Multiple sclerosis: Brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood

Christian Skulina††, Stephan Schmidt§, Klaus Dormair‡‡, Holger Babbe§, Axel Roers‖, Klaus Rajewsky††, Hartmut Wekerle†, Reinhard Hohlfeld†, and Norbert Goebels†‡,‡

1Institute for Clinical Neuroimmunology, Ludwig-Maximilians University, 81377 Munich, Germany; 2Max Planck Institute for Neurobiology, 82152 Martinsried, Germany; 3Department of Neurology, University of Bonn, 53127 Bonn, Germany; 4Department of Genetics and 5CBR Institute for Biomedical Research, Harvard Medical School, Boston, MA 02115; and 6Department of Dermatology, University of Cologne, 50931 Cologne, Germany

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We surveyed the T cell receptor repertoire in three separate compartments (brain, cerebrospinal fluid, and blood) of two multiple sclerosis patients who initially had diagnostic brain biopsies to clarify their unusual clinical presentation but were subsequently confirmed to have typical multiple sclerosis. One of the brain biopsy specimens had been previously investigated by microdissection and single-cell PCR to determine the clonal composition of brain-infiltrating T cells at the single-cell level. Using complementarity-determining region 3 spectratyping, we identified several identical, expanded CD8+ (but not CD4+) T cell clones in all three compartments. Some of the expanded CD8+ T cells also occurred in sorted CD38+ blood cells, suggesting that they were activated. Strikingly, some of the brain-infiltrating CD8+ T cell clones persisted for >5 years in the cerebrospinal fluid and/or blood and may thus contribute to the progression of the disease.

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classical experiments in an animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), suggested that myelin antigen-specific T cells play a crucial role because EAE could be transferred with CD4+ T cell lines specific for myelin basic protein (1). Obviously, such transfer experiments are impossible in human MS. Recently the possible contribution of CD8+ T cells has received increased attention, especially as effectors of the pathological immune reactions that damage the CNS of EAE animals and MS patients (2–6). Using molecular techniques, including single-cell PCR analysis of microdissected T cells, clonally expanded CD8+ (cytotoxic) T cells could be demonstrated in MS brain lesions at both perivascular and intraparenchymal sites (7). Another recent study found expanded CD8+ T cells in the cerebrospinal fluid (CSF) of MS patients (8). However, it is unclear whether the expanded CD8+ T cell populations observed at different tissue sites of MS patients are related and also relevant to the disease.

We had the opportunity to survey the T cell receptor (TCR) repertoire in three distinct tissue compartments (brain, CSF, and blood) of two MS patients who underwent a brain biopsy for diagnostic reasons. In one of these patients, the TCR repertoire had been previously investigated by microdissection and single-cell PCR analysis of brain-infiltrating T cells. Using complementarity-determining region 3 spectratyping, we identified several identical, expanded CD8+ T cell clones in all three compartments. Some of the expanded CD8+ T cells also occurred in sorted CD38+ blood cells, suggesting that they were activated. Strikingly, some of the brain-infiltrating CD8+ T cell clones persisted for >5 years in the cerebrospinal fluid and/or blood and may thus contribute to the progression of the disease.

Materials and Methods

Patients and Clinical Samples. Patient A (F.E., male) presented in 1996 with recurrent episodes of left-sided hemianopia at the age of 49 years. Cranial MRI suggested the presence of a malignant glioma in the right temporoooccipital lobe. Two weeks after the initial symptoms the lesion was completely removed. Part of the brain tissue was frozen in liquid nitrogen immediately after resection. Histopathological analysis showed a large area of inflammatory demyelination compatible with an MS lesion. CSF analysis revealed oligoclonal Ig bands. Within the subsequent 5 years, the patient experienced three relapses, which also supported the diagnosis of MS. Follow-up MRI confirmed the occurrence of new demyelinating lesions. The patient has been treated with IFN-β1a since the third relapse (1997) until submission of this paper. No further relapses have occurred during treatment. However, during the past years the clinical course has been slowly progressive. Patient A gave his informed consent to the use of brain material, CSF, and blood for research purposes. The blood and CSF samples analyzed in this study were taken 62 months after surgery. Another blood sample was analyzed in December 2003, i.e., 7 years after the brain biopsy. Patient A is seemingly homozygous for the haplotype HLA-A*0101, HLA-B*0801, HLA-Cw*0701, HLA-DRB1*0301, HLA-DRB3*0101, HLA-DQA1*05, HLA-DQB1*0201, because only one allele was detected for each of the loci analyzed.

Patient B (P.I., female) presented at the age of 32 with right-sided hemianopia in April 2001. Four weeks later she had a single generalized epileptic seizure, severe left-frontal headache, and nausea. MRI revealed four small lesions in the subcortical white matter and a large (4 × 2 × 2 cm) T2w-hyperintense region in the left occipitotemporal lobe, which suggested a low-grade glioma. Subsequently, the patient underwent diagnostic stereotactic brain biopsy. Histology showed inflammatory demyelination compatible with MS, and CSF analysis revealed oligoclonal Ig bands. Within the subsequent 11 months she suffered four additional relapses, which supported the diagnosis of MS. Blood and CSF samples analyzed in this study were taken in November 2001, 6 months after the brain biopsy. Patient B was typed as HLA-A*0301, - B*0702, 4002; Cw*0202, 0702; DRB1*1101, 1501; DRB3*0202, – DRB5*0101, – DQB1*0301, 0602.

Abbreviations: TCR, T cell receptor; BV, Vβ; BJ, Jβ; BC, Cβ; CSF, cerebrospinal fluid; CDR3, complementarity-determining region 3; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cell.

Data deposition: Sequence data for TCR -chains in Tables 2 and 3 have been deposited in the GenBank database (accession nos. AY534307–AY534315).

†To whom correspondence should be addressed: Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 28, CH-8091 Zurich, Switzerland. E-mail: norbert.goebels@usz.ch.

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3 sites and time points were identical both at the nucleotide and amino acid levels. If the population of TCR-BJ-specific primers and were directly sequenced (9). According to the recommendations of the suppliers.

Isolation of Peripheral Blood Mononuclear Cells (PBMC) and T Cell Subsets. PBMC were isolated with standard-density centrifugation, CD8⁺, CD4⁺, and CD38⁺ T cells were isolated from the PBMC with antibody-coated magnetic beads according to the manufacturer’s recommendations (Dynabeads, Dynal, Hamburg, Germany).

Isolation of RNA and cDNA Synthesis. Total RNA was extracted from brain, CSF, and blood cells with TRIZol-LS reagent (GIBCO/BRL). For cDNA synthesis, oligo(dT) and reverse transcriptase (SuperScript II, GIBCO/BRL) were used according to the recommendations of the suppliers.

CDR3 Spectratyping. CDR3 spectratype (“immunoscope”) analysis was performed essentially as described (9). In brief, cDNA was subjected to TCR-Vβ (TCR-BV) gene family-specific PCR in 26 separate reactions, each containing 1 of 26 BV family-specific and 1 Cβ (BC)-specific primer (10). With 1 of 13 fluorescence-labeled Jβ (BJ)-specific oligonucleotides (11), single-stranded runoff products were generated from each BV-specific PCR product. Transcripts were resolved on a sequencing gel, and fluorescence intensities were measured with an automated DNA sequencer (ABI377, Applied Biosystems). Clonal expansions appear as distinct peaks of a given CDR3 length above the Gaussian-like background of polyclonal cells. Expanded candidate BV–BJ3 subpopulations were subamplified from the initial BV–BC amplification product by using BV- and BJ-specific primers and were directly sequenced (9). If the population of TCR-β-chain mRNA molecules amplified by the BV primer combination was dominated by a single clonal transcript, the CDR3 sequence could be read by direct sequencing. For the detection of selected clones (as indicated on an oligoclonal background, clone-specific BJ primers, complementary to part of the J and part of the N-D-N region, were used. The sequence identity of the amplification product was determined by direct sequencing as above. The sequences of the clone-specific BJ primers were as follows: BV1–BJ2.3, 5’-AAAAATACCT-GCGATCTCCTCT-3’; BV9–BJ2.7, 5’-CCCCGAAGTACT-GCTGCCGAC-3’; BV22–BJ2.1, 5’-GAACGTCTATTGGT-TTCTCC-3’.

Immunohistochemistry, Microdissection, and Single-Cell PCR Analysis of Brain Tissue from Patient A. T cell infiltrates in the brain tissue of patient A had been previously analyzed by microdissection and single-cell PCR (7). In that study, TCR-BV sequences were amplified from single TCR-encoding, rearranged DNA molecules of brain-infiltrating T cells from perivascular or intraparenchymal locations (7). CD8⁺ T cells outnumbered CD4⁺ T cells by 5- to 6-fold in the parenchyma and by ~3-fold at perivascular locations.

CD4⁺ and CD8⁺ T cells were identified by staining with monoclonal antibodies (7). The cells were then mobilized from the surrounding tissue and transferred into PCR tubes by means of hydraulic micromanipulators. TCR-BV gene rearrangements were amplified by seminested PCR. Briefly, a first round of amplification was performed by using a mix of 24 BV gene family-specific and 8 external BJ-specific primers. In a second round of PCR, two to three of the same BV primers were used together with a mix of seven internal BJ-specific primers in separate reactions (7).

Of 24 rearrangements obtained from single parenchymal CD8⁺ cells of patient A, 17 (71%) originated from 9 different clones; only 7 sequences were unique (7). Forty-six of the 52 separate reactions (7). If the population of TCR-β-chain mRNA molecules amplified by the BV primer combination was dominated by a single clonal transcript, the CDR3 sequence could be read by direct sequencing. For the detection of selected clones (as indicated on an oligoclonal background, clone-specific BJ primers, complementary to part of the J and part of the N-D-N region, were used. The sequence identity of the amplification product was determined by direct sequencing as above. The sequences of the clone-specific BJ primers were as follows: BV1–BJ2.3, 5’-AAAAATACCT-GCGATCTCCTCT-3’; BV9–BJ2.7, 5’-CCCCGAAGTACT-GCTGCCGAC-3’; BV22–BJ2.1, 5’-GAACGTCTATTGGT-TTCTCC-3’.

Results

The brain tissue from patient A (the lesion had been surgically removed in 1996) was first analyzed by spectratype analysis of the TCR-BV CDR3 regions. Several peaks indicating clonally expanded T cell populations were observed. The peaks were compared to the TCR-BV sequences of expanded T cell clones previously amplified by single-cell PCR from microdissected brain-infiltrating CD8⁺ T cells (ref. 7; GenBank accession nos. AJ405646–AJ405745) and to clonal expansions detected by CDR3 spectratyping in CSF cells and PBMC subpopulations obtained 5 years after the brain biopsy. Corresponding peaks that occurred in two or more different samples were sequenced. Altogether, 13 TCR sequences were identified in this way (Tables 1 and 2). Nine of the 13 sequences derived by CDR3 spectratyping from brain tissue corresponded to one of the TCR sequence(s) previously identified by single-cell PCR (Table 1). Five of these nine sequences were also detected as clonal peaks in the CSF or blood of the same patient (Table 1). Specifically, the sequence BV1-CASS-ISGRK-DTQ-BJ2.3 was found in CD8⁺ and CD38⁺
(activated) blood cells and in the CSF; the sequence BV4-CS-VWEV-SGA-BJ2.6 was found in CD8+ blood cells and in the CSF; the sequence BV13.3-CASS-PGDRAQ-YNE-BJ2.1 was found in CD8+ blood cells and CSF; the sequence BV14-CASS-PLWEGGIG-NTE-BJ1.1 was found in CD8+ cells and CSF; and the sequence BV22-CASS-EGAGEH-NEQ-BJ2.1 was found in the CSF (Table 1).

Four of the TCR sequences that had been identified in the 1996 brain biopsy by CDR3 spectratyping did not correspond to any of the single-cell PCR-derived sequences (ref. 7 and Table 2). However, they could be traced in CSF cells obtained 5 years after the brain biopsy. One TCR sequence (BV9-CASS-QIRGVG-EQY-BJ2.7) also appeared in activated CD38+/H11001 T cells (Table 2).

Altogether, nine expanded CD8+ T cell clones from the MS brain lesion were tracked in the CSF, blood, or both (Tables 1 and 2). None of the clones was detected in the CD4+/H11001 PBMC population.

Additional blood samples from patient A were analyzed in December 2003, i.e., 7 years after the initial brain biopsy. CDR3 spectratyping of purified CD8+/H11001 T cells revealed a conspicuous TCR-BV1-BJ2.3 peak. The cDNA sequence of this peak was identical to the cDNA sequence coding for the BV1-CASS-ISGRK-DTQ-BJ2.3 sequence, which had been previously iden-

Table 2. TCR-BV sequences identified in brain tissue, CSF cells, and sorted peripheral blood cells (PBL) from Patient A by CDR3 spectratyping

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The N-D-N regions are shown in italics, +, completely readable sequence; (+), clonal identity clearly discernible on an oligoclonal background; (+)#, completely readable sequence with clone-specific primer; --, clone not identifiable by CDR3 spectratyping and/or direct sequencing. Note that the TCR sequences observed at different sites and time points were identical both at the nucleotide and amino acid levels.
Our present results shed new light on the possible pathogenic mechanisms of blood cells and in the CSF but not in the brain (Table 3). It has also been known for some time that the T cell repertoire expressed in the brain is similar to the peripheral immune compartments of this patient. The differences could have statistical reasons (sampling fluctuations) or could reflect systematic changes of the TCR repertoire over time (e.g., because of epitope spreading). In addition, the CSF and blood might contain clonally expanded bystander cells (see below). This must be taken into consideration when CSF or blood samples are used for immunological studies in MS patients.

It is noteworthy that patient A had been treated for many years with IFN-β when the follow-up CSF and blood samples were obtained. So far he has had no further clinical exacerbations during the treatment period. Our observation that more than half of the initially identified brain-infiltrating CD8+ T cells clones persisted during many years of treatment would be consistent with the assumed mechanism of action of IFN-β, which is thought to act as an antigen-nonspecific immunomodulatory agent (19). It often dampens, but does not completely halt, disease activity and would not be expected to eradicate (auto)antigen-specific T cells.

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The two MS patients from whom we obtained brain biopsies, blood, and CSF samples had had an unusual initial clinical presentation, necessitating a diagnostic brain biopsy. However, in both cases, the histological findings plus the subsequent clinical course and laboratory findings proved to be typical of MS and met the current diagnostic criteria (12). Obviously, our findings cannot be generalized to all MS patients, especially in view of the growing evidence for the pathological heterogeneity of MS (13). Furthermore, imaging studies suggest that the mechanisms of lesion genesis might be diverse (14). As regards the possible role of CD8+ T cells, it is noteworthy that a recently published study of 36 MS patients showed an overrepresentation of CD8+ T cells expressing particular TCR-BV chains in the CSF compared with blood cells in the majority of patients (8). Although TCR sequences were obtained in only a small number of these patients, the data indicate that CD8+ T cell expansions occur in the CSF of most MS patients (8).

CD8+ T cells are the predominating type of T cell in MS lesions (5, 15). It has also been known for some time that the T cell infiltrates of MS lesions express a limited repertoire of antigen receptors (TCR) (16). However, only very recently has it become technically possible to link the immunocytotoxic localization of CD8 with PCR analysis of the TCR expressed in individual, morphologically characterized CD8+ T cells (7, 17, 18). In MS patient A, the TCR repertoire had been previously studied with unprecedented precision by microdissection and single-cell PCR analysis of brain-infiltrating CD8+ T cells (7). When we compared the previously identified TCR sequences with the TCR sequences obtained 5 years later by CDR3 spectratyping analysis of CSF and blood cells, five of nine previously observed sequences recurred (Table 1). One of these T cell clones, which had been present in brain, CSF, and activated CD8+ blood T cell preparations, was again detectable as a conspicuous clonal expansion in CD8+ blood T cells 7 years after the biopsy.

It appears that the TCR repertoire expressed in the brain is similar to that in the peripheral immune compartments of this patient. The differences could have statistical reasons (sampling fluctuations) or could reflect systematic changes of the TCR repertoire over time (e.g., because of epitope spreading). In addition, the CSF and blood might contain clonally expanded bystander cells (see below). This must be taken into consideration when CSF or blood samples are used for immunological studies in MS patients.

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The slight pleocytosis that is often observed in the CSF of MS patients could be related to perivascular, meningeal, and/or parenchymal inflammation (23). The data obtained by microdissection and single-cell PCR of individual brain-infiltrating T cells clearly establish that some of the expanded CD8⁺ T cell clones occur in both brain lesions and CSF. Even T cell clones that deeply infiltrated the brain parenchyma could be found in the CSF (Table 1). Thus, the extent to which the CSF and blood mirror the immune cell repertoire of MS lesions seems to be greater than previously appreciated. This finding raises important questions. There could be a bidirectional traffic of CD8⁺ T cells between the CNS and peripheral compartments, or there could be unidirectional migration of CD8⁺ T cells from a constantly supplying peripheral source into the CNS. In the latter case, the CNS might act as a “sink” where the infiltrating T cells eventually die by apoptosis. Presently we cannot distinguish between these possibilities.

In principle, oligoclonal expansions of CD8⁺ can also occur in the normal immune system. For example, they can be sometimes observed in the blood of healthy elderly subjects (24), or they could nonspecifically infiltrate tissue lesions (25). However, their occurrence at different sites, striking persistence, and signs of their observation in the blood of healthy elderly subjects (24), or they could be unidirectional migration of CD8⁺ T cells between the CNS and peripheral compartments, or there could be a bidirectional traffic of CD8⁺ T cells from the CNS to blood and test them with large arrays of candidate antigens, including synthetic peptide libraries (39). To study antigen recognition by these T cells in vivo, it might be possible to construct transgenic mice that express a human TCR together with an appropriate MHC class II-restricted TCRs from myelin basic protein-specific CD4⁺ human T cell clones (40). Finally, our findings reemphasize the importance of cytotoxic mechanisms in myelin and axonal injury, calling for neuroprotective and repair strategies.

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