Expression of Human Recombination Activating Genes (RAG-1 and RAG-2) in Hodgkin’s Disease

By Hans Knecht, David J.L. Joske, Alice Emery-Goodman, Edith Bachmann, Fedor Bachmann, and Bernhard F. Odermatt

The differentiation status of Sternberg-Reed (SR) cells is still not well defined, primarily because of their scarcity in tumor biopsies of Hodgkin’s disease (HD). In this study we have determined the genomic differentiation status of SR cells by quantitation of recombination activating gene (RAG) expression. RAG genes are selectively transcribed in immature lymphoid cells. In B cells they are silent after genomic rearrangement has occurred, whereas in T cells they are downregulated during positive selection of double-positive thymocytes into single-positive cells. RNA from tumor biopsies either with numerous (11 cases) or with few SR cells (16 cases) was assessed by a sensitive reverse transcriptase polymerase chain reaction (RT-PCR) and the results compared with established positive and negative controls. In all except two cases levels of RAG expression were within the range of those determined in negative controls. In both positive cases and in the positive control RAG mRNA was further quantitated by competitive PCR. In cases with abundant SR cells RAG expression was still below that observed in 10^{-5} dilutions of positive controls. These results suggest that SR cells are derived from lymphoid cells, more differentiated than the pre-B or common thymocyte stage, which have already undergone genomic rearrangement. They show the value of assessing RAG expression by RT-PCR in the characterization of lymphoid malignancies.

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THE HUMAN recombinase activating genes (RAG-1 and RAG-2), which recently have been cloned, are necessary for the rearrangement of Ig and T-cell receptor (TCR) genes. RAG-1 and RAG-2 are expressed in early thymocytes, in acute lymphoblastic leukemia of T- and B-cell type (T-ALL and pre-B-ALL) but not in mature T- and B-cell malignancies or corresponding cell lines. Two findings suggest that RAG mRNA expression is a valuable new marker for determination of the genotypic maturation status of lymphoid cell populations.

There is now accumulating phenotypic and genotypic evidence that the putative tumor cells in Hodgkin’s disease (HD), the Sternberg-Reed (SR) cells, are of lymphoid origin. However, the maturation status of the SR cells is still a matter of discussion, particularly whether they derive from immature lymphoid cells or from already primed (rearranged) B cells or T cells. Because the assessment of RAG mRNA expression offers a new tool in the determination of the genotypic maturation status of lymphoid malignancies, we chose this approach in HD. Using a sensitive reverse transcription polymerase chain reaction (RT-PCR) we analyzed the RAG mRNA expression in 27 HD cases, including 6 cases with massive infiltration by SR cells. Furthermore, in positive cases and in T-ALL control case, RAG mRNA expression was quantitated by competitive PCR (C-PCR).

MATERIALS AND METHODS

Tissue selection. Snap-frozen tissue samples from 11 diagnostic lymph node biopsies with numerous SR cells (six cases with >25% SR cells, five cases with 10% to 25% SR cells), collected over a 9-year period and stored at −70°C, were analyzed together with 16 randomly chosen HD cases showing few SR cells. Histologic classification, DNA rearrangement studies, and Epstein-Barr virus (EBV) DNA status of all but one case have recently been reported in this journal.

RNA preparation. Total cytoplasmic RNA was isolated from fresh frozen tissue by caesium chloride centrifugation according to Chirgwin et al with minor modifications. RNA solutions were adjusted to a concentration of 100 ng/μL in Tris-EDTA, 5 mM MgCl2, 0.05 M Tris, pH 7.5, and stored at −70°C until used.

RT-PCR. RT-PCR was performed with the GeneAmp rTth reverse transcriptase RNA PCR Kit (Perkin Elmer Cetus, Norwalk, CT). The manufacturer’s amplification protocol was applied with the following modifications: reverse transcription was performed in a total volume of 10 μL with a final concentration of the downstream primer of 1.25 μM/L. Subsequent PCR was performed in a total volume of 50 μL with a final concentration of each primer of 0.25 μM/L and a final MgCl2 concentration of 2.5 mM. After 25 amplification cycles a sample of 10 μL was taken and replaced by fresh rTth DNA polymerase (final concentration 2.5 U/50 μL) diluted in PCR amplification buffer followed by a further 25 amplification cycles. The RT-PCR was run with a HYBAID intelligent heating block, model HIB10 (Hybaid Ltd, Teddington, Middlesex, UK). Technical details of prevention of contamination, gel preparation, and Southern transfer have been reported in detail elsewhere.

Cycling. Reverse transcription: 70°C for 5 minutes, followed by 60°C for 5 minutes, followed by 70°C for 10 minutes. (The optimal annealing temperature [Tm] for the downstream primer for the positive control reaction [DM152] is 70°C, the Tm for the downstream primer for RAG-1 and RAG-2 is 60°C.) PCR of cDNA: Pre-cycle; 95°C for 2 minutes. Cycles 1 to 25 and 26 to 50; strand separation at 93°C for 1 minute, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute. Between cycles 25 and 26 the reaction was kept at 72°C for 5 minutes.

Reverse transcription primers. DM152, identical to the interleukin-1α (IL-1α) primer from Wang et al, was used for the positive control reaction. HK32, identical to the interleukin-1α primer from Wang et al, was used for the positive control reaction. HK32, 5'-AGGATATACCAAACTGGT-3', was used for the positive control reaction. HK32, 5'-AGGATATACCAAACTGGT-3', was used for the positive control reaction.
homologue murine sequence at positions 1328 to 1327 (AT for CC) and at position 1319 (T for A).3,5

PCR primers. DM151, 5'-GTCTCTGAAATCAGAAATCCTT-CATC-3', is identical to the IL-1α 5' primer.3,5 HK31, 5'-GC-CATTGAGGAGCATGGAATT-3', corresponds to the RAG-1 sequences 2135 to 2155.2 HK28, 5'-TTGGCATATACCGGACA-T-3', corresponds to the RAG-2 sequences 1141 to 1160.3

Internal oligonucleotide probes. Specificity of the amplified products obtained with the HK31/32 set of primers was confirmed with a 32P-end labeled internal probe, HK32 Hyb, extending from position 2450 to 2470 (5'-GAGACAGTCCCTTCCATAGAT-3') of the RAG-1 gene. The specific internal oligonucleotide probe for amplified RAG-2 sequences, HK29 Hyb (5'-GAGTCTTCAAAGGGAGTGG-3') extends from position 1297 to 1278 of the RAG-2 sequence.3 Because this oligonucleotide sequence shows a complete homology between humans and mice5 it was also used as RT-PCR primer for a positive murine RAG-2 mRNA expression control. The concentration of the 32P-end labeled internal probes was 1 to 1.5 × 10^6 cpm/mL hybridization solution. The specificity of the amplification products obtained with either the HK31/32 set of primers (RAG-1) or the HK28/29 set of primers (RAG-2) is shown in Fig 1.

RAG-controls. Positive controls for RAG mRNA expression were RNA from a human T-ALL differentiation stage II (TdT+, CD1+, CD2+, CD3-, CD4-, CD5+, CD7+, CD8-) and RNA from mouse thymus. To confirm that the positive results were caused by cDNA amplification, RNA samples of the controls were also assessed by PCR without prior reverse transcription. As negative controls for RAG mRNA expression we used RNA from two cases of hairy cell leukemia (HCL), from a case of diffuse lymph node hyperplasia (LN react) and from the Namalwa cell line (Nam).

Fig 1. Specificity of RAG-1 and RAG-2 amplification products. Genomic DNA from T-ALL (AL), HCL (HC), and placenta (Pla') was amplified with the HK31/32 set of primers. Pla' and LTA (RAG-2 probe of 640 bp used in reference 4) was amplified with the HK28/29 set of primers. LTp is a small quantity of the unamplified RAG-2 probe used in reference 4 (RAG-2 plus). Genomic DNA from Raji (Ra) and Jijoye (JI) cell lines was amplified with sets of primers specific for the EBV BMRF 1 and LMP region, respectively. (A) Gel electrophoretic profile of the amplification products. (B) Southern blot of the gel shown in (A) hybridized with the RAG-1-specific internal oligonucleotide probe HK32 Hyb. (C) Southern blot shown in (B) was washed and rehybridized with the RAG-2-specific internal oligonucleotide probe HK29 Hyb.

Fig 2. Sensitivity of RT-PCR. Gradual dilutions of thymus RNA and of pAW 109 RNA copies were reverse transcribed and amplified with the HK31/32 and DM 151/152 sets of primers, respectively. Pr indicates input of corresponding primers only. Subscript black dot indicates RNA samples amplified by PCR without prior reverse transcription. (A1) Gel electrophoretic profile of the amplification products after 25 cycles. Specific bands are detectable in the thymus lanes 2, 20, and 200 ng. Note absence of specific bands in the pAW 109 lanes. (A2) Southern blot of the gel shown in (A1) hybridized with the RAG-1-specific internal oligonucleotide probe HK32 Hyb. Specific signals corresponding to the initial RNA concentration are identified. (B1) Gel electrophoretic profile of the amplification products after 50 cycles. Specific bands are now also seen in the control template lanes. The intensity of the 308-bp IL-1 bands corresponds well with the initial template concentration. (B2) Southern blot of the gel shown in (B1) hybridized with the RAG-1-specific internal oligonucleotide probe HK32 Hyb. Strong specific signals are seen in all three dilutions of thymic input RNA.

Quality control of target RNA samples was performed by successful RT-PCR with an HLA-DQa set of primers.

Quantitation of mRNA by C-PCR. The GeneAmp rTth reverse transcriptase RNA PCR Kit contain a positive synthetic control RNA template transcribed from an insert in the plasmid pAW 109. Reverse transcription of a given quantity of control RNA template followed by cDNA PCR results in generation of a 308-bp amplification product whose amount is directly proportional to the quantity of input RNA.22 In our system with 50 amplification cycles, 10^2 copies of the synthetic control RNA template (called interleukin-1 [IL-1]) still yielded a clearly detectable 308-bp amplification product on gel electrophoresis (Fig 2). However, the simultaneous amplification of this standard cDNA and target cDNA does not allow the determination of the approximate initial number of cDNA copies in the target sample because the amplification efficiency for each cDNA depends on the specific primer sequences and the length of the templates.

More accurate quantitation of mRNA is obtained by the C-PCR as shown by Gilliland et al.17 In this system serial dilutions of competitor DNA (differing from the cDNA of interest by a restriction site or a small intron) are coamplified (ie, in the same...
RESULTS

The pattern of RAG expression in controls and HD cases is summarized in Table 1. The highest level of RAG-1 mRNA was observed in mouse thymus and the human T-ALL case. In HD, only one case (with few SR cells) showed significant RAG-1 mRNA expression (Fig 3). Interestingly, this EBV DNA-positive case had a clonal rearrangement for $\lambda$ light chain. In this case and in the T-ALL control RAG-1 mRNA expression was quantitated by C-PCR. As shown in Fig 4 the T-ALL case had $>10^5$ specific cDNA templates transcribed per microgram RNA, the HD case had about $10^4$. All other HD cases, including those with numerous SR cells, lacked significant RAG expression. The mature B-cell line Nam, reported to be negative for RAG-1 expression when analyzed by Northern blotting, showed a weak RAG-1 expression (Table 1); this expression was still stronger than that identified in one HD case with abundant SR cells (Fig 5). In this case and in Nam cells transcription of RAG-1 mRNA was $<2 \times 10^3$ templates per microgram RNA as determined by C-PCR. In the six cases with abundant SR cells there was no correlation between RAG expression or Ig or TCR gene rearrangement and EBV DNA status: one case had no detectable EBV DNA, but clonal rearrangement for $\gamma_H$; five cases were without clonal rearrangements, three with only a few EBV genomes ($\leq 10^3$) and two with numerous viral copies ($\geq 10^4$). In our RT-PCR system RAG-2 expression was not observed at all in HD.

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>No. of Cases</th>
<th>cDNA Templates Transcribed</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-ALL</td>
<td>1</td>
<td>++ +</td>
</tr>
<tr>
<td>Mouse thymus</td>
<td>1</td>
<td>+ + + +</td>
</tr>
<tr>
<td>HCL</td>
<td>2</td>
<td>--</td>
</tr>
<tr>
<td>LN react</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td>Namalwa</td>
<td>1</td>
<td>+ --</td>
</tr>
<tr>
<td>HD SR cells</td>
<td>&gt;25%</td>
<td>6 -- (5) -- (6)</td>
</tr>
<tr>
<td></td>
<td>10% to 25%</td>
<td>5 --</td>
</tr>
<tr>
<td></td>
<td>&lt;10%</td>
<td>16 -- (15) -- (16)</td>
</tr>
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Numbers in parentheses refer to subsets of the total.

DISCUSSION

The results of this study indicate that RAG-1 and RAG-2 are typically not expressed in HD. We found that cases with massive infiltration by SR cells (>25%), when assessed with a very sensitive RT-PCR method, lacked RAG expression or showed expression levels inferior or equal to those of the negative controls. We conclude that RAG-1 and RAG-2 expression is very low or absent in SR cells. As shown by Northern blot analysis RAG-1 and RAG-2 are synergistically expressed in pre-B- and pre-T-cell lines, but not in mature B- and T-cell lines. Coexpression of RAG-1 and RAG-2 is also observed in cortical thymocytes during productive TCR rearrangement, but is downregulated in medullary thymocytes; RAG expression is terminated when double-positive thymocytes (CD4+,CD8+) undergo positive selection into single-positive cells (CD4+ or CD8+) through interaction of their TCR with the major histocompatibility complex. Analyzing a large series of acute leukemias with a sensitive RT-PCR method, Bories et al recently reported strong RAG expression in T-ALL and pre-B-ALL. However, no RAG expression was observed in slg+ ALL (Burkitt's type), chronic lymphocytic leukemia (B-CLL), and nodular lymphomas. These data suggest that RAG expression is a highly specific marker of the genotypic maturation status of both B and T cells. A dissociation between genotypic and phenotypic differentiation is observed in SR cells and HD-derived cell lines. Corresponding patterns are identified in EBV-infected pre-B-cell lines where germine configuration of the $\gamma$ genes is associated with surface markers of late B cells. In the light of these similarities and the recent identification of EBV DNA in SR cells, Stein et al
hypothesized that SR cells were derived from immature lymphoid cells infected by a viral pathogen inducing phenotypic maturation. However, the findings reported in the present study suggest that the SR cells in most HD cases are derived from more mature lymphoid cells that have already undergone the V(D)J rearrangement because: (1) no significant level of RAG expression was observed in cases with abundant SR cells; (2) in the one case with clonal JH rearrangement no EBV DNA potentially blocking the...
rearrangement machinery could be identified; (3) in three further cases, all nonclonal, with massive infiltration by SR cells (>10^4 per μg DNA). EBV genomes were scarce (≤10^2 per μg DNA) and therefore very unlikely to be a transforming element interacting with Ig gene rearrangement. In such cases outgrowth of clonal SR populations has been reported with disease progression. In the light of our findings this is best explained by Initially mature oligoclonal/polyclonal (rearranged) SR populations with subsequent growth advantage of one clone. Lack of RAG expression in SR cells is also consistent with a very early B-cell genotype before recombination activation as observed in the LyD9 cell line. However, it is quite unlikely that SR cells belong to this cell type because a characteristic SR cell marker (Ki-1) is not identified in fetal bone marrow and liver known to harbor such early lymphoid progenitors. The very low RAG expression identified in some cases (as little or less than that observed in the negative controls) probably results from illegitimate transcription and minimal DNA contamination. However, very low copy numbers of RAG mRNA could result either from the presence of single cells with a high RAG expression or from very few cells with low RAG expression. Recently, for example, it has been shown in mice that RAG expression is also observed in a very small population of mature, slgM-positive B cells (<0.1% of bone marrow cells). One case, characterized by a few SR cells, λ light chain gene rearrangement and significant RAG-1 expression remains unexplained. We have no means of detecting whether RAG transcription truly occurred in clonally rearranging SR cells, or if it was related to an early lymphoid clone unrelated to SR cells. In this case and the case with weak RAG expression the origin of the transcripts could be localized by in situ hybridization studies.

In summary, our study suggests that SR cells are derived from lymphoid cells beyond the pre-B or common thymocyte stage. It represents a novel approach in the characterization of the SR cells and should incite further analysis of HD cases with numerous SR cells and uncommon genotype.

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REFERENCES


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