Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients

Marc Jacobsen,1 Sabine Cepok,1 Elfriede Quak,1 Michael Happel,1 Rami Gaber,1 Andreas Ziegler,2 Sabine Schock,1 Wolfgang H. Oertel,1 Norbert Sommer1 and Bernhard Hemmer1

1Clinical Neuroimmunology Group, Department of Neurology, Philipps University, Rudolf-Bultmann Strasse 8 and 2Institute of Medical Biometry and Epidemiology, Philipps University, Bunsenstrasse 3, 35033 Marburg, Germany

Correspondence to: B. Hemmer, Clinical Neuroimmunology Group, Department of Neurology, Philipps University, Rudolf-Bultmann Strasse 8, 35033 Marburg, Germany
E-mail: hemmer@mailer.uni-marburg.de

Summary
Multiple sclerosis is a chronic inflammatory demyelinating disease of the CNS. Although the aetiology of multiple sclerosis is still unknown, it is widely believed that T cells play a central role in its pathogenesis. To identify and characterize disease-relevant T cells, we analysed CD4+ and CD8+ T cells freshly isolated from the CSF and peripheral blood of 36 multiple sclerosis patients for their T-cell receptor variable \( \beta \) (TCRBV) chain repertoire. In most patients, we found significant overexpression of individual TCRBV chains on CD8+ T cells from CSF compared with peripheral blood. In contrast, only a few multiple sclerosis patients showed differences between the two compartments in TCRBV expression on CD4+ T cells. The overexpression of specific TCRBV chains on CD8+ T cells was found to be stable over several months in selected patients and involved mainly T cells with a memory phenotype. In two patients studied, individual TCRBV chain overexpression was found to be caused by the expansion of T cell populations with identical or highly similar rearranged T-cell receptor \( \beta \)- and \( \alpha \)-chain sequences, which were not found among peripheral blood CD8+ T cells. Our findings demonstrate selective enrichment of memory CD8+ T cells in the CSF of multiple sclerosis patients, suggesting a role for these CD8+ T cells in the pathogenesis of multiple sclerosis. Our study provides a basis for future trials to identify disease-associated antigens and disease pathogenesis in multiple sclerosis.

Keywords: multiple sclerosis; neuroimmunology; T-cell receptors; clonal expansion

Abbreviations: EAE = experimental autoimmune encephalomyelitis; HLA = human leucocyte antigen; IgG = immunoglobulin G; MHC = major histocompatibility complex; TCC = human T-cell clone; TCR = T-cell receptor; TCRAV = T-cell receptor variable \( \alpha \) chain; TCRBV = T-cell receptor variable \( \beta \) chain

Introduction
Multiple sclerosis is a chronic inflammatory and demyelinating disease of the CNS and frequently leads to severe disability. The disease is characterized by white-matter inflammation with infiltrates of various immune cells, including CD4+ and CD8+ T cells, B cells, plasma cells and macrophages (Brosnan and Raine, 1996; Lucchinetti et al., 1996). Although the pathogenesis of multiple sclerosis has been investigated intensively during recent decades, the underlying cause is still unknown (Martin et al., 1992; Noseworthy, 1999). Several studies have focused on the humoral immune response in multiple sclerosis brain tissue and CSF. These studies identified a tissue-specific oligoclonal antibody response, which seems to be highly conserved among various brain lesions and to show little qualitative change over time in individual multiple sclerosis patients (Walsh and Tourtellotte, 1986). Similarly, B cells present in the brain and CSF of multiple sclerosis patients consist of a few expanded clones with signs of extensive hypermutation of the immunoglobulin variable region heavy-chain genes, which is probably driven by repeated antigen exposure (Owens et al., 1998; Baranzini et al., 1999; Colombo et al., 2000).

Less is known about the T-cell responses in multiple sclerosis patients, although T cells have been studied extensively (Ota et al., 1990; Pette et al., 1990; Martin et al., 1992; Utz et al., 1993). In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, a highly restricted encephalitogenic T-cell response was observed in some susceptible strains of mice (Zamvil and Steinman, 1990). These findings promoted studies that
focused on the analysis of T-cell receptor variable α (TCRAV) and T-cell receptor variable β (TCRBV) chains in multiple sclerosis. In contrast to EAE, in multiple sclerosis the T-cell receptor (TCR) repertoire of myelin-specific T cells was found to be highly heterogeneous, without significant differences between multiple sclerosis patients and healthy controls (Hafler et al., 1996). Other studies investigating the TCR repertoire of peripheral blood cells in multiple sclerosis patients have described skewing of TCRAV or TCRBV chains, mainly by the use of PCR (polymerase chain reaction)-based methods (Monteiro et al., 1996; Musette et al., 1996; Gran et al., 1998; Goebels et al., 2000). Focusing on CNS tissue, one group found that a limited number of TCRAV chains were rearranged in multiple sclerosis brain lesions, whereas another did not (Oksenberg et al., 1990; Wucherpfennig et al., 1992). Recently, oligoclonal expansion of CD8+ T cells in brain lesions of two multiple sclerosis patients was demonstrated by micromanipulation and single-cell PCR (Babbe et al., 2000). However, although innovative, this approach did not allow the investigation of either the TCR-α sequences or the phenotype of the infiltrating cells. Controversial observations have been reported on TCRBV and TCRAV chain expression by freshly isolated CSF T cells derived from multiple sclerosis patients (Lee et al., 1991; Birnbaum and van Ness, 1992; Usuku et al., 1996; Lozeron et al., 1998). Because of limitations in the experimental design used in these studies, it was not possible to do a differential analysis of CD4+ and CD8+ T cells, which differ substantially in their functional properties and TCRBV profiles. We investigated the repertoire of T cells in the CSF of multiple sclerosis patients, focusing on the two main T-cell subgroups of CD4+ and CD8+ T cells, by analysing TCR expression with a flow cytometric and DNA sequencing approach in a study involving multiple sclerosis patients and healthy controls.

Material and methods

Multiple sclerosis patients and healthy controls

Multiple sclerosis patients and healthy controls without a personal or family history of multiple sclerosis or other chronic inflammatory diseases were recruited at the Department of Neurology at the Philipps University of Marburg, Germany. In the multiple sclerosis group, only patients with clinically definite or laboratory-supported definite multiple sclerosis were included (Poser et al., 1983). Thirteen patients were male and 23 female. Their ages ranged from 18 to 66 years. Thirty-three patients had a relapsing–remitting course of disease, two had a secondary progressive course and one had a primary progressive course. At the time of the spinal tap, one patient was under treatment with cyclophosphamide and two patients had received steroids intravenously. The EDSS (Expanded Disability Status Score) ranged between 1 and 8.5 (mean 2.9). All but two patients had oligoclonal immunoglobulin G (IgG) bands in the CSF. All multiple sclerosis patients gave written informed consent. The control group consisted of 75 healthy subjects matched with the patient group for age and gender. The ethics committee of the University of Marburg approved the study.

HLA typing

Serological major histocompatibility complex (MHC) human leucocyte antigen (HLA) class I and molecular HLA class II typing was performed in 18 of the multiple sclerosis patients by the HLA laboratory of Philipps University, Marburg and the Stefan-Morsch Stiftung, Birkenfeld, Germany. The 18 patients expressed a broad spectrum of HLA class I and II molecules that differed from those of age-matched healthy controls only in the greater prevalence of HLA alleles B7 (allele frequency 25 versus 10%) and DR15/DR6 (allele frequency 35 versus 18%). The two multiple sclerosis patients who were analysed for CSF T-cell clonality had the following genotypes: HLA-A1, A24(9), B8, B44(12), Cw6, Bw4, Bw6 and HLA-DR3, -DR4, -DQB02, -DQB03 (Patient A.D.) and HLA-A3, -B7, -B35, -Bw6 and HLA-DR1, -DR15, -DQB05, -DQB06 (Patient K.H.).

Antibodies

The TCRBV antibody panel comprised TCRBV1-PE (clone BL37.2), BV2-PE (clone MB2D5), BV3-FITC (clone CH92), BV5.1-FITC (clone Immu157), BV5.2-FITC (clone 36213), BV5.3-PE (clone 3D11), BV6.7-FITC (clone OT145) (staining performed only in single patients), BV7-PE (clone ZOE), BV8-FITC (clone 56C5.2), BV9-PE (clone FIN9), BV11-FITC (clone C21), BV12-FITC (clone VER2.32.1), BV13.1-PE (clone Immu222), BV13.6-PE (clone JU74.3), BV14-PE (clone CAS1.13), BV16-FITC (clone TAMAYA1.2), BV17-FITC (clone E17.5F3), BV18-PE (clone BA62.6), BV20-PE (clone ELL1.4), BV21.3-FITC (clone IG125), BV22-FITC (clone Immu546), BV23-PE (clone AF23) (all from Immunotech now Becton Coulter), CD4-APC (clone RPA-T4; Becton Dickinson, NJ, USA, San Diego, Calif., USA) and CD8-PerCP (clone SK1; Becton Dickinson). For the additional characterization of T-cell populations that were expanded in the CSF, antibodies against CD45RA (clone 0584), CD45RO (clone CD28.2, Becton Dickinson) and CD8-PerCP were combined with the particular TCRBV antibodies. Isotype-specific control antibodies IgG-FITC (Becton Dickinson), IgG1-PE (Pharmingen), IgG1-PerCP (Becton Dickinson) and IgG (Becton Dickinson) were used to exclude the influence of non-specific binding. Overall, the TCRBV panel covered 64% of all known TCRs expressed on T cells (Muraro et al., 2000). The specificity of the antibodies has been confirmed recently by comparing the results of TCRBV chain PCR and flow cytometric analysis of human T-cell clones (TCCs) (Muraro et al., 2000).
Flow cytometry with whole blood
Fresh blood was diluted 1:1 with ice-cold PBS (phosphate-buffered saline) and 200 μl of the mixture was added to wells of a round-bottom 96-well plate (Nunc, Roskilde, Denmark). To avoid cross-contamination, each well that contained blood was left surrounded by empty wells. The plate was centrifuged at 200 g for 5 min, the supernatant was discarded and the top of the plate was dried briefly with a paper towel. The plate was then placed on ice and cell pellets were resuspended, adding the monoclonal antibody combinations directly from cluster tubes and mixing for 10 s with a multichannel pipette. After 30 min incubation on ice, erythrocytes were lysed in two steps using 200 μl of lysing solution (BD) according to the manufacturer’s instructions. The plate was centrifuged and the supernatant was discarded. After two additional washes with 200 μl of ice-cold PBS with 2% foetal calf serum, cells were transferred to 5 ml tubes (Falcon; BD) and analysed with a four-colour flow cytometer (FACSCalibur; BD). For each stained blood sample, at least 10,000 events were acquired based on a forward scatter/ sideways scatter lymphocyte gate.

Flow cytometry on CSF cells
Fresh CSF (10 ml, >4 leucocytes/μl, <1 h after the spinal tap) was spun at 200 g for 10 min, the supernatant was removed and the pellet was resuspended in PBS containing 5% foetal calf serum. Twenty microlitres of the cell suspension was added to each well of a 96-well plate. The plate was then centrifuged and the supernatant discarded as described above. Staining was performed as described for whole blood cells. No lysing step was applied to CSF cells since no red blood cells were contained in these specimens. Cells were then washed twice and analysed as described above.

Single-cell cloning and expansion, and RNA isolation
Freshly isolated CSF cells were centrifuged (as described above) and resuspended in IMDM (Iscove’s modified Dulbecco medium; Gibco) containing 5% human serum, 1% L-glutamine (Gibco) and 1% penicillin/streptomycin (Gibco). CSF cells were then diluted to 3, 10 and 20 cells/well of a 96-well microtitre plate (Nunc) and mixed with 50,000 allogenic APCs (antigen presenting cells), stimulated with Dynabeads CD3/CD28 T-cell expander (Dynal, Oslo, Norway) or phytohaemagglutinin (Sigma, Deisenhofen, Germany) following the manufacturer’s instructions, and incubated at 37°C under 5% CO2. The cells were restimulated with human recombinant IL-2 (interleukin 2; Diacclone, Besancon, France) every 7 days starting at Day 2 of culture. After 3 weeks, growing clones were screened for expanded TCRBV chains using flow cytometry (as described above). Half of the TCRBV-positive cells were further expanded in culture. The remaining cells were harvested for RNA isolation. RNA was isolated and reverse-transcribed following the kit supplier’s protocol (RNeasy Mini; Qiagen, Valencia, Calif., USA)

PCR and TCRBV sequencing
The cDNA was amplified using a set of primers specific for the TCRαV and TCRBV sequences, as described previously (Genevee et al., 1992). After the products had been visualized on agarose gel, they were purified using the QIAquick PCR purification kit (Qiagen) and sequenced using a DNA sequencing kit (PE Biosystems, Forster City, Calif., USA) on an ABI Prism 310 Genetic Analyser (PE Biosystems) following the manufacturer’s instructions. All amplification steps were performed on a PTC-200 Thermal Cycler (Biozym, Hessisch Oldendorf, Germany). TCCs (T-cell clones) expressing identical or highly similar TCRBV CDR3 regions were further analysed for TCRαV expression using the entire panel of TCRBV-specific primers. The TCRαV chain was then sequenced as described above. The nomenclature used for TCRBV-specific antibodies was that of Arden (Arden et al., 1995) and the TCR-αV and TCR-β sequences were analysed according to the nomenclature of Rowen and Folch (http://imgt.cnusc.fr:8104/; Rowen et al., 1996; Folch et al., 2000). The standard single-letter code is used for amino acid sequences.

Statistical analysis
TCRBV-chain staining was analysed by normalizing proportions with respect to at least two different parameters. The expression of each given TCRBV segment (TCRBVn) was determined on cells that stained for a main phenotypic marker (e.g. CD4). The percentage of expression was calculated according to the formula: (% cells expressing both main and TCRBVn/% all cells expressing main marker) (both TCRBVn-negative and -positive) × 100. For CD4+ cells, the formula would correspond to: % TCRBV+ cells = (% CD4+ TCRBV+ cells/% all CD4+ cells) × 100. To compare TCRBV expression in different compartments, the number of T cells that expressed a given TCRBV chain on CD4+ or CD8+ cells was determined in CSF and blood and the relative percentages were subtracted, i.e. for CD4 cells (% TCRBVn on CD4+ CSF) – (% TCRBVn on CD4+ blood). Positive values indicate relative enrichment of the given TCRBV in the CSF, whereas negative values indicate enrichment in blood. For statistical reasons, it is reasonable to assume that staining for CD4 or CD8 and the different TCRBV chains are independent events. Therefore, we compared the number of TCRBV+/CD4+ and TCRBV+/CD8+ T cells in both compartments by χ² analysis to detect significant differences in TCRBV expression. The χ² test is appropriate for the exclusion of false-positive results when a small amount of CSF cells is available for analysis. The same method was used for CD8+ T cells (Muraro et al., 2000). The χ² value for
significance was 18.5 since we used a global significance level of 5% in this study together with (i) a total of two hypotheses, (ii) 36 patients, (iii) 21 stainings and (iv) two cell types. The difference in TCRBV chain expression between blood T cells of multiple sclerosis patients and healthy controls was investigated using the Mann–Whitney U-test.

Results
No significant differences in TCRBV chain expression between peripheral blood T cells from multiple sclerosis patients and healthy controls

Thirty-six patients with clinically definite or laboratory-supported multiple sclerosis and 75 healthy controls matched for age, gender and ethnicity, with no personal or family history of multiple sclerosis or other chronic inflammatory disorders, were included in the study. The number of T cells expressing CD4+ or CD8+ and a particular TCRBV chain was analysed by four-colour flow cytometry (Fig. 1A). The mean percentages of CD4+ and CD8+ T cells expressing TCRBV chains were compared in peripheral blood from multiple sclerosis patients and healthy controls (Fig. 1B). Although the expression of TCRBV chains varied among individual multiple sclerosis patients and healthy controls, no TCRBV chain was expressed at significantly higher or lower levels in multiple sclerosis patients (smallest nominal $P = 0.058$). Taken together, these experiments did not provide evidence for significant differences in the expression of particular TCRBV chains between peripheral blood T cells of multiple sclerosis patients and healthy controls.

Fig. 1 TCRBV chain expression on peripheral blood T cells of multiple sclerosis patients and healthy controls. (A) Expression of TCRBV14 on CD4+ and CD8+ T cells of a multiple sclerosis patient determined by flow cytometry. The expression of TCRBV14 differed significantly between CD4+ (left) and CD8+ T cells (right). (B) Summary of TCRBV chain expression of 22 TCRBV chains on peripheral blood cells of 36 multiple sclerosis patients (black bars) and 75 age-matched healthy controls (grey bars). The analysis was performed on CD4+ (left) and CD8+ T cells (right). None of the TCRBV chains was overexpressed on peripheral blood T cells of multiple sclerosis patients in comparison with healthy controls. Bar charts display the mean level of expression and the standard error for each group.
TCRBV chain expression differs between CSF and peripheral blood T cells from multiple sclerosis patients

Next, we compared the TCRBV repertoire between CSF and peripheral blood from individual multiple sclerosis patients. Cells from peripheral blood and CSF were stained in parallel and the percentages of CD4+ and CD8+ T cells expressing each TCRBV chain were determined. The number of T cells in CSF and blood expressing CD4+ or CD8+ and each individual TCRBV chain was compared by $\chi^2$ analysis. As exemplified in Fig. 2A for Patient P.W., a highly significant difference was observed for CD8+ T cells, 7.7% of which expressed TCRBV2 in the peripheral blood and 16.4% in the CSF ($P < 10^{-6}$). In contrast, expression of TCRBV2 on CD4+...
Fig. 3 Summary of differential TCRBV chain expression on CD4+ and CD8+ T cells in CSF and peripheral blood of multiple sclerosis patients. A summary of all TCRBV chain analyses from 36 multiple sclerosis patients is shown. Each staining was performed and analysed as described in the caption of Fig. 2. TCRBV chains that were expressed at higher levels in CSF are indicated by arrows pointing upwards (cut-off $\chi^2 > 18.5$). Skewing on CD4+ T cells is indicated by white arrows on a black background and skewing on CD8+ T cells by black arrows on a grey background. Skewings of both subpopulations are marked by white arrows on a grey background. Dots indicate the absence of significant skewing of TCRBV chains. Empty squares indicate that no staining was performed or the quality of the staining was not sufficient for analysis. The boxes on the right summarize the results of TCRBV chain skewings for CD4+ and CD8+ T cells. Overall, we observed significant overexpression of TCRBV chains predominantly on CD8+ CSF T cells. TCRBV6.7 was not included because most patients were not analysed.

Fig. 4 Serial analysis of TCRBV chain expression on CD8+ T cells. The TCRBV expression on CSF and peripheral blood cells of Patient P.W. were analysed at three time points. TCRBV expression on CD8+ T cells (difference between CSF and peripheral blood) at months 0, 2 (+7 weeks) and 6 (+5 months) is shown. The entire panel of stainings was performed at time points 1 and 3. Because of the low number of cells during immunosuppressive treatment at time point 2, only one staining was performed. The higher expression of TCRBV2 and the lower expression of TCRBV13.1 on CD8+ T cells was confirmed at different time points.
T cells was found to be similar between CSF and peripheral blood \((P = 0.310)\). The analysis of all TCRBV chains in this patient showed significant differences in TCRBV chain expression \((P < 10^{-6})\), with two TCRBV chains on CD8+ and one chain on CD4+ T cells (Fig. 2B). Two skewings, TCRBV2 and 5.3, resulted in greater expression on CSF than peripheral blood T cells, demonstrating specific enrichment in the diseased organ compartment.

TCRBV chain expansion in CSF is predominantly observed on CD8+ T cells

Using this approach, we analysed TCRBV chain expression systematically on CSF and peripheral blood T cells from 36 multiple sclerosis patients by \(\chi^2\) analysis. The calculated \(P\) value taken as indicating significance was \(2.3 \times 10^{-5} \chi^2 = 18.5\) at a global significance level of 5% in this study, after adjusting for multiple testing. The results of the TCR expression analyses in all patients are shown in Fig. 3. Overall, most TCRBV chains were expressed similarly on both subpopulations of T cells in CSF and peripheral blood, demonstrating the power of this approach. However, for individual TCRBV chains we observed significant differences in TCRBV chain expression between blood and CSF T cells. In 22 of 36 patients (61%), an increase in TCRBV chain expression of at least one chain was detected on CSF T cells (Fig. 3), whereas 14 patients showed increased expression of one or more TCRBV chains in their peripheral blood (data not shown). Interestingly, when T cells were separated according to their phenotype, 27 TCRBV chains were significantly overexpressed on CD8+ CSF T cells, whereas only four chains were overexpressed on CD4+ CSF T cells. The difference between the number of skewings on CD8+ and CD4+ T cells was significant \((P < 0.001)\). Overall TCRBV chain skewings occurred with 16 of 21 TCRBV chains and no specific TCRBV chain was found to be skewed at a significantly higher frequency.

To investigate whether these expansions were stable over time, we analysed TCRBV repertoires in CSF and blood from five selected patients at different time points. In all donors, TCRBV expression profiles on CD8+ T cells remained stable over time. As shown for one of them (Fig. 4), CD8+ T cells expressing TCRBV2 were enriched in CSF compared with peripheral blood. When the patient was retested 7 weeks and 5 months later, we confirmed the increased expression of TCRBV2 on CD8+ CSF T cells, although the patient was treated with immunosuppressive and immunomodulatory drugs and the white cell count in CSF changed throughout the investigation.

Expanded CD8+ T cells express memory T-cell markers

To characterize further the expanded CD8+ T-cell populations in CSF, we performed additional flow cytometric analyses. The expression of CD28, CD45RA and CD45RO was analysed in peripheral blood and CSF on CD8+ T cells expressing the expanded TCRBV chain. CD8+ TCRBV+ cells were gated and the distribution of CD28 and CD45RA was determined. In general, the CD8+ TCRBV+ CSF T cells were mostly CD45RA−, CD45RO+ (data not shown for the latter) with a variable degree of CD28 expression (memory phenotype). In contrast, peripheral CD8+ TCRBV+ T cells contained higher numbers of CD45RA+CD28+ (naive phenotype) and fewer memory T cells (Fig. 5). Small amounts of CD8+CD45RA+CD28− T cells (effector phenotype) were observed, without significant differences between the two compartments.

Skewing in the CSF is caused by expansion of CD8+ T cells with similar or identical TCRs

Next, we wanted to determine the nature of the CSF CD8+ T-cell population expansion using a cloning and sequencing approach. This was necessary to separate CD4+ and CD8+ T cells and overcome the difficulties due to the low cell numbers available for analysis. Two patients with typical multiple sclerosis were selected for this analysis: multiple sclerosis Patient A.D., who had a very early disease state (18 years old, relapsing-remitting course, disease duration 6 months, >10 gadolinium-enhancing lesions, intrathecal...
IgG synthesis) and Patient K.H., who had a more advanced disease state (49 years old, relapsing–remitting course, disease duration 9 years, no gadolinium-enhancing lesions, intrathecal IgG synthesis). Patient A.D. showed overexpression of TCRBV22 and Patient K.H. overexpression of TCRBV17 on CD8+ CSF T cells. T cells were cloned from CSF and blood of both patients using cross-linking anti-CD3/CD28 antibodies or phytohaemagglutinin by limiting dilution assay to obtain clonal cultures. After in vitro expansion, several hundred TCCs from each patient were analysed by flow cytometry for the expression of CD8 TCRBV22 in Patient A.D. and TCRBV17 in Patient K.H. From Patient A.D., eight CD8+ and 10 CD4+ CSF clones expressing TCRBV22 reached cell numbers sufficient to isolate RNA for TCR-β chain analysis (Fig. 6). Three TCCs had the same rearranged TCR-β chain sequence (clonotype ADC14, ADC22 and ADC37 (clonotype 1) expressed TCRAV2 (IMGT), with identical TCRAJ regions (lower panel). Determination of the TCRBJ and AJ family (Jα and Jβ) was based on IMGT nomenclature. Clones with identical sequences are shown on a shaded background. NDN = diversity region.

### Table 1: TCR-β and TCR-α Sequences of CD8+ TCCs from Patient A.D.

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<th>AJ-region</th>
<th>Jα</th>
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<td>ADC14</td>
<td>C A V P</td>
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<tr>
<td>ADC22</td>
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<tr>
<td>ADC37</td>
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### Table 2: TCR-β and TCR-α Sequences of CD8+ TCCs from Patient K.H.

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<th>BJ-region</th>
<th>Jβ</th>
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<tr>
<td>ADC14</td>
<td>C A S S</td>
<td>E D S</td>
<td>S Y N E Q F F G P G</td>
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<tr>
<td>ADC22</td>
<td>C A S S</td>
<td>E D S</td>
<td>S Y N E Q F F G P G</td>
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<tr>
<td>ADC37</td>
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<td>E D S</td>
<td>S Y N E Q F F G P G</td>
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### Table 3: TCR-β and TCR-α Sequences of CD8+ TCCs from Patient K.H.

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<td>ADC31</td>
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<tr>
<td>ADC41</td>
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<td>A D S</td>
<td>S Y N E Q F F G P G</td>
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### Table 4: TCR-β and TCR-α Sequences of CD8+ TCCs from Patient A.D. TCR-β (upper panel) and TCR-α (lower panel) sequences of TCCs established from the CSF and peripheral blood (PB) are shown. Sequences of eight CSF CD8+ and 15 peripheral blood CD8+ TCCs are given. All clones expressed the TCRBV22 chain according to the nomenclature of Arden et al. (1995) and TCRBV2 according to the IMGT nomenclature (Rowen et al., 1996; Folch et al., 2000). TCCs ADC14, ADC22 and ADC37 (clonotype 1) expressed TCRAV2 (IMGT), with identical TCRAJ regions (lower panel). Determination of the TCRBJ and AJ family (Jα and Jβ) was based on IMGT nomenclature. Clones with identical sequences are shown on a shaded background. NDN = diversity region.

**Fig. 6** TCR-β and TCR-α sequences of CD8+ TCCs from Patient A.D. TCR-β (upper panel) and TCR-α (lower panel) sequences of TCCs established from the CSF and peripheral blood (PB) are shown. Sequences of eight CSF CD8+ and 15 peripheral blood CD8+ TCCs are given. All clones expressed the TCRBV22 chain according to the nomenclature of Arden et al. (1995) and TCRBV2 according to the IMGT nomenclature (Rowen et al., 1996; Folch et al., 2000). TCCs ADC14, ADC22 and ADC37 (clonotype 1) expressed TCRAV2 (IMGT), with identical TCRAJ regions (lower panel). Determination of the TCRBJ and AJ family (Jα and Jβ) was based on IMGT nomenclature. Clones with identical sequences are shown on a shaded background. NDN = diversity region.
A further two clones expressed identical TCR-β chains (clonotype 2), which differed from clonotype 1 by only one amino acid in the diversity (NDN) region. TCR-α analysis of clonotype 1 TCCs identified TCRAV2, with identical TCR-α chain rearrangements in all three TCCs (Fig. 6). In contrast, no similarities in the CDR3 region were detected among 10 CD4+ TCRBV22+ TCCs from the CSF of this patient (data not shown). To exclude the possibility that the oligoclonal expansion of CSF CD8+ T cells was solely a result of similar skewing in the peripheral blood, we analysed TCR-β sequences of 15 CD8+ TCRBV22+ TCCs established from the peripheral blood of this patient at the same time point. All TCR sequences were found to be different and none of the TCC had a similar TCR-β sequence compared with the CSF clonotypes (Fig. 6).

Similar results were obtained in Patient K.H. (Fig. 7). Eight CD8+ and 10 CD4+ TCCs expressing TCRBV17 were established from the CSF. Three of the CD8+ TCCs expressed identical TCR-β chain sequences (clonotype 3). TCR-α analysis demonstrated identical TCRAV and AJ sequences among the TCCs (TCRAV24, AJ41–1 according to immunogenetics nomenclature). We identified two additional TCCs that expressed identical TCR-β sequences (clonotype 4) without similarities to clonotype 3. A further two TCCs showed rearranged TCR-β chains with identical diversity and joining regions, although they differed by two amino acids in the TCRBV-NDN junction sequence (pair 1). In addition, these two clones expressed identical TCRAV and AJ sequences, with only one nucleotide exchange in the splicing region, resulting in a single amino acid change in the TCR.

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**Fig. 7** TCR-β and TCR-α sequences of CD8+ CSF TCCs from Patient K.H. TCR-β (upper panel) and TCR-α (lower panel) sequences of CD8+ TCCs established from the CSF are shown. All clones expressed the TCRBV17 chain according to the nomenclature of Arden et al. (1995) and TCRBV19 according to the IMGT nomenclature (Rowen et al., 1996; Folch et al., 2000). TCCs KHC12, KHC13 and KHC39 expressed TRAV24 (IMGT), with identical joining regions. TCCs KHC1 and KHC4 expressed TRAV1–2 (IMGT), with identical AJ chains but one nucleotide exchange in the joining region. Clones with identical sequences are shown on a shaded background; similar sequences are shown in shaded boxes with sequence differences underlined. NDN = diversity region.
chain. In contrast, TCCs derived from cell populations that were not enriched in CSF according to the TCRBV expansion profile (e.g. CD4+ TCRBV17+ and CD8+ TCRBV1+ or TCRBV2+ or TCRBV8+) differed in their rearranged TCR-chain sequences (Fig. 8).

**Discussion**

It is generally accepted that T cells are involved in the pathogenesis of multiple sclerosis. This hypothesis is supported by several findings. Activated T cells are present at high density in CNS lesions and also in the CSF of multiple sclerosis patients (Brosnan and Raine, 1996; Lucchinetti et al., 1996). Linkage and association studies have shown that the HLA locus coding for molecules involved in T-cell recognition is important for the development of multiple sclerosis (Olerup and Hillert, 1991). EAE, an animal model of multiple sclerosis, is transferred passively by myelin-specific T cells but not by other compartments of the immune system (Wekerle et al., 1994). Although indirect evidence points to T cells as mediators of the disease, little is known about the nature of the T-cell response in the CNS of multiple sclerosis patients.

To investigate this question, we systematically studied peripheral blood T cells from a large number of multiple sclerosis patients and a group of healthy controls matched for age and gender. This study did not reveal significant differences between multiple sclerosis patients and healthy controls in the expression of TCRBV chains on peripheral blood T cells. This finding contrasts with those of some previous studies, which showed a difference in TCRBV chain expression in multiple sclerosis patients using PCR-based semiquantitative methods (Monteiro et al., 1996; Musette et al., 1996; Gran et al., 1998). The diverging results may be explained in part by differences in the detection method and the lack of age-matched controls in some of these studies. Furthermore, we investigated TCRBV chain expression on peripheral blood and CSF T cells from multiple sclerosis patients. TCRBV chain expression analysis without in vitro manipulation of T cells revealed highly significant differences in TCRBV chain expression between peripheral blood and CSF cells. These differences were found to be persistent in

<table>
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<th>Phenotype</th>
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<th>NDN</th>
<th>BJ-region</th>
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**Fig. 8** TCR-β sequences of control and CSF TCCs from Patient K.H. CD8+ and CD4+ CSF TCCs with non-enriched TCRBV chains are shown. No clonotypes and no similarities in the TCRBV chains were found. NDN = diversity region.
individual patients. The most striking observation was the specific enrichment of CD8+ memory T cells in the CSF. In contrast, skewing was observed only rarely on CD4+ T cells. In two patients studied, we demonstrated oligoclonal expansion as the cause of the accumulation of CD8+ T cells in the CSF. Interestingly, in one patient, five of eight CD8+ T CCs from the CSF expressed a highly similar TCRBV chain, which differed only by one amino acid. In the second patient, we found identical sequences in three of eight T CCs. Two additional T CCs showed identical TCR-β sequences and two T CCs had highly similar TCR sequences. Neither CD4+ CSF-derived T CCs nor CD8+ blood T CCs expressing the same TCRBV chain showed clonal or oligoclonal expansion. Although these findings are based only on the analysis of two patients at a single time point and do not demonstrate clonal persistence formally, they are in line with a recently published histopathological study in two multiple sclerosis patients (Babbe et al., 2000). These authors identified oligoclonal T-cell expansions in different brain lesions of multiple sclerosis patients that involved predominantly CD8+ T cells.

Taking the results of both studies together, we conclude that (i) expansion of CD8+ T cells in CSF is a common finding in multiple sclerosis rather than an exception, (ii) CD8+ T cell expansions seem to be stable over time in individual patients and (iii) at least in some patients, the expansion of the CD8+ T-cell population is due to clonal or oligoclonal expansion.

However, the driving antigen for these cells is uncertain. The fact that these cells are specifically enriched in the CNS suggests that they are stimulated by brain-resident target antigens. Theoretically, three different sources are possible: (i) a self antigen that is abundant mainly in the brain, (ii) an immune cell-derived epitope and (iii) an infectious agent that persists in the CNS.

On the basis of findings in EAE, myelin antigens have been implicated in the pathogenesis of multiple sclerosis. Recently, EAE was established with CD8+ myelin-specific T cells, demonstrating that MHC class I-restricted T cells can mediate autoimmunity in the CNS (Huseby et al., 2001; Sun et al., 2001). CD8+ T cells specific for myelin antigen have been isolated from humans, but frequency determination in multiple sclerosis patients versus controls has not so far suggested a role of those cells in the pathogenesis of multiple sclerosis. However, these studies have been hampered by difficulty of presenting myelin antigens via the MHC class I pathway and problems in generating CD8+ antigen-specific T CCs from human donors in vitro. Nevertheless, it is possible that CD8+ T cells in the CNS of multiple sclerosis patients are driven by a self antigen. Alternatively, the clonal expansion of T cells may consist of regulatory T cells, which control CD4+ myelin-specific T cells in the CNS. Although this hypothesis cannot be ruled out, the possibility that a polyclonal CD4+ response in the brain is controlled by clonal or oligoclonal CD8+ populations seems to be less likely. Finally, the CD8+ CSF expansion could indeed target a non-self class I-dependent antigen, such as a viral epitope, that is resident in the brain tissue of patients with multiple sclerosis. Epidemiological studies (Sibley et al., 1985; Kurtzke, 1997) and the therapeutic effect of type-I interferons (Johnson and Panitch, 1999) support the role of infectious agents, particularly viral antigens, in the pathogenesis of multiple sclerosis (Sibley, 1985). However, direct evidence for a multiple sclerosis-associated pathogen is lacking (Meinl, 1999). Nevertheless, the possibility of a brain-resident infectious agent as a target for a CD8+ T-cell response in the CNS has to be considered.

Besides the implications of this study for dissecting the T-cell response in the CNS of multiple sclerosis patients, the identification of persistently expanded CD8+ T-cell populations in the CNS of multiple sclerosis patients provides for the first time an opportunity to identify, isolate and characterize these cells. Using novel techniques to analyse T-cell recognition (Hemmer et al., 1997, 1999; Hiemstra et al., 1997), it may be possible to identify their nominal antigen and define the target antigen responsible for the accumulation of CD8+ T cells in the CSF. This may help to clarify the nature of the CNS T-cell response in multiple sclerosis and allow specific immunotherapies to be designed.

Acknowledgements
We wish to thank Annette Hehenkamp, Rainer Holzbach, Dr Steffi Jaekel, Sabine Lnenicka and Kathrin Seibert for technical assistance and Stefan Nessler for comments on the manuscript. B.H. is a Heisenberg Fellow of the Deutsche Forschungsgemeinschaft. The study was supported by the Deutsche Forschungsgemeinschaft (HE 2386/2-1 and 4-1).

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Received June 18, 2001. Revised October 15, 2001. Accepted October 15, 2001