Rearranged Antigen Receptor Genes in Hodgkin’s Disease

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Despite intensive efforts using a wide variety of approaches, the cellular lineage and clonality of the abnormal cells of Hodgkin’s disease have remained an enigma. In the present study, cell separation techniques that enriched for Reed-Sternberg cells and their variants were used to generate sufficient percentages of abnormal cells to allow detection of rearrangements in these cell fractions. DNA from the involved tissues of eight Hodgkin’s disease patients was subjected to Southern blot analysis to detect rearrangements of T cell antigen receptor genes and immunoglobulin genes. Immunoglobulin gene rearrangements were found in three of five cases in which Reed-Sternberg cells and their variants were enriched by cell separation techniques to cell frequencies >1%. Rearrangements of immunoglobulin heavy chain genes occurred in two cases, and a λ light chain gene rearrangement occurred in a third case. Rearrangements were not detected in lymphocyte fractions or in unseparated cells prepared from the same tissues. The putative Hodgkin’s cell line, L428, also contained rearrangements of immunoglobulin heavy and κ and λ light chain genes and, in addition, harbored a single T cell receptor β gene rearrangement. These findings indicate that Reed-Sternberg cell-enriched fractions contain clonal cell populations and provide a lead, at the molecular genetic level, to a possible lymphoid derivation of the Reed-Sternberg cell.

The application of monoclonal antibody phenotyping analysis, cytogenetics, and molecular genetics has identified virtually all non-Hodgkin’s lymphomas as clonal neoplasms of lymphocytes. In contrast, little is known regarding the cellular derivation, or even clonality, of the Reed-Sternberg cells of Hodgkin’s disease (reviewed in refs. 8 and 9). Immunological and cytochemical analyses of Hodgkin’s disease tissues have fostered an array of conflicting findings that have led to many alternative speculations. On the basis of these investigations, Hodgkin’s disease has variously been suggested to be a neoplasm of B cells, T cells, monocytes/macrophages, interdigitating reticulum cells, or myeloid cells. Consequently, no definitive features have emerged to classify precisely the lineage of Reed-Sternberg cells.

Cell biology investigations of Hodgkin’s disease have been hampered by several natural factors. One prominent obstacle has been the relative paucity of Reed-Sternberg cells in Hodgkin’s disease and a vast predominance of supposedly “normal” cellular elements—lymphocytes, plasma cells, histiocytes, and eosinophils. Thus, unlike most non-Hodgkin’s lymphomas, the presumed neoplastic cells of Hodgkin’s disease are a minor fraction of the total cell number, often <1%, which is generally insufficient for functional studies, cytogenetics, or molecular genetic analysis.

Moreover, it has been remarkably difficult to generate and maintain bona fide, continuous Reed-Sternberg cell lines. Although several cell lines have been cultured, some of these either have not been immortalized or were contaminated by cells derived from other sources. One cell line, L428, derived by Diehl and colleagues from the pleural effusion of a patient with previously treated nodular sclerosing Hodgkin’s disease has been maintained for >6 years. Whether the L428 cell is indeed the direct descendant of a Reed-Sternberg cell, a secondary neoplasm, or a lymphoblastoid cell is not known, since the primary Reed-Sternberg cells from that patient have not been genetically compared with the L428 cell line. The L428 cell remains a possible candidate as a Reed-Sternberg cell, however, since it carries karyotypic abnormalities and shares an immunoreactive surface membrane protein with Reed-Sternberg cells (Ki-1 or HeF-1) and has a phenotype slightly reminiscent of the Reed-Sternberg cell.

The focus of the present study was to determine the clonality and lineage of Reed-Sternberg cells by molecular genetic analysis of their immunoglobulin and T cell receptor genes. The success of this approach holds promise in that it is sensitive, provides direct evidence of clonality without necessitating cytogenetics, and can be used to identify lineage-related markers without gene expression. Because cells from Hodgkin’s disease might be separable by physical methods, our strategy was to apply cell separation procedures to enrich the percentage of Reed-Sternberg cells to threshold levels needed to detect gene rearrangements by Southern blot analysis. By isolating Reed-Sternberg cells, we have begun to explore the molecular biology of Hodgkin’s disease.

MATERIALS AND METHODS

Tissues and cells. Involved lymph nodes and spleens were obtained from eight patients with nodular sclerosing Hodgkin’s disease (Table 1). Cases were unselected, and no attempt was made to restrict the study to cases with high percentages of Reed-Sternberg cells. Cases of lymphocyte-depleted Hodgkin’s disease were excluded from analysis because of the high likelihood that many may actually be non-Hodgkin’s lymphomas. Cases of mixed cellularity or lymphocyte-predominant Hodgkin’s disease were not available. All patients were advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent.

Fresh tissue samples were finely minced in RPMI 1640 culture medium (GIBCO, Grand Island, NY) and subsequently digested...
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with collagenase type IV 1.0 mg/mL (Sigma Chemical, St Louis) for 30 to 90 minutes at room temperature with constant stirring. Cell viabilities throughout all procedures were consistently >90%. Cytocentrifuge slides of the cell suspensions were prepared for morphological and immunostaining analyses.

Cell suspensions were fractionated by Ficoll-Hypaque (Lymphoprep, Accurate Chemical and Scientific, Westbury, NY) or Percoll (Pharmacia, Piscataway, NJ) density-gradient centrifugation or by elutriation. Ficoll-Hypaque separation was performed by layering 8 mL of suspended cells (10^7/mL) over 4 mL of Ficoll-Hypaque in a 15-mL conical tube for 30 minutes at 400 g at room temperature. Both the interface and pellets were saved for analysis. Percoll solutions were prepared in accordance with the instructions of the manufacturer using 45% to 60% isosmotic Percoll solutions with a density ranging from 1.050 to 1.076 g/mL. An aliquot of 1 mL of 0.1 mg/mL (Sigma Chemical, St Louis) collagenase type IV was added to the slurry of cells. After incubation for 30 to 90 minutes at room temperature with constant stirring, cell separation experiments, cell suspensions were prepared by centrifugation at 5 x 10^7/mL was spun through 9 mL of gradient for 20 minutes at 400 g at room temperature. A control gradient containing standardized beads was simultaneously run to determine density positions. Morphological analysis of Wright’s-stained cytocentrifuge slides was used to evaluate the optimal density range for separation of Reed-Sternberg cells and variants from the remaining cell population. Once this density range was determined, a two-step discontinuous isosmotic Percoll gradient was performed, which trapped the enriched Reed-Sternberg cell fraction between the selected densities (1.051 and 1.069 g/mL). A large volume of cells (20 to 25 mL at 5 x 10^7/mL) was separated using the discontinuous Percoll gradient.

The splenic cells obtained from Hodgkin’s patients were also separated by counterflow centrifugal elutriation with a Beckman JE-6B elutriation system and rotor as previously described, with the exception that the separation media was Dulbecco’s phosphate-buffered saline (DPBS) without calcium and magnesium (Biofluids, Rockville, MD). Prior to elutriation, 4 to 8 x 10^8 spleen cells were incubated at 37°C in 40 mL of DPBS containing 1 mg/mL of deoxyribonuclease (Sigma) for 30 minutes. The cells were then loaded into the elutriation rotor, which was spinning at 1,960 rpm, at a flow rate of 8 mL/min. One hundred fifty milliliters were collected at this flow rate and constituted the first fraction. Subsequently, the cells in fractions 2 through 6, each containing 150 mL, were collected at flow rates of 11, 13.6, 16.4, 18.0, and 19.2 mL/min, respectively. The last fraction, number 7, was comprised of cells that eluted at the final flow rate after the centrifuge was stopped.

Samples from appropriate fractions were viably stored over liquid nitrogen as described. Cytocentrifuge slides were prepared and used for differential counts (1,000 cells) and immunostaining (see below). DNA was extracted from the majority of cells (1 to 3.5 x 10^7 cells) as outlined below.

Cell lines used in these studies were L428 (V. Diehl, University of Cologne) and the African Burkitt lymphoma, Raji (American Type Culture Collection, Rockville, MD). Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient from a normal individual.

Immunophenotyping. Cytocentrifuge preparations were stained with monoclonal antibodies using an indirect avidin-biotin-peroxidase complex (ABC).26 The percentage of positive cells was determined by scoring 500 to 1,000 cells per slide.

Monoclonal antibodies and their sources included Leu-1, Leu-2, Leu-3, Leu-4, and Leu-M1 (Becton Dickinson Monoclonal Antibodies, Sunnyvale, CA); B-1, B-4, (Coulter Immunology, Hialeah, FL); Hefi-1 (Dr Richard Fisher, Loyola University); and anti-Tac (Dr Thomas Waldmann, National Cancer Institute).

Gene rearrangement analysis. High-mol-wt DNA was extracted from unseparated specimens, Reed-Sternberg cell-enriched fractions, Reed-Sternberg cell-depleted fractions, and the L428 cell line24 by a standard phenol-chloroform ethanol precipitation technique.35 When only small numbers of cells (<40 x 10^3) were available, DNA was extracted by ultracentrifugation with a guanidine thiocyanate-cesium chloride gradient.24 DNA was subjected to restriction fragment digestion, Southern transfer, and probed with genomic clones of human immunoglobulin Jκ, Jγ, Cκ, Cγ, T cell γ gene J region (Jγ) and cDNA of the T cell receptor β subunit constant region (Cβ), and a full-length cDNA (Tγ) as detailed in previous reports.33,35 For each sample, BamH1, EcoR1, and HindIII-digested DNAs were probed with Jκ and Cκ, BamH1 digests with Cβ, and EcoR1 digests with Cγ, Jγ, and Tγ probes were hybridized with blots containing L428 DNA digested with BamH1, EcoR1, and HindIII.

For Southern blot sensitivity analysis varying proportions of DNA obtained from Raji cells were mixed with DNA from normal polyclonal splenic lymphocytes. DNA was digested with EcoR1, and conventionally prepared Southern blots were probed with Jκ.

RESULTS

Cell separation. Unfractionated cell suspensions of tissues involved by Hodgkin’s disease contained <1% Reed-Sternberg cells and variants, except case 4, which was comprised of 4% abnormal cells (Table 1). Percentages displayed in Table 1 represent counts determined by morphological analysis of cytocentrifuge preparations, which were confirmed in all instances by parallel cytocentrifuge slides stained with Leu-M1,35 Tac33,35 and Hefi-1.28 In each case, most normal cells were lymphocytes that expressed either T or B cell surface markers. Reed-Sternberg cells and their variants were unreactive with all anti-T cell antibodies, B-1, and B-4. In the elutriation cell separation experiments, Reed-Sternberg cells and their variants increased in fractions 5 through 7; thus, these fractions were combined in case 2 with a yield of 6.6% Hodgkin’s cells. Enriched fractions of Reed-Sternberg cells were isolated from discontinuous Percoll gradients in cases 1, 4, 5 and 7 (Figs 1 and 2) using predetermined optimal density ranges (Fig 1); the results of large-scale gradient centrifugation separations are listed in Table 1.

Leu-M1 and Hefi-1 staining confirmed the percentages of Reed-Sternberg cells and variants in the enriched fractions (Fig 3). Lymphocyte fractions contained <0.1% Leu-M1- or Hefi-1-positive cells in all cases. As previously demonstrated by other researchers,40,41 Reed-Sternberg cells often formed

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*Percoll gradient; †, elutriation; ‡, Ficoll-Hypaque gradient.
IgH, Igκ, Igκ, immunoglobulin heavy, k and λ chain genes; Tγ, T cell receptor β chain gene; G, germline; R, rearranged; RR, two rearrangements; ND, not done (separations not performed due to insufficient cells).
Fig 1. Percoll gradient centrifugation. A cell suspension of a lymph node involved by Hodgkin's disease was centrifuged through a continuous Percoll gradient as described in the Materials and Methods Section. Cell samples were taken from several densities, and the optimal density for enrichment of Reed-Sternberg cells and variants (RS) was determined by morphological analysis of cytocentrifuge preparations. In this example (case 5), peak RS separation was obtained between 1.061 and 1.069 g/ml.

Fig 2. Density gradient separation of Reed-Sternberg cells and variants (case 5). A cell suspension of an involved lymph node, which contained 0.04% Reed-Sternberg cells and variants, was fractionated by centrifugation through a discontinuous Percoll gradient. (A) Lymphocytes (>99%) were recovered from the pellet (density >1.069 g/mL) (Original magnification x400; current magnification x263; Wright's stain). (B) Reed-Sternberg cells and their mononuclear and multinuclear variants were highly enriched in the interface at density 1.069 g/mL (original magnification x400; current magnification x270, Wright's stain).

Fig 3. Immunostaining of cytocentrifuge preparations of Reed-Sternberg cell-enriched fraction (case 5). (A) Spontaneous adherence of T cells to mononuclear variants of Reed-Sternberg cells. Reed-Sternberg cells and variants did not react with the anti-T cell antibody Leu-4 (Leu-4, ABC; original magnification x1,000 current magnification x6,500). (B) Leu-M1 staining of a mononuclear variant of a Reed-Sternberg cell reveals elaborate dendritic processes that engulf adherent T cells (Leu-M1, ABC; original magnification x1,000, current magnification x6,500).

spontaneous rosettes with autologous T cells (Fig 3A). Dendritic processes elaborated by Reed-Sternberg cells and their variants were stained by Leu-M1 and appeared to engulf adjacent T cells (Fig 3B). The functional meaning of this intercellular activity is not known.

Gene rearrangement. DNA obtained from intact, unseparated, Hodgkin's disease samples was initially screened for gene rearrangements. As in previous studies using this approach, no rearrangements were detected (Fig 4). Therefore, subsequent analyses were performed with DNA from fractionated cell populations. Sufficient DNA (usually >100 μg) was extracted from fractions of interest to allow analysis with each probe and multiple restriction enzymes. In three of
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Fig 4. Southern analysis of immunoglobulin k genes from unfractionated Hodgkin's tissues. Filter hybridization of BamHI digests of genomic DNA obtained from cases 1 through 4 showed no evidence of k gene rearrangement. Case numbers are shown above each lane. Adjacent dashes indicate the germline BamHI restriction fragment size (12 kilobase, kb) hybridizing with the C, probe.

The eight cases (cases 2, 6, and 7), immunoglobulin gene rearrangements were identified in the Reed-Sternberg cell-enriched fractions (Fig 5, Table I). In contrast, in these same cases, rearrangements were not apparent in either the unseparated cells or in the Reed-Sternberg–depleted (lymphocyte) fractions (Fig 5, Table I). Thus, detectable gene rearrangements were specifically associated with fractions containing >1% Reed-Sternberg cells and variants. No Tα gene rearrangements were detected in the cases.

The Reed-Sternberg cell-enriched fraction in case 2 showed one rearranged λ light chain allele, but no rearrangements of immunoglobulin heavy chain, k light chain, or Tα genes were seen. Although light chain gene rearrangements are found in B cells, it is unusual to find a light chain gene rearrangement without a concomitant heavy chain gene rearrangement. This finding might be attributable to a technical factor, such as comigration of rearranged and germline bands, but no JH rearrangements were detected despite digestion with BamHI, EcoRI, and HindIII. Potentially, deletion of a substantial portion of the JH region during rearrangement might diminish the sensitivity of detection of JH hybridization. Simultaneous hybridization with the same labeled probe on the same filter, however, demonstrated rearrangements in cases 6 and 7 (Fig 5, Table I). Alternatively, the λ gene rearrangement in case 2 may have resulted from chromosomal breakage or DNA deletion within a hybridizing restriction fragment rather than variable region gene joining. No cytogenetics were available, and sequence analysis would be required to resolve this point.

Cases 6 and 7 both displayed JH rearrangements and no light chain or Tα gene rearrangements. Again, nondetection of rearrangements might be due to differential sensitivities of each probe. Because heavy chain genes are known to rearrange prior to light chain genes in pre-B cells, our findings may imply a pre-B cell stage of development. This cannot be concluded with certainty, however, since one cannot reliably exclude the possible existence of a light chain gene rearrangement in such a minor cell population.

L428 cells were found to contain gene rearrangements that included one heavy chain, one k light chain, one λ light chain, and one Tα chain gene (Fig 6). Restriction fragment analysis of the Tα and JH genes showed only germline hybridizing bands.

To estimate the sensitivity of Southern blot analysis for the detection of rearrangements in rare clones, a dilution experiment was performed. The B cell (Burkitt's) lymphoma line, Raji, carries two rearranged JH genes as seen in EcoRI-digested DNA. When known amounts of Raji DNA were

Fig 5. Immunoglobulin gene rearrangements in Reed-Sternberg cell-enriched fractions. Cell types were separated as outlined in Table I, and DNA was extracted and analyzed for gene rearrangement in Southern blots. Case numbers are at top. In case 5, no rearrangements of the immunoglobulin heavy chain gene were detected in either the lymphocyte or Reed-Sternberg cell-enriched (RS) fractions. The RS fraction from case 6 contained two nongermline bands (arrows) hybridizing with JH in HindIII-digested DNA. In contrast, no rearrangements were seen in either the unseparated cells or lymphocyte fractions from case 6. In case 2, the RS fraction exhibited a single rearrangement of the λ gene (arrow), whereas no λ rearrangements were detectable in unseparated cells; germline sizes (-).
Fig 6. Gene rearrangements in the L428 Hodgkin's cell line. By Southern analysis, single gene rearrangements were detected for the $\beta$ subunit of the T cell antigen receptor ($C_\beta$), immunoglobulin heavy chain ($J_H$), $\kappa$ light chain ($J_\kappa$), and $\lambda$ light chain ($C_\lambda$) (arrows). Location of germline restriction fragments (-).

mixed with DNA obtained from normal polyclonal lymphocytes and digested with EcoRI, nongermline bands contributed by Raji cells could still be detected when Raji DNA was diluted to only 2% of the total DNA (Fig 7). Therefore, clones with a frequency of as little as 2% of the cell population are detectable by Southern analysis, thus confirming the findings in Reed-Sternberg cell-enriched fractions.

**DISCUSSION**

By enriching for Reed-Sternberg cells and their variants using cell separation techniques, we uncovered immunoglobulin gene rearrangements in Hodgkin's disease. In each of the three primary cases in which rearrangements were detected, Reed-Sternberg cells and variants were enriched to account for $>1\%$ of the cell population, a level that was shown to be near the threshold of sensitivity for Southern analysis. Rearrangements were not observed in any of the samples containing $<1\%$ Reed-Sternberg cells and variants. Thus, lack of detection of rearrangements in Hodgkin's disease tissue may be a consequence of subthreshold percentages of Reed-Sternberg cells. It should be emphasized that cases studied here were unselected, yet were unequivocally diagnostic examples of Hodgkin's disease. No attempt was made to examine tissues with high percentages of Reed-Sternberg cells specifically, a procedure that might permit inadvertent inclusion of some non-Hodgkin's large cell lymphomas due to difficulties inherent in differential diagnosis. Furthermore, analyses were performed on lymph nodes and spleens involved by Hodgkin's disease, in which the diagnosis is most accurately made. With this approach, we avoided ambiguities regarding circulating neoplastic cells that might well be secondary non-Hodgkin's lymphoma or leukemia arising in a patient with Hodgkin's disease. Based on the findings reported here, evidence that rearrangements occurred in the Reed-Sternberg cells per se is circumstantial, since cell fractions were not purified to homogeneity. For example, although unlikely, a minor clonal B cell or plasma cell population with a density or volume comparable to Reed-Sternberg cells may have been concomitantly enriched in the Reed-Sternberg-containing fractions. Documentation of the gene rearrangements of Reed-Sternberg cells will necessitate the elimination of extraneous cells by further cell purifications. An additional finding of the present study is that no gene rearrangements were identified in the lymphocyte fractions of lymph nodes containing Hodgkin's disease. Thus, no significant clonal population was contained among these cells. Furthermore, the lymphocyte fraction served as a germline control for each patient to guard against restriction fragment length polymorphism.

Our findings indicate a heterogeneity of rearranged genes among cases of Hodgkin's disease. Rearrangement of immunoglobulin heavy but not light chain genes occurred in two cases (6 and 7), and the converse was seen in case 2. Two
cases (4 and 5) with fractions containing relatively abundant Reed-Sternberg cells had no demonstrable rearrangements for immunoglobulin or T cell receptor β chain genes. The inconsistent rearrangement pattern from case to case may simply be technical; thus, rearrangements in minor cell populations might remain undetected. Alternatively, rearrangements may not have occurred, perhaps as a result of incomplete attempts at B cell differentiation. Cells might be arrested at a pre-B cell stage in which immunoglobulin heavy but not light chains rearrange. Pre-B cells, however, contain terminal deoxynucleotidyl transferase, and this enzyme is not seen in Hodgkin’s disease. Rearrangements resulting from chromosomal breaks rather than immunoglobulin variable region gene recombination could give rise to the Southern blot pattern we observed. Whether chromosomal damage might account for rearrangements in the present cases is not known since cytogenetics were not performed, but some reported examples of Hodgkin’s disease carry abnormalities of the long arm of chromosome 14, where the immunoglobulin heavy chain gene is located.

By identifying immunoglobulin gene rearrangements, our molecular genetic analysis of the Reed-Sternberg-enriched cell fractions demonstrated minor clonal cell populations in Hodgkin’s disease. This finding, coupled with earlier reports of clonal chromosomal abnormalities, argues that Reed-Sternberg cells are clonally expanded in Hodgkin’s disease. Whether the clonal cells detected by either approach are indeed the Reed-Sternberg cells or some other cellular component of Hodgkin’s disease associated with Reed-Sternberg cells remains to be determined, however.

Our study of Reed-Sternberg cell-enriched fractions corroborates the demonstration by Weiss and associates of immunoglobulin gene rearrangements in cases of Hodgkin’s disease with abundant Reed-Sternberg cells. In that study, as in the present report, rearranged heavy chain genes were found without accompanying light chain gene rearrangements in some cases, a feature that deserves further investigation. In another study, rearranged Tα genes in four cases but no immunoglobulin gene rearrangements were reported from a series of eight cases of Hodgkin’s disease. In that study, however, Tα rearrangements were detected with a probe contaminated with variable and joining gene sequences that could produce numerous additional germline bands, which are difficult to interpret without matched germline control tissue from the same patient. Rearrangements of either Tα or immunoglobulin genes were also identified by Knowles and colleagues in 3 of 15 cases of Hodgkin’s disease, but it was concluded that rearrangements were attributable to clonal lymphoid cells rather than Reed-Sternberg cells.

No direct evidence was provided, however, to substantiate such an interpretation. As shown in the present report, lack of rearrangements as observed by others would be expected in a study of unseparated samples. Furthermore, the actual percentage of Reed-Sternberg cells in a specimen is extremely low and probably less than would appear by estimation from a single histological section. Thus, without direct cell counting of the sample, cases with apparently “numerous” Reed-Sternberg cells might well contain an insufficient percentage of abnormal cells for detection of rearrangements by Southern blot. Finally, when a differential diagnosis of Hodgkin’s non-Hodgkin’s lymphoma must be made, it is premature at present to derive a diagnosis of Hodgkin’s disease based on the absence of rearrangement as suggested by Knowles and colleagues. This precaution should be seriously considered in light of the issues that affect the interpretation of gene rearrangements in a rare cell subpopulation, as well as findings reported here and elsewhere.

Rearrangements of immunoglobulin heavy and κ and λ light chain genes in the L428 cell line suggest a B cell lineage for this cell. The usual hierarchy of light chain gene rearrangement has been violated, since κ light chain gene analysis demonstrated a single band of germline configuration. Thus, one of the two κ alleles has been neither rearranged nor deleted, as would be expected in a B cell with a rearranged λ gene. An explanation for this unexpected rearrangement of light chain genes might be that, since the Cκ locus is polymorphic, the single nongermline Cκ hybridizing band obtained with EcoRI is a restriction fragment length polymorphism. The size of the EcoRI fragment (7.0 kilobase, kb) however, is not the size of any of the known polymorphic EcoRI fragments (8, 13, 18, and 23 kb) and therefore can be considered a consequence of rearrangement of the λ gene locus. In addition, L428 carries one rearranged Tα gene, an occasional feature of B cell neoplasms. Tα gene rearrangements and germline immunoglobulin genes were found in a T cell line, CO, derived from a patient with Hodgkin’s disease, but it is not known whether this cell line represents the tumor. The unusual molecular genetic characteristics of L428 elude precise classification of cellular lineage, but its rearrangement of immunoglobulin genes is a feature shared with our primary cases of Hodgkin’s disease; together, these findings might provide a clue as to the derivation of the abnormal cells of Hodgkin’s disease.

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