	8	7	4	4/4	—	2	1	3	4/4	—
8	10	4	9	4	1	1	7/4	—	1	3	5/4
9	9	7	9	7	8/7	—	—	1	2	—	7/7
Monoclonal											
10	9	9	6	6	—	—	6/6	—	—	—	6/6
11	7	7	6	6	—	—	6/6	—	—	—	6/6
12	18	18	6	6	—	—	6/6	—	6/6; deletion of 23 bp with frame shift	—	—

*Sequences were compared with known germ-line sequences in a data bank (GenBank, release 87). Numbers following a slant line are the numbers of identical sequences. A difference of 2 nucleotides or less from the germ-line sequence in the data bank was considered to represent a low degree of deviation; a difference of 3 to 12 nucleotides, a medium degree; and a difference of more than 12 nucleotides, a high degree.

†As revealed by 6 percent polyacrylamide-gel electrophoresis.

‡Differences in the length of the amplification products could not be determined, but the sequences differed.

Table 2. Clinical, Histologic, and Immunohistologic Features of 12 Patients with Classic Hodgkin’s Disease Whose Reed–Sternberg Cells Were Positive for CD20 and CD30 and Negative for CD3 and T-Cell Receptor β .*

PATIENT NO.	AGE (YR)/ SEX	DISEASE STAGE†	DISEASE SUBTYPE	REED–STERNBERG CELLS			
				EBV- POSITIVE‡	% OF ALL CELLS	% IN MITOSIS	CD15§
1	29/F	IIA	NS	No	1.2	2.9¶	+
2	59/M	IIIA	NS	No	2.0	0.7¶	+/-
3	29/F	IIA	NS	No	1.8	3.0	+
4	7/M	IIeA	NS	No	1.9	2.7	+
5	64/M	IVA	MC	Yes	1.4	1.2	—
6	53/M	IIA	MC	Yes	2.4	3.3¶	+/-
7	50/M	IIA	MC	Yes	1.7	3.3	+
8	39/M	IIIB	MC	No	1.0	5.3	—
9	12/F	IIA	MC	No	2.0	7.0	+
10	13/F	IVB	MC	Yes	1.3	12.3	—
11	64/F	IIIB	MC	Yes	0.8	9.3	—
12	53/M	IIB	MC	Yes	4.0	9.0	—

*NS denotes nodular-sclerosing type, MC mixed-cellularity type, and EBV Epstein–Barr virus.

†According to the Ann Arbor classification system of disease stages.

‡As determined by staining with antibodies against latent membrane protein and by in situ hybridization with EBER probes.

§Plus signs denote the presence of CD15-positive cells, minus signs the presence of CD15-negative cells, and both signs together the presence of both CD15-positive and CD15-negative cells.

¶No cells in this sample were in anaphase or telophase.

monoclonal gene rearrangements that we studied had somatic mutations. In each patient, all the Reed–Sternberg cells isolated had the same distinctive mutations. The heterogeneous pattern of the V_H gene mutations in five of the six patients with polyclonal Hodgkin’s disease indicates that in a given tissue sample the Reed–Sternberg cells were not only unrelated, but also probably derived from B cells in different stages of maturation; those without somatic mutations could correspond to B cells that had not yet entered the germinal center, whereas those with V_H gene mutations may have originated from memory B cells that had left the germinal center.³⁷

If the Reed–Sternberg cell is indeed the neoplastic component of Hodgkin’s disease, then our finding of polyclonal Reed–Sternberg cells conflicts with current concepts of tumorigenesis. Monoclonal neoplasms grow through continuous mitotic divisions and give rise to identical progeny. In the case of polyclonal Reed–Sternberg cells, the mechanism of cell growth must differ. Quantitative and qualitative analyses of mitosis indeed revealed that the mitotic index was far lower in the patients with polyclonal cells than in those with monoclonal cells. Moreover, three of the six patients with polyclonal Reed–Stern-

berg cells, but none of the three with monoclonal Reed-Sternberg cells, lacked late mitotic figures, suggesting a disturbance of the mitotic process, of cytokinesis, or both. These observations suggest that polyclonal populations of Reed-Sternberg cells arise from the continuous recruitment of unrelated B lymphocytes. Such a mechanism would be predicated on the susceptibility of certain B cells to be transformed into Reed-Sternberg cells (a process perhaps mediated by genetic instability); a transforming agent or agents, such as EBV; and an immune defect that impairs the elimination of aberrant cells. There is evidence of each of these elements in Hodgkin's disease.³⁸⁻⁴²

The classification of Hodgkin's disease as polyclonal or monoclonal may have clinical implications. For example, the patients with polyclonal Reed-Sternberg cells may respond better to chemotherapy⁴³ than those with monoclonal Reed-Sternberg cells. Our study shows that the presence of B-cell markers on Reed-Sternberg cells does not constitute an example of aberrant gene expression, but indicates a real relation between those cells and B cells. We therefore conclude that there are B-cell types of Hodgkin's disease and that some of them contain polyclonal populations of Reed-Sternberg cells. Further studies of single cells may clarify the origin of Reed-Sternberg cells that express T-cell antigens or of those that lack both B-cell and T-cell antigens.

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CORRECTION

Clonality of Reed–Sternberg Cells in Hodgkin’s Disease

To the Editor: In 1995 we reported in the *Journal* the results of a molecular analysis of single Reed–Sternberg cells in biopsy specimens from patients with Hodgkin’s disease.¹ Amplified immunoglobulin heavy-chain gene sequences bore the hallmarks of B cells, and in 6 of the 12 cases we studied, there was evidence of the presence of monoclonal Reed–Sternberg cells — that is, identical immunoglobulin-gene rearrangements in several different Reed–Sternberg cells. Unexpectedly, we also found polyclonal Reed–Sternberg cells (i.e., cells with unrelated immunoglobulin-gene rearrangements). Other groups have also found polyclonal Reed–Sternberg cells,² but some investigators indicated that the cells in individual cases were derived from a single precursor.³

We have continued to study the clonal nature of Reed–Sternberg cells, and we believe that we can now satisfactorily account for these conflicting data. First, it is clear that Reed–Sternberg cells show a high degree of immunoglobulin-gene somatic mutations, which means that family-specific V_H primers rather than consensus primers are required for the polymerase chain reaction that allows identification of clonal immunoglobulin-gene rearrangements. Second, when Reed–Sternberg cells (or other cells) are isolated, precautions must be taken to avoid aspirating medium that contains DNA leached from the polyclonal reactive B cells present in the tissue sample.

Unless scrupulous attention is paid to these technical aspects of the method, there is a risk that artifactual results may suggest a polyclonal proliferative process in Hodgkin’s disease. In our original cases, and in 25 cases analyzed more recently, we did not find polyclonal immunoglobulin sequences in Reed–Sternberg cells, and we are therefore persuaded that these cells are of monoclonal origin.

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