Hodgkin and Reed-Sternberg Cells Do Not Carry T-Cell Receptor γ Gene Rearrangements: Evidence From Single-Cell Polymerase Chain Reaction Examination

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Hodgkin and Reed-Sternberg (H&RS) cells are generally accepted to be the neoplastic cells of Hodgkin’s disease (HD), even though they represent only a minority of the cellular infiltrate in affected tissues. Recent immunologic studies and Southern blot analyses of DNA extracted from whole lymph node tissue favored, but did not convincingly prove a lymphoid origin of H&RS cells. To detect rearrangements of the T-cell receptor chain (TCRγ) genes at the single-cell level as an indication of early T-cell lymphoid differentiation, we isolated H&RS cells by micromanipulation from cytospin preparations of fresh biopsy material. TCRγ chain rearrangement was detected by polymerase chain reaction using four “forward primers” that were constructed corresponding to all four V families and two “reverse primers” corresponding to consensus sequences of J segments. Rearrangements of all V families in combination with the different J segments were detected in human peripheral blood and tonsillar T cells. Although rearrangements of TCRγ chain genes were shown in single cells of 10 of 10 T-cell leukemias, no rearrangement of these genes was found in single H&RS cells from 13 consecutive patients with HD. Our results indicate that H&RS cells from the vast majority of cases are not derived from T cells. This finding may have implications for the pathogenesis of HD and the development of more effective treatment regimens.

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The Diagnosis of Hodgkin’s disease (HD) is based on the characteristic histologic finding of typical mononuclear Hodgkin (H) and multinucleated Reed-Sternberg (RS) cells that are surrounded by a mixed lymphohistiocytic infiltrate. Hodgkin and Reed-Sternberg (H&RS) cells are believed to represent the malignant cell population in HD because of their ability to disseminate, their multinuclearity, and the results of cytogenetic studies showing aneuploidy and clonality. In contrast to non-Hodgkin’s lymphomas that have been shown to be clonal proliferations of either T or B lymphocytes by immunohistology and molecular genetics, there is an ongoing debate on the cellular origin of H&RS cells in HD.

Functional studies and molecular genetic analyses have yielded conflicting results. This is mainly due to the fact that H&RS cells represent only a small minority in affected tissues. Based on morphologic and immunologic studies, nearly all hematopoietic cells have been proposed as the normal counterpart of these cells. Similarly, investigations of Hodgkin’s-derived cell lines by immunologic and molecular biologic methods have also not been conclusive. Because of presence of surface markers usually characteristic for B and T lymphocytes, a lymphoid origin of H&RS cells has been suggested by some investigators. In addition, molecular genetic studies have attempted to resolve controversies concerning the clonality, origin, and lineage of H&RS cells. Somatic recombinations of genes coding for Ig and T-cell receptor (TCR) molecules occur early in B- and T-cell ontogeny and are a prerequisite for functionally active molecules in mature lymphocytes. Evidence for clonal rearrangements was found in less than 15% of HD-affected lymph nodes by Southern blot analysis for TCR and even more rarely for Ig. The vast majority of samples showed germline genes or polyclonal rearrangements. Because of the scarcity of H&RS cells in involved tissues, the lack of detectable rearrangements could be attributed to the low sensitivity of Southern blot analysis, which is not able to detect clonal populations that make up less than 1% to 5% of a tissue sample. On the other hand, the rearrangements that were found in some cases of HD-affected tissue could conceivably be caused by oligoclonal proliferations of the lymphoid infiltrate and not the H&RS cells.

Because attempts to obtain pure populations of sufficient numbers of H&RS cells have not met with success, only studies at the single-cell level can be expected to solve the question of whether H&RS cells are really of lymphoid origin. Single-cell polymerase chain reaction (PCR) has been shown to be a powerful tool in determining the gene expression profile of H&RS cells. We have used single-cell PCR for the detection of rearrangements of the Ig heavy chain and the TCRγ chain in isolated H&RS cells to resolve the question of whether these cells represent bona fide lymphoid cells.

Materials and Methods

Patients. Patient data are summarized in Table 1 (and Roth et al10). Small parts of lymph node biopsies from 17 consecutive patients performed for diagnostic reasons were used for H&RS cell isolation after obtaining the patients’ informed consent according to the guidelines of the local ethics committee. Samples from 13 patients were available for this study.

Immunohistology. Immunohistology was performed on fresh frozen tumor tissue in 9 cases as described elsewhere and on paraffin-embedded tissues in the remaining 8 cases. From the quick-frozen tissues, serial frozen sections of about 1 cm in area and 4 to 6 μm in thickness were air-dried overnight, fixed in acetone for 10 minutes at room temperature, and immunostained immediately or stored at...
-20°C for up to 1 week. The following antibodies were used on frozen sections: anti-IgM (clone Rl/69) and CD 20 (L26) from Dakopatts (Copenhagen, Denmark); CD3 (SK7), anti-TCR-α/β/β (clone WT31), and anti-TCR-α/β-1 (clone 11F2) from Becton Dickinson (San Jose, CA); anti-pan-TCR-α/β (clone BMA031) and anti-TCR-β-chain (clone 6 TCS 1) from T Cell Sciences (Cambridge, MA); CD30 (HRS-4) was supplied by one of the investigators (M.P.). Negative controls were performed in each individual case without the primary antibody. Stained B and T lymphocytes served as intrinsic positive controls. To highlight the neoplastic compartment, CD30 staining was performed in each case.

**Isolation of H&RS cells.** Lymph nodes were prepared for H&RS cell isolation as previously described. Briefly, lymph node tissue was dissected into small pieces with sterile scalpels, suspended in phosphate-buffered saline (PBS), and further processed by density centrifugation on a Ficoll Hypaque (Pharmacia, Freiburg, Germany) gradient. Interphase cells were fixed in 3% wt/vol paraformaldehyde and cytocentrifuged onto glass slides. Staining for the presence of CD30 antigen was performed by the APAAP assay using the monoclonal antibody HRS-4. H&RS cells were identified by morphologic criteria and positive staining for the CD30 antigen. Single H&RS cells were picked with glass capillaries using a micromanipulator (Eppendorf, Hamburg, Germany), transferred into reaction tubes, and submitted to PCR.

**PCR for TCRγ and β-actin genes.** For the amplification of TCRγ gene rearrangements, four different V-family-specific primers and two J-family-specific primers corresponding to published sequences were used: Vγ(5’TCTTCAACTTTGGAAGGGAAGA3’), corresponding to Vγ5 families 1-8), Vγ(5’GGTCACTTACGACACCTCA3’), corresponding to Vγ9), Vγ(5’TCTCAGCTATCAGTCCCCACGG3’ corresponding to Vγ10), Vγ(5’CAGACCGCAGAAAATAGGG3’, corresponding to Vγ11), Jγ(5’CAAGTGTTGTTGCTCAGACTGCAAA3’), and Jγ(5’GTGACTATGAGG(GT)TAGTCCCC3’). PCR reactions were performed in a volume of 10 μL in a PCR buffer containing 0.25 μmol/L of each of the three primers and 0.5 μmol/L of a 1:1:1 mix of all 5’ primers, 200 μmol/L of each dNTP, 15 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.01% gelatin, and 1 U Taq polymerase (Boehringer Mannheim, Mannheim, Germany). After adding buffer, primers, and polymerase, the reaction tubes were submitted to UV radiation for 3 minutes before adding cells or DNA template. The samples were subjected to 40 temperature cycles (95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes) using a thermal cycler (Biometra, Göttingen, Germany). A 1:50 dilution of the PCR reaction product was amplified for another 40 cycles. For this second amplification, only one of the V- and J-specific primers (0.25 μmol/L) was used for each PCR reaction. All experiments were performed using a reaction tube without DNA template as a negative control as well as appropriate positive controls (cells of the T-cell leukemia line L 735). The reaction products were separated on an agarose gel (2%) and transferred to nylon membrane (Boehringer Mannheim). For hybridization, 32P end-labeled internal oligonucleotides were used: Vγ(5’CAGGGAGGGGAAAAGCCCAACAGC3’), Vγ(5’CAGCCCCCCTGGGAATGTTGGTG3’), Vγ(5’CGGCACCTCAGCCAGGATGG3’), and Vγ(5’GAGGAGCTCCATCAAAGGGCCTT3’). A sequence of the β-actin gene was amplified from single H&RS cells in parallel using two primers (5’ATCATGTTTGTACCTCACC3’ and 5’CATCCCCCTCCTGCAAGACTCAAC3’); two rounds of 30 PCR cycles with 95°C for 40 seconds, 55°C for 1 minute, and 72°C for 3 minutes; and hybridization at 52°C with an 32P end-labeled oligonucleotide (5’GACCTGCTGCCGGCACTGACTGAC3’).

**RESULTS**

**Immunohistology.** Careful microscopic screening failed to show any histomorphologic clue for cytopenosis or surface expression of IgM, CD20, CD3, TCR-α/β, or TCR-γ/δ within the neoplastic population. Mononuclear Hodgkin cells and the Reed-Sternberg giant cells (both cell types essentially CD30+) were scattered throughout the lesions. Because of direct cell/cell contact between H&RS cells and lymphocytes, densely packed T-cell rosettes often lead to an immunoprecipitate (eg, of CD3 antibody) at the interface of surface membranes, giving rise to a linear staining at the cell surface of H&RS cells. This pattern was not accepted as indicative for genuine surface expression of the detected antigen by the tumor cells.

**Validity of the experimental approach.** In the DNA ex-
tracted from peripheral blood lymphocytes (PBLs) of normal donors and tonsillar lymphocytes, rearrangements of all four Vγ families were shown by TCRγPCR (Fig 1), corresponding to a polyclonal pattern of TCR gene rearrangement. The PCR products were approximately 330 bp in length. Amplification of DNA from a T-cell line (Jurkat) yielded two bands, corresponding to a biallelic rearrangement of the TCRγ gene (data not shown). Rearranged DNA (Vγ1/Jγ) could be amplified in 9 of 10 reaction tubes with 1 cell of the T-cell line L735 each and in 9 of 10 reactions with 3 L735 cells each and visualized after hybridization with an internal oligonucleotide (Fig 2A and B). Similarly, single cells prepared from bone marrow or peripheral blood of eight T-cell acute lymphoblastic leukemias (T-ALL), one T-cell chronic lymphocytic leukemia (T-CLL), and one Sezary syndrome were isolated by micromanipulation from cytocentrifugation slides. Rearrangements were detected in all cases (10/10). Biallelic rearrangements of the TCRγ gene were detected in 6 patients. The following Vγ and Jγ genes were found to be rearranged: Vγ1 in 3 cases, Vγ2 in 5 cases, Vγ3 in 4 cases, Vγ4 in 4 cases, J in 12 cases, and Jp in 4 cases. After determining the individual rearrangement pattern, amplified DNA from all patients was separated on a single gel and hybridized to a mixture of all four internal hybridization probes (Fig 3).

The results from experiments with single cells of T-cell leukemias, Sezary’s syndrome, and tonsillar lymphocytes, as well as similar experiments using a T-cell line (L735) and PBLs, show that the method is sensitive and reliably detects rearrangements of the TCRγ genes in single cells of known lymphoid origin.
Single-cell PCR on H&RS cells. TCRγ PCR (13/17 cases; Table 1) was performed in reaction tubes containing 3 isolated H&RS cells for each of the TCRγV PCRs. Each reaction was repeated four times. β-Actin PCR from single H&RS cells as well as TCRγPCR from T cells (CD7+, CD2+) picked from the same lymph node suspension and L735 single cells, respectively, served as positive controls. After hybridization to an internal oligonucleotide, no signals were observed in the reactions performed on H&RS cells. In contrast, β-actin DNA could be amplified from single H&RS cells of all 13 cases of HD included in this study (Fig 4) and rearranged TCRγ genes were successfully amplified from single surrounding lymphocytes of the same cases (data not shown). This shows that the TCRγ genes were not rearranged in the H&RS cells examined by us.

DISCUSSION

Rearrangements of the T-cell receptor genes are characteristic of lymphoid cells of T-cell lineage and occur early enough in lymphoid ontogeny to be detectable in all known lymphoid neoplasms of T-cell origin. Our failure to detect these rearrangements in all H&RS cells examined provides evidence that HD does not represent a clonal proliferation of T lymphocytes or T-lymphoid precursors. Our experience with frozen sections of lymph nodes affected by HD showed that cryostat sections cannot be used as a source of DNA exclusively derived from H&RS cells, because of a high risk that DNA from the reactive cells is contaminated over the frozen section by the cryostat knife. In addition, it cannot be excluded that a reactive cell lying underneath the H&RS cells is picked and transferred together with the H&RS cells into the reaction tube. Therefore, we had to restrict our study to viable H&RS cells prepared from single-cell suspensions of fresh biopsy material, even though this limited the number of cases available for PCR analysis. Our results show that the methodologic approach described here is useful in determining the clonality and origin of lymphocytes and related cells from tissues in which the cells of interest represent only a minority of the cellular environment.

The marker for T-cell commitment chosen by us (the γ-
chain of the TCR complex) is usually rearranged early during lymphoid development, long before other genes (eg, the β chain of the TCR) become functional. Our PCR strategy is highly sensitive and reliable, because we could show the presence of rearranged TCR genes in single neoplastic cells from cases with T-cell leukemia, T-CLL, and Sezary's syndrome. By picking 3 instead of 1 cell per reaction and repeating each experiment four times, we have provided a "safety margin" to correct for the possible failure of the PCR. Positive controls were performed in each single-cell experiment. Also, DNA PCR from single cells has been successfully used by us to show the presence of Ig heavy chain gene rearrangements in B-cell non-Hodgkin's lymphoma and of Epstein-Barr virus DNA in H&RS cells. We conclude from our studies that a rearrangement of the TCRγ gene occurs only rarely, if ever, in H&RS cells. Therefore, the neoplastic cells in HD in B and T antigen-negative cases do not seem to originate from T cells. However, because only complete rearrangements without major mutations (which have been described for IgH, but not for TCR genes) or deletions are detected with our PCR approach, we cannot exclude the possibility that H&RS cells are derived from cells that have an incomplete or illegitimate rearrangement of the TCR genes.

Immunohistology did not lend any support to the concept of a B- or T-lymphoid nature of the H&RS cells in the series of consecutive cases described here, because neither T- nor B-cell antigens (including TCR proteins) were expressed on the H&RS cell surface. So far, we were not able to examine a case of HD with T-cell antigen receptor expression, an obviously rare subentity of HD. Apart from the typical nodular paragranuloma subtype that is regarded to be a B-cell neoplasm, no consistency with regards to B- or T-cell markers has been observed for the other subtypes. This finding led an expert panel to refrain from a definite statement on the exact nature of the tumor cells in mixed cellularity, nodular sclerosis, and lymphocyte depletion. However, some immunologic studies have been interpreted as support for the hypothesis that H&RS cells are derived from lymphocytes and that HD presents a bona fide malignant lymphoma. It must be kept in mind that expression of lymphoid surface markers has been observed in a number of hematologic neoplasms that are definitely not of lymphoid origin.

The problem with previous results obtained using molecular biologic methods is that the detection of TCR and IgH gene rearrangements in DNA extracted from whole lymph node tissue involved by HD may simply be caused by the presence of oligoclonal lymphoid populations with appropriate rearrangements and cannot necessarily be attributed to the presence of these rearrangements in the H&RS cells themselves. This is also true for a recent report that suggested a B-cell origin in some cases of classical HD, but used whole sections for the investigation of IgH rearrangements. The controversy surrounding a possible B-cell origin for H&RS cells of some cases of classical HD can only be solved by single-cell PCR, which so far has failed to show rearranged IgH genes in H&RS cells lacking B- or T-antigen expression. The approach presented here is able to yield conclusive results because only H&RS cells are subjected to PCR. The single-cell PCR examination of T lymphocytes surrounding H&RS cells will show whether the clonal patterns observed by some investigators are derived from oligoclonal populations within the inflammatory infiltrate.

The lack of detectable TCRγ rearrangements and the results from studies of the expression profile of single H&RS cells at the mRNA level point towards the derivation of H&RS cells from hematopoietic cells that have not entered the pathway of T-lymphocyte differentiation but are capable of using gene expression programs characteristic of different hematopoietic lineages.

In summary, we have found no evidence to support the hypothesis that classical HD represents a malignant disease of T-cell origin. Examination of further cases of HD, including atypical and rare cases with T- and B-cell antigen expression, as well as single-cell examination of "bystander" lymphocytes from HD-affected lymph nodes, will show whether this is true for all cases of HD or whether there are subgroups that can be defined on the basis of molecular studies.

NOTE ADDED IN PROOF

While this report was in press, Küppers et al (Proc Natl Acad Sci USA 91:10962, 1994) and Delabie et al (Blood 84:3291, 1994) reported on clonal Ig gene rearrangements in Hodgkin and Reed-Sternberg cells of a total of 5 cases of lymphocyte-predominant and 2 cases of other subtypes of Hodgkin's disease.

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