CD8$^+$ T Cells in Hodgkin’s Disease Tumor Tissue Are a Polyclonal Population with Limited Clonal Expansion but Little Evidence of Selection by Antigen

Klaus Willenbrock,* Axel Roers,† Birgit Blöbaum,* Klaus Rajewsky,† and Martin-Leo Hansmann*

From the Institute for Pathology,* University of Frankfurt, Frankfurt am Main; and the Institute for Genetics,† University of Cologne, Cologne, Germany

A minor component (about 25%) of lymphocytes in Hodgkin’s disease (HD) are CD8$^+$ T cells. It is unclear whether the presence of these cells reflects an antitumor cytotoxic response. The goal of the present study was to investigate clonal composition and the T cell receptor (TCR) β repertoire of the CD8$^+$ T cell population in HD. Single CD8$^+$ cells were micromanipulated from frozen tissue sections of lymph nodes affected by primary HD and subjected to single target amplification of TCRβ gene rearrangements. Sequence analysis of the V region genes revealed the presence of expanded CD8$^+$ T cell clones in all three cases analyzed. Most of these clonal expansions accounted for less than 10% of the CD8$^+$ T cell population. In one case, 30% of the CD8$^+$ T cells belonged to one or two clones. Comparison of V region sequences, however, did not provide evidence that the micromanipulated CD8$^+$ cells were sampled from a population that was selected for particular antigen specificities. No obvious biases in TCR Vβ and Jβ gene segment usage or CDR3 length distribution were found. Similarities of CDR3 amino acid sequences as found in selected CDR3 structures were rare. These results suggest that, like CD4$^+$ T cells, CD8$^+$ T cells may also be recruited into the tumor tissue in an antigen-nonspecific manner. (Am J Pathol 2000, 157:171–175)

In classical Hodgkin’s disease (HD), the B-cell-derived tumor cells with characteristic morphology known as Hodgkin-Reed-Sternberg (HRS) cells account for only a small fraction of the cells in the tumor. They are surrounded by a polymorphous infiltrate composed mainly of T lymphocytes but also of a variable admixture of histiocytes, eosinophils, and plasma cells. Most T cells in HD tumor tissue are CD4$^+$. These cells densely surround HRS cells. The finding that HRS cells secrete the CD4$^+$ T cell attractant thymus and activation-related chemokine (TARC) and the polyclonal nature of the CD4$^+$ T cells rosetting around HRS cells have lent support to the view that CD4$^+$ T cells are recruited into the tumor tissue in an antigen-nonspecific fashion. Besides CD4$^+$ T cells, a variable fraction of cells in HD tumor tissue are CD8$^+$ cytotoxic T lymphocytes (CTL) expressing granzyme B and perforin.

In 40% of HD cases, HRS cells carry Epstein-Barr virus (EBV). EBV$^+$ HD is associated with a higher percentage of CD8$^+$ cells, resulting in a lower CD4/CD8 ratio in these cases. CD8$^+$ cells were found to contribute a mean fraction of 21% of the reactive lymphocytes in EBV$^+$ and 34% in EBV$^+$ HD. Surprisingly, a high percentage of activated CD8$^+$ cells in lymph nodes infiltrated with HD has recently been shown to be associated with a poor prognosis.

The question whether CTL in lymph nodes infiltrated by HD represent an antitumor cytotoxic immune response or, alternatively, are a nonspecifically attracted bystander population remains unresolved. The present study addresses this issue by analyzing clonal composition and T cell receptor (TCR) repertoire of CD8$^+$ cells in HD-affected lymph nodes from three cases (2 EBV$^+$, 1 EBV$^-$) of primary disease. Single CD8$^+$ cells were micromanipulated from sections of tumor tissue. TCRβ gene rearrangements were amplified from these single cells using mixtures of Vβ-family- and Jβ-specific primers. The gene rearrangements were analyzed with respect to clonal identity, Vβ and Jβ gene segment usage and shared CDR3 amino acid motifs.

Supported by Deutsche Krebshilfe, Mildred Scheel Stiftung, and Deutsche Forschungsgemeinschaft through SFB 502.

Accepted for publication April 14, 2000.

K. W. and A. R. contributed equally to this work.

Address reprint requests to Klaus Willenbrock, Senckenbergisches Institut für Pathologie, Universitätssklinik Frankfurt, Theodor Stern Kai 7, D 60590 Frankfurt am Main, Germany. E-mail: Willenbrock@em.uni-frankfurt.de.
**Materials and Methods**

**Tissue Samples**

Cervical lymph nodes removed for diagnostic purposes at primary manifestation of HD were analyzed. Clinical and histopathological data are summarized in Table 1. EBV status was determined by EBV-encoded RNA in situ hybridization as described.13

**Immunostaining and Micromanipulation**

Immunostaining of frozen tissue sections was performed as described,14 using monoclonal antibodies against CD30 (BerH2, Dako, Glostrup, Denmark), CD8 (DK25, Dako), and CD20 (L26, Dako). Alkaline phosphatase was developed using Fast Red TR (Sigma, Deisenhofen, Germany). Single cells were collected by micromanipulation as described14 and transferred into polymerase chain reaction (PCR) tubes. For each case, two different areas showing tumor infiltration were chosen for micromanipulation.

**Amplification of TCR Vβ Gene Rearrangements and Sequence Analysis**

Amplification of TCR Vβ gene rearrangements was performed as described.7 Briefly, micromanipulated cells were incubated with proteinase K. A first round of PCR was carried out in the same tube using a mix of 25 Vβ family- and 7 Jβ-specific primers. A second round of amplification was carried out in 96-well plates adding 1 μl of the first-round reaction to each of 8 reaction mixtures, each containing 2 to 5 of the 25 Vβ primers and a mixture of internal Jβ primers.

PCR products were directly sequenced using the Ready Reaction dRhodamine cycle sequencing kit (Perkin Elmer, Foster City, CA) as recommended by the manufacturer. Sequences of Vβ gene rearrangements were deposited in the European Molecular Biology Laboratory database under accession numbers AJ403708-AJ403865.

**Results**

**Tumor Tissue**

In cases 1 and 3, HRS cells were positive for EBV-encoded RNA as determined by in situ hybridization (data not shown). CD8+ cells were markedly more frequent in the EBV+ cases, with case 1 containing the highest numbers. In this case, CD8+ cells were scattered throughout the tumor nodules and were often found close to the HRS cells, sometimes even in direct contact with them (Figure 1A). CD8+ cells in case 2 were found mostly at the periphery of the nodules close to and within the fibrotic bands, but rarely if ever close to HRS cells, or in the center of the tumor nodules where most HRS cells resided (Figure 1B). The CD8+ cells of case 3 seemed to be scattered randomly over the tissue and were seldom found in close contact with HRS cells.

**Micromanipulation and PCR Analysis of CD8+ T Cells in HD**

Areas showing infiltration by HRS cells were identified in CD30-stained sections. Single CD8+ cells from an adjacent section were micromanipulated and transferred into PCR tubes. TCRβ gene rearrangements were amplified from these single cells using mixtures of Vβ-family- and Jβ-specific primers and were directly sequenced. In each of the cases analyzed, single B cells were micromanipulated as negative controls from adjacent sections stained for CD20. Aliquots of the buffer covering the section during the micromanipulation procedure (buffer samples, Table 1) and PCR tubes containing PCR reagents but no cell (water controls, Table 1) served as additional controls. Single CD3+ T cells from periph-
eral blood of a healthy donor were flow-cytometrically sorted into PCR tubes and analyzed as a control for the efficiency of single target amplification of TCR gene rearrangements.

Table 1 shows an overview of the single cell PCR results. Twenty-four percent of the micromanipulated T cells yielded a specific PCR product. This PCR efficiency fits with our earlier results.7 None of the negative control samples, but 23 of 32 (71%) of the sorted single CD3/H11001/H11002/H9251/H9252 TCR T cells, yielded a specific product, demonstrating the high efficiency of single cell PCR. The rearrangements obtained from sorted T cells were all unrelated to each other and to the rearrangements amplified from micromanipulated cells.

Clonal Composition of the CD8 T Cell Population in HD

In cases 1, 2, and 3, micromanipulated CD8 T cells yielded 45, 43, and 32 TCR gene rearrangements, respectively, 39, 30, and 25 of which were potentially functional. In each of the three cases, clonal expansions were detected by amplification of identical gene rearrangements from different CD8 T cells. In case 1, four clones were identified with 3 or 2 members in the sample. Among the 43 gene rearrangements in case 2, nine belonged to one clone (clone 1). Five identical nonfunc-

tional (out-of-frame) gene rearrangements may represent either a second clone (clone 2) or, more likely, were amplified from the second allele of the TCR-β locus of clone 1. Two additional clones were represented with two members each. In case 3, three pairs of identical gene rearrangements were detected among the 32 TCR V region sequences obtained in total.

Sequence Comparison of Potentially Functional TCRβ Gene Rearrangements

Rearrangements of functional Vβ gene segments which were rearranged in frame and devoid of stop codons in CDR3, thus potentially functional, might be subject to selection by antigen. Therefore, potentially functional gene rearrangements from CD8 T cells from HD tissue were compared with each other and with a control collection of 196 potentially functional Vβ gene rearrangements amplified from sorted single T cells from peripheral blood of four healthy donors.

Figures 2 and 3 show the distribution of the Vβ and Jβ gene segments used in potentially functional TCRβ gene rearrangements of CD8 T cells from the three cases of HD and the control T cells. Clonally related sequences were counted only once. A broad spectrum of Vβ gene segments was observed in the different cases. However, a significant number of Vβ segment combinations were shared among the cases. These shared combinations were further analyzed to determine if they were derived from the same T cell clone. The results showed that several common Vβ segment combinations were present in all three cases, indicating a common antigenic stimulation.
segments was used by the CD8⁺ T cells. Given the relatively small number of sequences, no striking over-representation of particular Vβ gene families was observed. The usage of Jβ gene segments in the rearrangements from micromanipulated CD8⁺ T cells was similar to that determined in the control collection and in accordance with published data on Jβ gene segment usage in peripheral blood T cells of healthy individuals.15,16

Selection of a T cell population for a common antigen specificity may reflect itself in identical or similar CDR3 amino acid sequence motifs in clonally independent T cells. To compare CDR3 sequences of gene rearrangements from CD8⁺ T cells of each case, deduced amino acid sequences were aligned according to the conserved motifs of the Vβ or the Jβ gene segments flanking CDR3. In particular, those rearrangements were compared which used either the same Vβ or Jβ gene segment or which had a CDR3 of identical length. For comparison, amino acids were grouped into hydrophobic (L, I, F, M, V, A), polar (Q, N, T, S, Y), acidic (D, E), basic (R, K, H), and small nonpolar, predominantly found in turns (G, P). However, no striking similarities or dominant motifs were found except for a pair of unique rearrangements amplified from cells of case 3. These sequences were both BV3S1 - BJ2.2 rearrangements, the CDR3s had the same length and three amino acids encoded by N nucleotides (nucleotides randomly inserted at junctions during gene rearrangement, printed in italics) were identical (CASSLSGRPGQGAAGELFFG; CASSLSGRARDRGRLGEFFG).

Discussion

In support of the concept of immunosurveillance, which postulates a critical role for the immune system in the elimination of tumor cells,17 clonal expansions of CTL specific for antigens presented by the tumor cells have been detected in various malignancies.18,19 In contrast to other solid tumors where infiltrating T cells usually are a minor component, T cells account for most of the tumor mass in HD. CD4⁺ T cells predominate; however, they do not seem to be involved in an antitumor response.7,8 The relevance of the 10 to 25% of CD8⁺ T cells,20 which are usually found interspersed among the CD4⁺ T cells, remains unknown. These cells might represent a cytotoxic response directed against the HRS cell population. Whether HRS cells are capable of presenting antigens peptides on MHC class I molecules and should, therefore, be susceptible to lysis by CTL specific for, eg, EBV-derived peptides or other tumor antigens.26 Direct comparison of the CTL repertoire of peripheral blood with that of the tumor infiltrated lymph node and determination of the antigen specificity of CTL clones expanded in situ might elucidate the nature of the CD8⁺ T cells in HD tissue.

Acknowledgments

We thank Ralf Küppers for helpful discussion and for critically reading the manuscript, Tilman Spieker for performing the in situ hybridization, and Christiane Gerhard and Tanja Schaffer for excellent sequencing work.

References


