CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery

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Rasmussen encephalitis (RE) is a rare neurological disorder of childhood characterized by uni-hemispheric inflammation, progressive neurological deficits and intractable focal epilepsy. Destruction of neurons and astrocytes by cytotoxic CD8 T cells has been proposed as a pathogenic mechanism underlying this enigmatic disorder. We tested this hypothesis by analysing the clonal composition and T-cell receptor (TCR) repertoire of CD4+ and CD8+ T cells using complementarity determining region 3 (CDR3) spectratyping from peripheral blood and corresponding CNS specimens. Severe perturbations of the TCR repertoire were found in brain infiltrates from all specimens (n = 5). Clonal expansions, as evidenced by peripheral blood analysis (n = 14), belonged to the CD8+ T-cell subset, while CD4+ cells showed normal distributions. Some of those expansions were analysed in the respective CNS specimens by histochemistry. The stainings showed Vβ specific T cells containing the cytotoxic molecule granzyme B and lying in close appositions to NeuN+ neurons and GFAP+ astrocytes. Analysis of corresponding CNS/blood specimens revealed overlapping but also CNS-restricted expansions of certain TCR clonotypes suggesting expansions of T cells within the target organ itself. Longitudinal analysis of peripheral blood samples (n = 5) demonstrated dominance but also longitudinal persistence of specific CD8 T-cell clones over time. The Vβ/Jβ usage, length of the CDR3, and biochemical characteristics of the CDR3 amino acids suggested high similarities putatively related to common driving antigen(s) without shared clones. Taken together, our data strongly support the hypothesis of an antigen-driven MHC class-I restricted, CD8+ T cell-mediated attack against neurons and astrocytes in the CNS dominating the pathogenesis in RE.

Keywords: Rasmussen encephalitis; CDR3 spectratyping; CD8 cytotoxicity; clonal expansion

Abbreviations: CDR3 = complementarity determining region 3; HD = healthy donor; NDN = D segment with flanking N sequence; TCR = T-cell receptor; PBMC = peripheral blood mononuclear cells; RE = Rasmussen encephalitis
Introduction

Rasmussen encephalitis (RE) is a very rare chronic progressive neurological disorder affecting mostly children and associated with hemispheric atrophy, focal epilepsy, intellectual decline and progressive neurological deficits (Rasmussen et al., 1958; Bien et al., 2005; Dubeau et al., 2007). The aetiology and pathogenesis of this severely disabling inflammatory disease are still enigmatic. An intriguing feature of RE is the restriction of the inflammatory process to one brain hemisphere, setting it apart from any other inflammatory disease of the CNS. Histopathological findings in RE comprise lymphocytic infiltrates, microglial nodules, neuronal and astrocytic loss, and gliosis of the affected hemisphere (Robitaille, 1991; Farrell et al., 1995). The hypothesis of RE as a primarily antibody-driven attack against neuronal structures [e.g. the glutamate receptor GluR3 (Rogers et al., 1994)] could not be confirmed in larger cohorts (Wiendl et al., 2001; Watson et al., 2004). Active brain inflammatory lesions contain large numbers of T lymphocytes, which are recruited early within the lesions suggesting that a T cell-dependent immune response contributes to the onset and evolution of the disease (Farrell et al., 1995). Moreover, the histopathological observation of granzyme B-containing CD8+ T cells in direct apposition to MHC class I positive neurons raised the hypothesis of a CD8+ T-cell mediated neuronal attack as a key pathogenetic mechanism underlying RE (Bien et al., 2002). Apart from neuronal cell death, CD8 cells may also be responsible for the degeneration of astrocytes found in RE lesions (Bauer et al., 2007).

The antigens of these brain-infiltrating lymphocytes are still unknown. It is not even clear yet, whether the CNS-directed T-cell response is focused towards particular antigens. Experimentally, this could be proven by demonstrating that individual clones are expanded in the tissue (Dornmair et al., 2003). This was possible in multiple sclerosis (Oksenberg et al., 1993; Skulina et al., 2004; Junker et al., 2007), but so far, only one study on the T-cell receptor (TCR) repertoire in RE has been published (Li et al., 1997). No information is available regarding the putatively pathogenic CD8+ T cells and longitudinal behaviour of T cells in RE patients.

Here we provide a detailed analysis of the TCR repertoire from RE patients’ brain biopsy specimens as well as from time-matched and non-time-matched peripheral blood samples. CD4+ and CD8+ T-cell CDR3 spectratypes and sequences of dominant expansions in the CNS were determined in CNS samples of RE patients and compared with their peripheral T-cell repertoire, if available in serial peripheral blood samples. Additionally, morphological studies have been performed using immunohistochemistry and visualizing expanded T-cell populations in the brain specimens, their position and granzyme B positivity. We provide strong evidence for an antigen-driven MHC class-I restricted, CD8+ T cell-mediated attack against antigens presented on neurons and astrocytes in the CNS as the prevalent pathogenic process in RE.

Materials and Methods

Patients and diagnosis

The local Ethics Committee (Ethikkommission der Universität Bonn) approved all studies and clinical investigations were conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants or their parents or legal guardians. Fourteen patients (age 6–59, mean age 11) diagnosed with RE according to the typical clinical, MRI and neuropathological findings as proposed in (Bien et al., 2005) were enrolled in this study. From all RE patients, peripheral blood samples were obtained when patients were not receiving immunotherapy. Serial blood samples were available from five patients all receiving either tacrolimus or i.v. immunoglobulin treatment when these follow-up samples were taken. CNS tissue was obtained during neurosurgical operations (diagnostic open brain biopsies or functional hemispherectomies) from 12 RE patients. Material from all patients was paraffin-embedded. From five of these 12 patients, parts of the brain samples were cryoprotected in addition. As controls, peripheral blood was obtained from healthy volunteers and CNS tissue from autopsies in persons without known neurological disease, stroke victims and neurological tissue from glioblastoma patients (Neuropathology University Wuerzburg, Prof. Roggendorf).

Immune cell purification and isolation

Peripheral blood mononuclear cells (PBMC) were isolated via density gradient centrifugation using lymphocyte separation medium (PAA Laboratories, Linz, Austria) within 24 h after blood draw. CD4+ or CD8+ T cells were negatively isolated from PBMC using magnetic bead isolation (negative isolation procedure, MACS®, Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer’s instructions. Purified T-cell subsets were over 95% pure as controlled by flow cytometry analysis.

Immunohistochemistry

Four-micrometre thick paraffin sections were studied by light-microscopy after staining according to established techniques using antibodies to the lymphocytic markers CD3, CD8, granzyme B, CD68, the neuronal marker NeuN and the astrocytic marker glial fibrillary acidic protein (Bauer et al., 2007). Densities of CD3+ and CD8+ cells were determined as described earlier (Bien et al., 2002). When clonal expansions of Vβ2 and Vβ3 were found in the brains of patients #03 and #10, 10-μm sections of cryoconserved brain material of these patients were double-stained immunohistochemically for light microscopy (antibodies to other Vβ families for immunohistochemistry use are either not available or do not give reliable staining results as reported by others (Bosboom et al., 2001) or tested in our own laboratory). For the Vβ chains, the following antibodies were used: (1) Vβ2 (mouse-IgG antibody, clone MPB2D5, from Beckman Coulter, Marseille, France, dilution 1:50) and (2) Vβ3 (mouse-IgM, clone CH92, from Beckman Coulter, 1:100). Co-stainings were done using the following antibodies: (A) CD8 (mouse-IgG, clone CB/144B, from Dakocytomation, Glostrup, Denmark, 1:50); (B) CD3 (rabbit-IgG, clone SP7, from Lab Vision, Fremont, CA, USA, 1:500); (C) NeuN (mouse-IgG, clone A60, from Millipore, Billerica, MA, USA, 1:50); (D) glial fibrillary acid protein (GFAP, mouse-IgG,
RNA isolation

RNA from peripheral blood T cells was isolated using standard methods (Quiagen RNeasy kit; Quiagen, Hilden, Germany) according to the manufacturer’s instructions. Isolation of RNA from CNS specimens was performed from cryo-preserved CNS tissue of patients with the confirmed clinical and histopathological diagnosis of RE (see above).

To acquire statistically significant data, we included \(1 \times 10^6\) CD8+ T cells in the analysis of each sample. We quantified the number of CD8+ T cells in 4 \(\mu\)m sections of each RE sample by immunohistochemistry. From this number the total amount of CNS tissue (as \(50\, \mu\)m slices) was calculated to be used for RNA-isolation (\(\sim 10^5\) CD8+ T cells) to make sure that we included roughly the same number of cells in each analysis. Slices were directly transferred into TriZol (Invitrogen, Mannheim, Germany), homogenized and then processed according to the manufacturer’s protocol.

CDR3 spectratyping

For the CDR3-spectratyping, we used the V\(\beta\) forward primers as described in Monteiro et al. (1996), the J\(\beta\) reverse primers as described previously (Puiseux et al., 1994), and two different C\(\gamma\) reverse primers: ‘SpTy\(\beta\)-out’ (Junker et al., 2007) and ‘C\(\gamma\)-R’ (Monteiro et al., 1996). The V\(\gamma\) nomenclature according to Arden et al. (1995) is used throughout the manuscript.

For the peripheral blood derived samples, the following protocol was applied: First, the cDNA was used in 25 V\(\beta\)-C\(\gamma\) reactions: 1.25 \(\mu\)l 10 pmol/\(\mu\)l V\(\beta\) primer, 1.25 \(\mu\)l 10 pmol/\(\mu\)l C\(\gamma\)-R primer, 0.5 \(\mu\)l cDNA, 0.25 \(\mu\)l 2.5 mM dNTPs (Promega, Mannheim, Germany), 2.5 \(\mu\)l 10x buffer, 0.1 \(\mu\)l TaqPolymerase (both Applied Biosystems), 19.15 \(\mu\)l DEPC-H\(\text{H}_2\)O. PCR conditions: 94°C, 6 min; 94°C, 1 min, 59°C, 1 min, 72°C, 1 min (40 x); 72°C, 7 min. After these first-round PCRs, every PCR product was subjected to 13 individual V\(\beta\)-J\(\gamma\)-‘run-off’ reactions (modified from Pannetier et al., 1993; Baltiwalla et al., 1996) with 13 5'-fluorescence-tagged J\(\gamma\) primers to differentiate between individual TCR J\(\gamma\)-regions and also with a 5'-fluorescence-tagged C\(\gamma\)-R primer. The length of these fluorescence-labelled PCR products was then analysed on an ABI 3130 genetic analyser (Applied Biosystems), applying a module for fragment analysis. 500-ROX (Applied Biosystems) was the internal standard in each sample.

In the case of the CNS-samples, we used a more sensitive protocol (Junker et al., 2007) to compensate for the low T-cell numbers in the CNS specimens. Briefly, we introduced a semi-nested pre-amplification PCR step before the PCR described above. There, we used the same forward primers as above, but employed the C\(\gamma\)-specific reverse primer ‘SpTy\(\beta\)-out’ (10 pmol/\(\mu\)l) that hybridizes downstream of C\(\gamma\)-R, but upstream of the RT-primer C\(\gamma\)-RT. Samples were incubated for 5 min at 94°C. Then PCR was run for 30 cycles of: 94°C, 1 min, 56°C, 1 min, 72°C, 1 min, followed by an incubation of 72°C, 10 min. From this PCR, 1 \(\mu\)l reaction was used as template in the protocol mentioned before. NED-tagged primers were bought from Applied Biosystems (Foster City, CA, USA) and all other primers were provided by Metabion (Martinsried, Germany).

Data analysis

Data were processed by GeneMarker® software (SoftGenetics, State College, PA, USA). For all PCR products of V\(\beta\)- and C\(\gamma\)-primers, or V\(\beta\)- and J\(\gamma\)-primers, respectively, we plotted the peak intensities versus the fragment lengths. Unskewed repertoires yield Gaussian length distribution, whereas skewed repertoires show distortions (Pannetier et al., 1995). To evaluate the relative skew of the repertoires, we used the data obtained from blood of the healthy control persons as a standard: for each V\(\beta\)-C\(\gamma\)-combination, we measured the average fragment lengths, i.e. we determined the positions of the maxima of the Gauss distribution. At these length positions, the peak intensity of the corresponding V\(\beta\)-C\(\gamma\) product from the patients was defined as ‘1.0’. A V\(\beta\)-C\(\gamma\) product from a patient was considered skewed, if a peak exceeded the intensity on this semi-quantitative scale for a factor of greater than two. If the factor was between 1.0 and 2.0, the repertoire was considered slightly disturbed. Very high peaks were reamplified with unlabelled V\(\beta\) and J\(\gamma\) primer and the PCR products were sequenced directly. If a peak was not high enough for direct sequencing, the PCR product was sequenced after using the TA cloning kit pGEM-T (Promega).

Results

For patients’ demographical and sample collection data, see Table 1.

Basic immunopathology of brain specimens

For comprehensive quantitative immunopathological work-up, paraffin-embedded specimens were available for analysis. They revealed a mean density of 20 CD8+ T cells/mm² on 4 \(\mu\)m sections. A mean of 84% of them was CD8+, and 42% was granzyme B positive, respectively. The mean proportion of
CD8+ cells lying in close apposition to neurons was 5%. CD68+ cells were observed at a density of 147/mm², 1% showing macrophage morphology (the rest to be considered microglial cells, partly forming nodules). These figures are highly similar to those obtained from a previous RE cohort (Bien et al., 2002).

There was neuronal and astrocytic cell loss (not quantified).

Expanded T-cell clones infiltrate the CNS

We analysed the CDR3 length distribution for all Vβ families by CDR3 spectratyping from cryoprotected biopsy samples of five RE patients. In all patients, the TCR Vβ repertoire of the infiltrating T cells revealed clonal perturbations. Some examples are shown in Fig. 1A. Oligoclonally expanded T-cell clones were detected in the CNS of all patients (Fig. 1B). In 15–50% of the assessed Vβ families we detected oligoclonal expansions.

We also analysed various CNS control specimens. The first group represented CNS tissue specimens, in which neuropathological alterations were absent. In four out of five cases, we could not get a sufficient spectratyping signal due to the very small number of T cells in the CNS of these non-inflammatory controls (only few Vβ families were detectable). The fifth case, however, showed the complete Vβ repertoire with few clonal expansions (<20% of the assessed Vβ families, data not shown). Additionally, we analysed CNS specimens from three stroke patients. These samples are rich in mononuclear cells, but the accumulation is random and without any role for antigen-specific T cells. The TCR repertoire of the invading T cells in the detectable Vβ families was normally distributed (data not shown). As a third control group, we analysed tissue specimens from glioblastoma patients (n = 5). These samples showed a very strong PCR signal due to the high amount of T cells in the tissue. Several of the Vβ families showed signs of clonal expansions (examples shown in Fig. 1C), which is in line with previous studies (Ebato et al., 1993). Interestingly, there were identical peaks in different patients (Fig. 1C, Vβ13.1).

Inter-individual analysis of T-cell expansions as analysed by Vβ/Jβ spectratyping suggested similarities in the antigen specificity of the expanded T-cell clones. To characterize the expanded clones, the TCR β-chains were sequenced. The deduced amino acid sequences of the CDR3 regions of CNS expanded T-cell clones are listed in Fig. 2, Supplementary Table 1. In the five patients, with CNS and peripheral blood samples available, no shared ‘public’ clones were found. The Vβ-usage and the CDR3 lengths and amino acid sequences were diverse, except some similarities (Vβ/Jβ/CDR3) between the Vβ18 clones of patients #01 and #07 (Fig. 2A). Between, both chains are of the Vβ18 family, thus they carry identical CDR1 and CDR2 loops, their CDR3 loops are of the same length and consist exclusively of small or hydrophilic amino acids (with the exception of a single leucine in patient #07). Thus, even if, for example, there was a Vβ11-Jβ2.7 expansion in the CNS of patients #01, #04 and #10, with identical lengths of the CDR3 regions, the amino acids of the NDN regions did not show high homology (Fig. 2B). This is most strikingly evident from the positions of the arginine residues that introduce permanent positive charges into the CDR3 loop.

Skewed TCR repertoire of peripheral blood CD8T cells parallels the repertoire in brain

To assess whether the clonal expansions of T cells were restricted exclusively to the CNS or were also present in the periphery, we purified CD4+ and CD8+ T cells from the peripheral blood of RE

### Table 1 Clinical details of the patient cohort

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Gender</th>
<th>Age at onset of neurological symptoms (prodromal stage) (years)</th>
<th>Age at onset of acute stage (years)</th>
<th>Peripheral blood sampling (months after onset of acute stage)</th>
<th>Brain biopsy (months after onset of acute stage)</th>
<th>Number of CD8+ T cells/mm² in brain parenchyma</th>
</tr>
</thead>
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<tr>
<td>#01</td>
<td>M</td>
<td>10.8</td>
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<td>5.7</td>
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<td>12</td>
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<td>5.0</td>
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<td>11</td>
<td>3</td>
</tr>
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<td>2.9</td>
<td>2</td>
<td>0</td>
<td>n.a.</td>
</tr>
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<td>4.5</td>
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<td>4</td>
</tr>
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<td>6.1</td>
<td>26</td>
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<td>n.a.</td>
</tr>
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<td>18.1</td>
<td>7</td>
<td>6</td>
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<td>6.5</td>
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<td>50.6</td>
<td>–4</td>
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<td>n.a.</td>
</tr>
<tr>
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<td>F</td>
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<td>11.4</td>
<td>28</td>
<td>3</td>
<td>n.a.</td>
</tr>
<tr>
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<td>15.5</td>
<td>36</td>
<td>10</td>
<td>n.a.</td>
</tr>
<tr>
<td>#13</td>
<td>F</td>
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<td>28</td>
<td>n.a.</td>
<td>n.a.</td>
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<td>4.5</td>
<td>21</td>
<td>8</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

*Patients with available corresponding brain tissue and blood samples.*
patients \((n=14)\) and from healthy volunteers as controls \((n=7)\) were analysed for their TCR repertoire. CD8+ T cells from RE patients showed strong skewings (Fig. 3A), with several clonal expansions of many V\(\beta\) families. In contrast, the repertoire of CD4+ T cells revealed only marginal deviations from the profile found in healthy individuals. As expected, spectratyping of CD4+ and CD8+ T cells in healthy volunteers revealed repertoire patterns that closely resembled Gaussian CDR3 length distributions of all V\(\beta\)s (data not shown). Figure 3B summarizes the results of the repertoire analysis of CD8+ T cells from all RE patients. Strong clonal expansions in peripheral blood were detected in all patients (red dots). Inter-individual comparison of CDR3 length distributions among all RE patients in our cohort revealed that peripheral clonal expansions of some V\(\beta\) segments were more prevalent than others, i.e. 8 of 14 patients had clonal expansions in V\(\beta\)11 and V\(\beta\)15, whereas disturbances in the V\(\beta\)1 or V\(\beta\)7 segments were never detected (Fig. 3B).

Correspondence between TCR repertoire in CNS and peripheral blood

In all five RE patients with brain samples available, we also had access to blood samples. This allowed us to compare the TCR repertoire in CNS infiltrating versus peripheral blood derived CD4+ and CD8+ T-cell populations. We observed shared expansions (patient #01: 12%, #03: 10%, #04: 25%, #07: 23%, #10: 45%) with the same V\(\beta\) segment and identical CDR3 length in CNS and parallel peripheral blood samples within the CD8+ T-cell
subset, but never in the CD4+ population (Fig. 3B). To make sure that these clones in the CNS and periphery are identical, we sequenced exemplarily the respective clones in the patients #01 and #10. These clones turned out to have the same CDR3 nucleotide sequence in the CNS and in the peripheral CD8+ T-cell compartment (one example is given for patient #01, Vβ11Jβ2.7, Fig. 3B). Apart from the similarities of TCR expansions shared between CNS samples and peripheral blood, analysis of five patients with parallel CNS and blood samples revealed numerous clonal expansions restricted to either the CNS (23–59%) or the periphery (18–45%).

Morphological studies of expanded T-cell populations in CNS specimens

To confirm the presence of clonally expanded CD8+ T-cell clones in CNS and to support their putative pathogenic role, we exemplarily performed immunohistochemical double stainings for T-cell markers and Vβ2 and Vβ3 families which were clonally expanded in the CNS of patients #03 and #10. These clones turned out to have the same CDR3 nucleotide sequence in the CNS and in the peripheral CD8+ T-cell compartment (one example is given for patient #01, Vβ11Jβ2.7, Fig. 3B). Apart from the similarities of TCR expansions shared between CNS samples and peripheral blood, analysis of five patients with parallel CNS and blood samples revealed numerous clonal expansions restricted to either the CNS (23–59%) or the periphery (18–45%).

Persistence of the CD8 TCR repertoire in peripheral blood over time

To further support the hypothesis that CD8+ T cells play a key role for the self-perpetuating nature of the CNS immunopathology, we next investigated whether individual T-cell expansions persist over time or would be subject to change. Spectratyping analysis of CD8+ T cells was therefore performed on five RE patients at several time points after the first blood sample was taken (9–25 months). We found that particular CD8+ T-cell clones persisted in individual RE patients over periods of more than 1 year. The relative height of the peaks, however, varied over time, indicating fluctuations in absolute numbers in peripheral blood. This pattern was observed in all five patients. Exemplary results of three patients are shown in Fig. 6A. As an additional point, there was no significant correlation between disease onset and strength of the clonal expansions.

One patient (#07) underwent immune ablation and stem cell reconstitution with CD34+ haematopoetic stem cells from her identical twin. Accompanying immune reconstitution of this patient, the long-term persisting CD8+ T-cell clones reappeared within a period of a few months after ablation (data not shown).

Figure 2 Comparison of clonal CNS expansions in different patients. Clonally expanded T cells from the CNS of RE patients were analysed by CDR3 spectratyping and sequencing of the PCR products. The deduced amino acid sequences of the CDR3 are shown below the spectratyping profiles (NDN region in bold). The peak numbers are the exact length of the PCR fragment (in bp). (A) Vβ18 in the CNS of two patients (#01, #07) as well as Vβ18Jβ1.5 in the CNS of patient #01 and Vβ18Jβ2.7 in the CNS of patient #07. (B) Vβ11 and Vβ11Jβ2.7 in the CNS of three patients (#01, #04, #10).
Figure 3 The TCR repertoire of peripheral blood CD8+ but not CD4+ T cells is skewed in RE patients PBMC from 14 RE patients, magnetically separated into CD4+ and CD8+ T cells, were subjected to spectratyping analysis. (A) Representative example of the peripheral TCR repertoire of a RE patient (#10). White bars represent CD4+ T cells, black bars CD8+ T cells. The x-axis shows the CDR3 length of each possible TCR in the assessed Vβ regions, while the y-axis shows the normalized peak height (for the calculation see Materials and methods). (B) The x-axis shows the Vβ region of observed peripheral expansions of CD8+ T cells, the y-axis shows the number of 14 RE patients. An expansion is indicated by a red dot. Patients, where a CNS sample was available, are tinted grey. If an expansion (same Vβ and CDR3 length) also exists in the corresponding CNS sample (Fig. 1B), the dot is marked with a black line. An empty black line represents an expansion in the CNS, which is not present in the periphery. The amino acid sequence of the CDR3 of one exemplary matching clone is given (NDN region in red).
Figure 6B demonstrates the persistence of an expanded T-cell clone in the peripheral CD8+ T-cell compartment of patient #01 (time period of 15 months), confirmed by sequencing. This identical clone was also strongly expanded in two independent areas of the CNS from the same patient: one part of the biopsy comprised mainly cortical tissue, the other one subcortical white matter (both within the superior frontal gyrus), and both showed typical histopathological characteristics of active RE. Interestingly, the TCR repertoires from these two brain regions in patient #01 were identical, suggesting a homogenous clonal distribution in the affected hemisphere.

Discussion

Destruction of neurons and astrocytes by cytotoxic CD8+ T cells has been proposed as pathogenic mechanism underlying RE.

We tested this hypothesis by analysing the clonal composition and TCR repertoire of CD4+ and CD8+ T cells using CDR3 spectratyping from peripheral blood and corresponding CNS specimens. We show here that the existing clonal expansions in RE patients, at least those in peripheral blood, are predominantly due to CD8+ cells and that they may persist for at least 1–2 years.
All CNS specimens from RE patients showed severe perturbations of the TCR repertoire. All patients showed clonal T-cell expansions, which strongly supports the hypothesis of an antigen-driven, T cell-mediated autoimmune process in contrast to a secondary immune response. This has been shown in many other autoimmune diseases and it confirms previous findings in RE (Li et al., 1997).

Upon exemplary morphological assessment of expanded Vβ families in the brains of two patients, T cells expressing these Vβ families were readily found. These lymphocytes contained the cytotoxic molecule granzyme B and were found in close appositions to neurons and astrocytes (Figs 4 and 5) thereby extending previous pathogenetic findings on RE T cells in general to lymphocytes with individually overexpressed Vβ clones (Bien et al., 2002; Bauer et al., 2007).

Brain specimens from stroke victims showed normal distribution of the TCR repertoire. This is expected due to the unspecific T-cell influx occurring on the site of a stroke lesion. In the one case of non-pathological CNS autopsy tissues allowing us to get an adequate PCR signal we found slight TCR perturbations, which is not surprising, because the person was 64 years of age, where age-related clonal expansions are quite common (Messaoudi et al., 2004). As an even more meaningful control experiment, we investigated tissue specimens from glioblastoma patients. It has been published previously that lymphocytes in glioma tissue show skewed distributions of the TCR repertoire (Ebato et al., 1993) and even contain the same amino acids in the NDN region in some cases (Ebato et al., 1994). In our analyses we also found several clonal expansions in the glioblastoma patients’ CNS, confirming the above studies. Strikingly, we found matching Vβ13.1 peaks in two patients (Fig. 1C). Of note, it is known that Vβ13.1 is preferentially expanded in glioma patients (Ebato et al., 1993). Both, healthy control subjects and glioma patients confirmed the results expected from previous studies and therefore provided evidence that our method may reveal clonal expansions without introducing particular biases.

When we detected peaks similar in Vβ/Jβ composition and CDR3 length in several patients, we sequenced their CDR3 regions. Although there are some similarities (Fig. 2A), the majority of the clonal expansions are too different to be able to assume common triggers. However, thus far identical TCR expansions have only been found in very few diseases, e.g. paroxysmal nocturnal haemoglobinuria (PNH) (Gargiulo et al., 2007), where the finding of identical TCRs (so called public clones) can be considered as an unambiguous indication for the recognition of similar epitopes, albeit it is probable that these TCRs do not...
recognize the same peptide under the given different MHC restrictions, especially because the patients in this study did not share HLA genes. Shared (public) clones with the same CDR3, however, can only be assumed if one of the HLA genes of different individuals matches. In the mouse system with a given inbred identical MHC locus, such public clones against single peptides have been observed (Menezes et al., 2007). Similar findings in humans are so far very scarce. On the other hand, the similarity in the Vβ/Jβ composition and CDR3 length (Fig. 2A) should be considered as too high to be explained by pure coincidence in the composition of these expansions, as the theoretical size of the T-cell repertoire is \( \sim 10^{19} \) (Davis and Bjorkman, 1988).

Surprisingly, we found very strong clonal expansions exclusively in the peripheral CD8+ T-cell compartment of RE patients providing evidence for an ongoing CD8+ T cell-mediated immune reaction in the periphery of all RE patients. Interestingly, the expansions showed skewed Vβ segments. While this bias towards certain Vβ segments could be interpreted as an indication of common antigenic triggering leading to similar expansions in different patients, the Vβ segment alone cannot prove antigenic similarities. Also, it has been shown that CD8+ T cells are more prone to antigen-driven or spontaneous age-related expansions than CD4+ T cells. This is especially observed in older aged human individuals (Messaudy et al., 2004). The mean age of RE patients at the time of the analysis was 11 years; age-related expansions therefore seem highly unlikely as an explanation of our data. As expected, the peripheral TCR repertoire of the CD8+ T cells of healthy individuals was normally distributed with only insignificant aberrations that reached a very small fraction of the patients’ disturbances, if they occurred at all.

We found that 10–45% of the clonal expansions in the brains of RE patients were shared with the peripheral CD8+ T-cell repertoire, while there were no shared expansions within the CD4+ T-cell compartment. Of note, many shared TCR sequences were validated by DNA sequencing (exemplary in Fig. 6B). In addition to the low number of parenchymal CD4+ T cells in RE biopsy specimens, these data provide very strong evidence that the CNS infiltrating, oligoclhonally expanded T-cell clones are CD8+ T cells.

We also found several expansions that were restricted to the brain or to the periphery in all five patients. This is not surprising, since the peripheral TCR repertoire in inflammatory CNS disorder only partly reflects the repertoire in the CNS. Examples from other chronic progressive inflammatory CNS disorders, i.e. multiple sclerosis has revealed similar findings (Skulina et al., 2004).

We found that several CD8+ T-cell expansions persisted for up to 15 months. The fact that these CD8+ T-cell clones stay expanded for more than 1 year strongly supports their assumed pathogenic role in RE. We speculate that these cells (re)encounter their specific antigen during this time because T cells that are not confronted with their antigen for a certain time after an expansion will be subject to regulatory mechanisms to ensure homeostasis, mainly over the Fas/FasL pathway (reviewed by Lenardo et al., 1999).

In the case of the stem cell transplantation, the re-occurrence of the clones might possibly be explained by assuming that T cells residing in the CNS are not sufficiently eradicated by the immunablative regimen. The detection of expanded T-cell clones in peripheral blood thus could indicate either a strong persisting memory response or, much more likely, hint towards an ongoing exposure to the yet unknown antigen(s). Of note, the peripheral TCR repertoire of CD4+ and CD8+ T cells in the patient’s identical twin was completely normally distributed (data not shown), proving that RE is not (solely) of genetic origin, but dependent on an exogenous trigger, which might possibly be a virus. Also, the application of various immunotherapeutic regimes (e.g. intravenous immunoglobulins or tacrolimus) did not seem to affect the persistence of expanded individual T-cell clones in the patients whom we examined over a longer period of time. An alternative explanation, which we cannot rule out completely, might be that the persistence of these clones results from this therapy. However, it seems highly unlikely that drugs used for immuno regulatory purposes promote the survival of specific clones.

Our observations clearly favour the hypothesis of an ongoing pathogenic process triggering survival or continuous activation of pathogenic CD8+ T-cell clones detectable in the peripheral blood of RE patients. Exposure to either CNS-derived autoantigen(s) or a persisting viral infection would be compatible with such a hypothesis.

It is tempting to speculate that in RE patients the common trigger of the focused, dominant CD8+ response is a similar antigen, which persists over a long period of time and sustains the stimulus necessary for the perseverance of these putatively pathogenic clones. The long-term persistence (or re-occurrence) of putatively pathogenic T-cell clones despite therapy (or even immunosuppression) may indicate an ongoing exposure of the immune system to the antigenic trigger. This trigger (autoantigen or virus) could very well reside within the CNS. The finding of identical TCR clones between the CNS and peripheral blood compartment in patients with matching CNS-blood samples is in line with this assumption. Furthermore, TCR clones were found between individual patients demonstrating high biochemical similarities in their CDR3, albeit not fully matching on the CDR3 amino acid level. The antigenic specificity of these CNS-infiltrating CD8+ T cells in RE patients, however, remains elusive at present.

Taken together, our findings support the assumption that local clonal expansions of CD8+ T cells represent a pathogenic feature of active RE lesions. Partly, expanded, putatively pathogenic CD8+ T-cell clones can also be found in the peripheral blood of RE patients, where a severely perturbated TCR repertoire of CD8 but not CD4 cells TCR repertoire is constantly found in all patients.

We provide strong evidence for an antigen-driven MHC class-I restricted, CD8+ T cell-mediated attack against antigens presented on neurons and astrocytes in the CNS dominating the pathogenesis in RE. While further work is warranted to characterize the nature of the recognized antigenic structure(s) by putatively pathogenic T-cell clones, our study has significant implications relating to our understanding of RE pathogenesis.

Supplementary material

Supplementary material is available at Brain online.
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