Detection of Clonal Hodgkin and Reed-Sternberg Cells With Identical Somatically Mutated and Rearranged VH Genes in Different Biopsies in Relapsed Hodgkin’s Disease

By Martina Vockerodt, Marta Soares, Holger Kanzler, Ralf Küppers, Dieter Kube, Martin-Leo Hansmann, Volker Diehl, and Hans Tesch

Hodgkin’s disease (HD) represents a malignant lymphoma in which the putative malignant Hodgkin and Reed-Sternberg (H-RS) cells are rare and surrounded by abundant reactive cells. Single-cell analyses showed that H-RS cells regularly bear clonal Ig gene rearrangements. However, there is little information on the clinical evolution of HD in a given patient. In this study, we used the single-cell polymerase chain reaction (PCR) to identify H-RS cells with clonal Ig gene rearrangements in biopsy specimens of patients with relapsed HD. The obtained clonal variable region heavy-chain (VH) gene rearrangements were used to construct tumor-clone-specific oligonucleotides spanning the complementarity determining region (CDR) III and somatically mutated areas in the rearranged VH gene. A number of biopsies were obtained during a period of 3 years from two HD patients. H-RS cells with identical VH rearrangements were detected in two separate infiltrated lymph nodes from one patient with nodular sclerosis HD. In a second patient with mixed cellularity HD subtype, clonal VH rearrangements with identical sequences were detected in infiltrated spleen and two lymph node biopsies. Despite the high sensitivity of the PCR method used (one clonal cell in 10⁵ mononuclear cells), residual H-RS cells were not found in peripheral blood, leukapheresis material, purified CD34⁺ stem cells or bone marrow. The results show that different specimens from relapsed patients suffering from classical HD carry the same clonotypic IgH rearrangements with identical somatic mutations, demonstrating the persistence and the dissemination of a clonal tumor cell population. Thus, PCR assays with CDRIII-specific probes derived from clonal H-RS cells are of clinical importance in monitoring the dissemination of HD and tumor progression and could be useful for analysis of minimal residual disease after autologous stem cell transplantation.

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MATERIALS AND METHODS

Patient Samples

Patient no. 1. In December 1986, a 22-year-old woman presented with HD of the nodular sclerosis subtype. Clinical staging showed stage...
IIA with a bulky mediastinal tumor. The patient was treated according to the HD1 protocol of the German Hodgkin Study Group with two cycles of COPP/ABVD (cyclophosphamide, vincristine, procarbazine, prednisone, adriamycin, bleomycin, vinblastine, dacarbazine) followed by consolidating radiotherapy. The patient went into complete remission.

In June 1992, a first relapse was diagnosed with involvement of bone marrow and enlarged mediastinal, inguinal, axillary, and iliac lymph nodes. The treatment included four cycles of salvage chemotherapy, under which the patient achieved complete remission. In February 1995, a second relapse occurred with enlarged retroperitoneal and mediastinal lymph nodes. Histological examination confirmed the relapse in an inguinal lymph node. The patient was again treated with salvage chemotherapy and went into partial remission.

In January 1996, a peripheral stem cell apheresis was performed. In February 1996, the patient relapsed again with enlarged cervical lymph nodes and suspicious liver and bone marrow infiltration. Histological examination showed HD infiltration in a cervical lymph node. The patient was again treated with polychemotherapy and went into partial remission.

**Patient no. 2.** In March 1990, a 28-year-old woman was diagnosed with HD mixed cellularity subtype, stage IJV. The patient presented with enlarged supravacular, axillary, mediastinal and inguinal lymph nodes, and pulmonary lesions. The patient was treated with three cycles of MOPP/ABVD (nitrogen mustard, vincristine, procarbazine, prednisone, adriamycin, bleomycin, vinblastine, dacarbazine), one cycle of COPP/ABVD, and consolidating radiotherapy and went into complete remission.

In September 1993, a first relapse occurred with mesenteric and paraaortal lymph nodes. The patient was treated with four cycles of salvage chemotherapy, followed by high-dose chemotherne and autologous stem cell transplantation. In July 1995, a second relapse was detected with mesenteric and axillary lymph nodes and spleen involvement. A laparotomy with splenectomy was performed and histological examination confirmed the infiltration of the spleen and the mesenteric lymph node with H-RS cells. The patient was subsequently treated with involved field radiotherapy. In June 1996, progressive lymphomas were detected in the mediastinum, the axilla, and the paraaortal region, but not in the bone marrow. After treatment with polychemotherapy, the patient achieved partial remission.

**Cell and Tissue Samples**

Granulocyte colony-stimulating factor (G-CSF)–mobilized leukapheresis material, heparinized peripheral blood mononuclear cells (PBMCs), and bone marrow aspirates were collected from the patients. Frozen and paraffin-embedded tissue sections were obtained from the Department of Pathology of the University of Cologne (Cologne, Germany). Peripheral blood was also obtained from healthy volunteers, and paraffin-embedded tissue sections were obtained from the Department of Pathology of the University of Cologne (Cologne, Germany). DNA was isolated from the cell mixtures containing 10 to 104 L428 cells as described above.

**Sequence Analysis**

PCR products were gel-purified and directly sequenced using the Ready Reaction DyexDeoxy Terminator cycle sequencing kit (Perkin Elmer, Weiterstadt, Germany). Sequence products were analyzed on an automatic sequencing system (ABI377; Applied Biosystems, Weiterstadt, Germany) and compared with the Genbank data library using DNAsis software (Version V3.6; Pharmacia).

**DNA Preparation**

High molecular weight DNA was isolated using a DNA extraction kit (Qiamp Blood/Tissue kit; Qiagen, Hilden, Germany). For paraffin-embedded tissue sections, first incubated with xylol to remove paraffin and incubated overnight with proteinase K. A as a control for cross-contamination of the analyzed samples with PCR products, DNA extraction of mononuclear cells from healthy volunteers or tissue sections from irrelevant donors was performed in parallel to all patient samples.

**Estimation of the Assay Sensitivity**

Cells of the HD-derived cell line L428 were mixed with 107 PBMCs of a healthy volunteer and serial dilutions of lymphoma cells in 106 PBMCs were performed. DNA was isolated from the cell mixture containing 10 to 104 L428 cells as described above.

**Clone-Specific PCR and Southern Blot**

For each V<sub>H</sub> gene, one forward primer, spanning a somatically mutated area of the sequenced V<sub>H</sub> gene (CDRI for patient no. 1 and CDRII for patient no. 2), and two reverse CDRIII-specific primers were designed. For L428 cells, the V<sub>H</sub>SFRI was used for amplification. The primer sequences and annealing temperatures were as follows: L428rev1, 5′ CCGGGAGACAACTCCCCCCTCATC 3′; and L428rev2, 5′ AACCTCCCCCATCTGACTATG 3′ (1.5 mmol/L MgCl<sub>2</sub>; annealing temperature, 63°C); NSfor, 5′ TACACCTTCACAACCATGGTC3′; NSrev1, 5′ GAAGTCAGTGAGTACATCCATA 3′; and NSrev2, 5′ ATCCATATAATTACACAGGTGGC 3′ (2 mmol/L MgCl<sub>2</sub>; annealing temperature, 61°C); MCfor, 5′ GAGACTTCAAGGACAGCTCACTCAT3′; MCrev2, 5′ GTGTGGACAGTTGTGATCCATGCA 3′ (1.5 mmol/L MgCl<sub>2</sub>; annealing temperature, 63°C). The first and second round of amplification was performed as described, using 1 µg of DNA and 0.125 µmol/L of the primers mentioned above. After DNA amplification for 40 cycles, 1 µL of the PCR product was reamplified for 40 cycles using the same forward primer and the corresponding nested CDRIII reverse primer. The reactions were performed in five replicates to avoid false-negative results in cases when the amount of target molecules was at the detection limit of 1 tumor cell in 10<sup>6</sup> mononuclear cells. All PCR products were separated on a 2% agarose gel and then transferred to a nylon membrane (Hybond-N<sup>+</sup>; Amersham Buchler, Braunschweig, Germany) using an alkali transfer buffer (0.4 mol/L NaOH/1.0 mol/L NaCl). Hybridization with a fluorescein isothiocyanate (FITC)-labeled oligonucleotide was performed at 42°C overnight. The following oligonucleotides were used for hybridization: L428tern, 5′...
CACCAACTGTTGCATCTTGTTGG 3

8

; NSintern, 5

AAA TACTCA-

CAGACGTTTAAAGAC 3

8

; and MCintern, 5

GTGCGGACGA TTT-

CA TGGGGACA 3

8

. The washing steps were performed at 42°C with

5

3

SSC/0.1% sodium dodecyl sulfate (SDS) for 5 minutes twice. The
detection of specific PCR products was performed following the
protocol for the ECL detection system (Amersham Buchler). Filters
were exposed to x-ray film (AR, Kodak, Integra Bioscience, Fernwald,
Germany) for 10 seconds to 10 minutes.

RESULTS

Estimation of PCR Sensitivity

The detection limit of the PCR approach was estimated by
mixing PBMCs of healthy volunteers with different amounts of
cells from the HD cell line L428. This cell line harbors a
rearranged VH 5 gene (M.V., unpublished data).

DNA samples of 1 µg containing the equivalent of 0.2 to 200
L428 cells in 2 × 10^5 PBMCs were analyzed by seminested
PCR with a V H 5 FRI primer and two nested CDRIII-specific
primers (L428rev1/rev2). Reactions containing water or PBMC
DNA of a healthy volunteer served as negative controls.
Southern blot hybridization with a specific internal oligonucleo-
tide (L428intern) and ECL visualization was performed with all
reactions to show specificity of the amplificates. As shown in
Fig 1, specific amplification products of 296 bp were visible on
ethidium bromide-stained agarose gels and the corresponding
Southern blot at a dilution corresponding to 1 tumor cell in 10^5
mononuclear cells after the first round of amplification with the
primers V H 5 and L428rev1. The second amplification cycle
with the nested CDRIII-specific primer L428rev2 led to an
increase of the amount of specific PCR products corresponding
to the dilution steps of 10^2 to 10^5 but did not increase the

Table 1. Summary of Single-Cell Analysis of Two Cases for V Gene Rearrangements

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Experiment No.</th>
<th>Cells Positive for V Rearrangements After PCR With Different Forward Primers</th>
<th>PCR Products</th>
<th>Rearrangements</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1a</td>
<td>ND 13/17 —* ND 13 13 13 V H 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1b</td>
<td>ND 7/14 —* 7/14 7 7 7 V H 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>LN 0/13</td>
<td>0/13 —* 3/13 3 3 3 V H 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>LN 0/7</td>
<td>0/7 —* 3/7 3 3 3 V H 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>spleen 0/10</td>
<td>0/10 —* 2/10 2 2 2 V H 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1a†</td>
<td>ND 0/8 T cells —* ND 8 8 5 V H 3,3 V H 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1b</td>
<td>ND 0/8 T cells —* 0/8 T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>LN 0/4 buffer</td>
<td>0/4 T cells —* 0/10 buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>LN 0/6 T cells</td>
<td>0/6 T cells —* 0/6 T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>spleen 0/5 T cells</td>
<td>0/5 T cells —* 0/5 T cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

H-RS cells were analyzed together with B and T cells coded in a blinded fashion from two patients with HD. The number of PCR products obtained and sequenced from H-RS and T cells for the first and the second experiment is indicated. Clonally independent V H gene rearrangements of various V H gene families were obtained from control B cells (not shown). Repeated rearrangements indicate clonally related rearrangements; unique rearrangements indicate rearrangements amplified once and not related to any other rearrangement. The sequences obtained from H-RS cells of both patients were deposited in the Genbank under the accession numbers AJ 007389 and AJ 007390.

Abbreviations: LN, lymph node; ND, not determined.

*PCR was performed either with V H FRI or with V H FRII primers.
†In this experiment, a B-cell–specific antibody was eventually used for the “T-cell staining.”
sensitivity to the dilution step of $10^{-6}$, because the dilution of $10^{-6}$ did not contain any target molecules for amplification. The same results were found for five replicates (not shown).

Identification of Tumor-Specific V<sub>H</sub> Rearrangements in Single H-RS Cells of HD Patients

Two independent micromanipulations and single-cell PCRs (experiment no. 1a/b) were performed of single CD30<sup>+</sup> H-RS cells and control CD3<sup>+</sup> T cells picked from a frozen section of an inguinal lymph node that was biopsied in March 1995 (LN 3/95) from patient no. 1 (Table 1). Overall, 20 of 31 H-RS cells yielded PCR products with VH 1 primers. Sequence analysis showed an identical sequence for all H-RS cells (Fig 2A). The heavy-chain rearrangement (NSVH1) is potentially functional and uses a J<sub>H</sub> 4 gene. Comparison to the most homologous germline gene DP14 showed that the rearranged gene is highly mutated. A D<sub>H</sub> gene could not be identified. A second micromanipulation was necessary, because clonally unrelated IgH rearrangements were obtained from six of eight T cells in experiment no. 1a. We speculate that a specific antibody for B-cell antigens was erroneously used for the “T-cell staining.” In experiment no. 1b, all control CD3<sup>+</sup> T cells and buffer controls were negative for V<sub>H</sub> gene rearrangements.

As shown in Table 1, 13 CD15<sup>+</sup> H-RS cells were analyzed by single-cell PCR picked from a frozen section of a mediastinal lymph node (LN 7/95) that was biopsied in July 1995 from patient no. 2 (Table 1, experiment no. 2). Because the use of V<sub>H</sub> and V<sub>k</sub> family-specific FRI primers did not lead to any amplification product after the second round of PCR, another set of single cells, which was picked at the same day from the HD-infiltrated frozen lymph node section (LN 7/95) and from a frozen HD-infiltrated spleen section (SP 7/95), was analyzed by single-cell PCR with VH family-specific FRII primers (Table 1, experiment no. 2”). One of seven micromanipulated CD15<sup>+</sup> H-RS cells from the lymph node H-RS cell and 1 of 10 CD30<sup>+</sup> H-RS cells from the spleen section led to an amplified product with the V<sub>H</sub>1 FRII primer. Comparison to the most homologous germline gene (DP-14<sup>22</sup>) showed that this highly mutated...
rearrangement (MCVH1) is rearranged out of frame to the JH3 gene (Fig 2B). We assume that the MCVH1 gene from other H-RS cells was either not amplified or amplified only with reduced efficiency because of mutations at the primer binding sites. Therefore, we screened all micromanipulated PCR product-negative cells again using a clone-specific primer in combination with the JH-Mix for the second round of PCR. Three PCR products were each obtained from experiment no. 28 and experiment no. 288 with identical DNA sequences.

Detection of Clonal H-RS Cells in Different HD Infiltrated Tissues

Twenty-two DNA samples of two HD patients obtained from different tissue and blood samples and different time points of the disease were analyzed using a seminested PCR approach with a clone-specific forward and two nested CDRIII-specific reverse primers followed by Southern blot hybridization with an internal oligonucleotide. In Fig 3, the analyzed DNA samples are depicted schematically. In Fig 4, representative agarose gel electrophoreses and the corresponding Southern blot hybridization of PCR products from the two patients are shown.

Patient no. 1. Fourteen DNA samples (from March 1994 to May 1997) were analyzed. The specific DNA fragment of 249 bp was detected in the DNA sample of the HD-infiltrated lymph node tissue (LN 7/95) and in cervical lymph node taken in February 1996 (LN 2/96) (Fig 4A and B). Sequence analysis of the two obtained PCR products showed identical sequences as compared with the H-RS cells (NSVH1) analyzed by single-cell PCR (Fig 2A). The amplification of DNA obtained from the bone marrow aspirate (BM 3/95) and apheresis material (AP 3/95) taken during the second relapse led to unspecific amplification products of different size (Fig 4A and B). No specific PCR products were detected in apheresis material collected in January 1996 (AP 1/96) and in the bone marrow aspirate (BM 3/96). All bone marrow and blood samples taken at different time intervals remained negative in the PCR analysis.

Patient no. 2. Eight DNA samples (from February 1994 to July 1996) were analyzed. PCR analysis of the DNA sample from fresh spleen cells (SP 7/95) and an axillary lymph node (LN 7/96) showed the presence of the specific tumor-specific amplification product (Fig 4C and D). Sequence comparison presented in Fig 2B showed identical sequences for the DNA sequences obtained from the spleen (SP 7/95), the lymph node tissue (LN 7/96), and micromanipulated single H-RS cells from spleen and lymph node cells (MCVH1). No specific amplification products were found in DNA extracted from apheresis material (AP 9/95), bone marrow aspirate (BM 6/96), and various blood samples obtained at different times of the disease.

DISCUSSION

There is now a general agreement that the putative malignant H-RS cell belongs in most cases of HD to a clonal population of mature B cells. However, little is known about the clinical evolution of the tumor clone in patients with HD in the course of the disease.

We analyzed whether CDRIII-specific DNA probes derived from clonal H-RS cells could be used as a diagnostic marker to follow-up the dissemination of HD in different biopsy specimens over a time period of several years. Our results show that independently derived specimen from relapsed patients suffering from classical HD carry the same clonotypic IgH rearrangements with identical somatic mutations, demonstrating the
persistence and the dissemination of a clonal tumor cell population.

To establish H-RS cell-specific DNA probes, single H-RS cells were micromanipulated from frozen lymph node sections and a spleen section and single-cell PCR was performed to detect rearranged V genes. In both cases, clonally related, highly mutated VH region genes were amplified from single H-RS cells. The detection of rearranged Ig genes in H-RS cells is consistent with earlier studies, where clonally related, highly mutated VH rearrangements were found.5,7,9-12

We find it noteworthy that the clonal rearrangement of single H-RS cells of patient no. 2 was amplified from CD15+H-RS cells of a mediastinal lymph node as well as from CD30+H-RS cells of the spleen, demonstrating an extensive dissemination of this tumor clone. The detection of rearranged Ig genes in H-RS cells is consistent with earlier studies, where clonally related, highly mutated VH rearrangements were found.5,7,9-12

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The detection limit of our PCR and Southern blot approach was estimated in a dilution assay with the HD-derived cell line L428. Our assay could detect only one tumor cell as well as from CD30+H-RS cells of the spleen, demonstrating an extensive dissemination of this tumor clone. The detection of rearranged Ig genes in H-RS cells is consistent with earlier studies, where clonally related, highly mutated VH rearrangements were found.5,7,9-12

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The results of the present report show that the same tumor clone is present at different time points and in different lesions. Sequence analyses showed identical sequences amplified from DNA samples of tissue sections as in single H-RS cells in both patients. This confirms the expansion of clonal cells, which are present in the analyzed tissues with minimum of 1 tumor cell surrounded by 10⁵ nonmalignant cells. There was no indication for ongoing mutations in these VH genes in the dominant tumor clones as described for LP-HD.10-12 Thus, the chemotherapy after the second relapse neither eliminated this tumor clone and prevented the dissemination to other compartments of the lymphatic system nor was the DNA of the analyzed part of the V-region genes altered by point mutations, deletions, or insertions.

The absence of additional mutations within V-region genes under chemotherapy shows that the clone-specific PCR of V region genes is a suitable assay to analyze dissemination and persistence as well as tumor progression in HD patients. Strikingly, in our study, PCR positivity was found in lymph node or spleen samples, whereas at the same time cells of the

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**Fig 3.** PCR-based monitoring of clonal H-RS cells in different blood and tissue samples from two patients with relapsed HD for 3 years from March 1994 to May 1997. Collection of samples analyzed from a patient with NS-HD (patient no. 1) and from a patient with MC-HD (patient no. 2). (□) Specific PCR products were amplified from these samples with clone-specific primers. (○) No PCR products were detected in the DNA samples. (*) Single-cell PCR was performed from a frozen tissue section. Abbreviations: BM, bone marrow aspirate; LN, lymph node tissue; AP, apheresis material; LI, liver tissue; SP, spleen cells; PB, peripheral blood; CD34+, MACS-separated CD34+ cells.
tumor clone were undetectable in bone marrow and apheresis material of both patients. The absence of tumor cells in these samples even at the point of relapse demonstrates that H-RS cells may home primarily to lymphatic organs. It still remains unclear whether the spreading of H-RS cells throughout the body takes place in the blood or in the lymphatic system. The studies of Wolf et al have provided strong evidence that clonogenic cells can circulate in peripheral blood, because they established the cell line L1236 from peripheral blood of an HD patient. However, the dissemination of H-RS cells in bone
marrow and peripheral blood seems to be a rare event in advanced stages of tumor progression.

Upon relapse, both patients were scheduled for high-dose chemotherapy and autologous stem cell transplantation, which may improve the rates of complete remission and disease-free survival.37-39 A concern with the treatment protocols used is that residual tumor cells may be transferred through the stem cell harvests.19-21 It is unknown whether stem cell harvests from the peripheral blood of HD patients might contain lymphoma cells that can lead to a relapse. In the current study, we analyzed four apheresis materials and two preparations of MACS-purified CD34+ cells by PCR and found no contaminating tumor cells, indicating that the tumor burden in the transplants was below the detection limit of our PCR assay of 1 malignant cell in 105 reactive cells. The amplified CDRIII of clonal H-RS cells could now be used as an individual tumor marker to screen stem cell harvests for residual H-RS cells.

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