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Increased activation of Iba1⁺ microglia in pediatric epilepsy patients with Rasmussen's encephalitis compared with cortical dysplasia and tuberous sclerosis complex

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Abstract

Microgliosis is prominent in Rasmussen's encephalitis (RE), a disease with severe seizure activity. However, it is unclear if microglial activation is similar with different histopathologic substrates. Iba1-immunolabelled microglial activation was assessed in neocortex from pediatric epilepsy surgery patients with RE (n=8), cortical dysplasia (CD; n=6) and tuberous sclerosis complex (TSC; n=6). Microglial reactivity was increased, in severely affected RE areas (29% labeling) compared with minimally affected areas of RE cases (15%) and cases of TSC (14%) and CD (12%). There was no qualitative association of Iba1 immunolabelling with the presence of CD8⁺ cytotoxic T-cells and no statistical association with clinical epilepsy variables, such as seizure duration or frequency. Iba1 appears to be an excellent marker for detecting extensive microglial activation in patients with RE. In addition, this study supports the notion that Iba1-labeled microglial activation is increased in patients with active RE, compared with cases of CD and TSC.

Keywords

Seizures; inflammation; astrocytes; surgery; cytomegalic neurons

Introduction

A syndrome of severe epilepsy and progressive hemispheric dysfunction, usually occurring in children, is named Rasmussen's encephalitis (RE), after Theodore Rasmussen (Rasmussen et

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al., 1958). The origin of the disease is unknown. Suggestions have been made implicating a viral infection of the brain or an autoimmune process as the cause, but no apparent consensus as to etiology has been reached (Rasmussen, 1978; Bien et al., 2005; Power et al., 1990; Rogers et al., 1994; Vinters et al., 1993; Farrell et al., 1995). T-cells dominate the inflammatory response, and lately cytotoxic Tcell attack on neurons and astrocytes has been proposed as being a key pathogenetic mechanism (Bien et al., 2002; Bauer et al., 2007). Interestingly Pardo et al. (2004) described a “significant heterogeneity in the stages of cortical pathology and the multifocal nature of the disease”, demonstrating various stages of inflammation present in the brain of patients with RE (Pardo et al., 2004). The pathologic change in RE is almost exclusively confined to one cerebral hemisphere, though given that the “unaffected” cerebral hemisphere is only rarely biopsed, the degree of its involvement may be underestimated (Larner et al., 1995; Hart et al., 1998). Signs of bilateral inflammatory changes in both cerebral hemispheres have been described in a few patients with RE (Robitaille, 1991; Chinchilla et al., 1994). Pathologic findings can range from an active disease stage with inflammation and the presence of microglial nodules, lymphocytic perivascular cuffing, neuronophagia, astrocytic activation, and glial scarring, to more subtle changes with various levels of neuronal loss and some gliosis, and moderate perivascular inflammation with or without microglial nodules. Whether the inflammatory changes observed in the brain in RE are primary or a secondary reactive response to chronic seizures is still not clarified (Antel et al., 1996).

Extensive microglial activation is a well described phenomenon in RE and a hallmark of this disease (Banati et al., 1999). Reactive microglia display multiple changes as a consequence of their activation, including altered morphology, cellular proliferation, change in antigen expression and synthesis of a variety of active molecules (Streit et al., 1999). To study microglial activation in RE, antibodies against ionized calcium binding adaptor molecule 1 (Iba1), an EF hand calcium binding molecule, were applied as an immunohistochemical marker. The general function of the Iba1 molecule is not well described, although it appears to be involved in membrane ruffling believed to be related to cell motility and phagocytosis by microglia/macrophages and activation of microglia (Imai et al., 2002; Ohsawa et al., 2000). In the brain, Iba1 is considered a specific marker for microglia and macrophages, and was applied in this study due to the excellent detailing of microglial morphology in immunohistochemistry (Imai et al., 1996).

The purpose of this study was to determine whether microglial reactivity in RE was present uniformly in the neocortex of the affected cerebral hemisphere or distributed in a more inconsistent pattern. Additionally the level of microglial reactivity in the neocortex was compared in cases of RE and control cases of cortical dysplasia (CD) and tuberous sclerosis complex (TSC), two other diseases known to cause epilepsy in children that are associated with less prominent inflammatory changes in the cortex (Boer et al., 2006; Vinters et al., 2006).

Materials and Methods

Human brain specimens

In the course of surgical treatment of patients included in this study, tissue samples of brain resections were obtained from the Department of Neurosurgery and transferred to the Neuropathology laboratory at the University of California, Los Angeles. All patients or their parents signed written research informed consent and this study was approved by the UCLA Institutional Review Board (IRB). The basis for surgical treatment for all patients was intractable epilepsy resistant to pharmacological therapy. Surgical treatment involved removal of brain regions generating seizures in all patients except one, where the focus was too large and only diagnostic biopsies were performed (see Table 1). Most cases involved large cortical resections including hemispherectomy. Tissue samples from 8 patients diagnosed with RE

were included in this study. Control samples encompassed neocortical tissue from 6 patients with cortical dysplasia (5 Palmini type IIb and 1 type Ib) and 6 tubers and immediately surrounding tissue from patients with tuberous sclerosis complex (patient clinical characteristics summarized in Table 1) (Palmini et al., 2004).

Clinical Data

Clinical data was abstracted from the medical record for comparison between patient groups and immunostaining features. Clinical data included: gender, age at seizure onset, age at surgery, seizure duration, seizure frequency, type of operation performed (e.g. hemispherectomy, multilobar resections, lobar or focal), side resected, and seizure outcome. Seizure frequency before surgery was recorded as daily or weekly. Seizure control post-surgery was recorded as seizure free or not seizure free. Minimal follow-up was at least one year from surgery for seizure outcome data. Seizure duration was calculated as the interval (in years) from age at seizure onset to age at surgery. For the statistical analysis, clinical data and percent of Iba1 immunostaining (see below) were entered into a statistical program (StatView). Statistical comparisons used t-tests, Chi-square, or ANOVA as appropriate. A priori p-values were set at $P < 0.05$ to be considered statistically significant.

Diagnosis of tuberous sclerosis and Rasmussen encephalitis

Neuropathological findings in brain tissue resections from TSC patients included those previously detailed by Metha et al. (2008): abundant cytomegalic/dysmorphic neurons and balloon cells in cortex or subcortical white matter, calcinosis and rare findings of Ki-67 positive cells (Mehta et al., 2008). Patients were clinically diagnosed with TSC according to the 1998 revised diagnostic criteria for TSC, which classifies features of TSC as major and minor according to Roach et al. (1998) (Roach et al., 1998; Vinters et al., 2006). Genetic analyses of TSC specimens are in progress, but were not available for data analysis.

Establishing the diagnosis of RE was based on the combination of clinical, electrophysiological, and morphological tests as proposed by Bien et al. (2005). Two sets of criteria are applied in ascertaining the diagnosis of RE. The first set consists of focal seizures (with or without *epilepsia partialis continua* (EPC)) and unilateral cortical deficit(s) determined clinically, unihemispheric EEG slowing with or without the presence of epileptiform activity and unilateral seizure, and finally progressive unihemispheric focal cortical atrophy with either grey or white matter T2/FLAIR hyperintense signal, or hyperintense signal or atrophy of the ipsilateral caudate head visualized via MRI. If not all criteria in the first set are present, the conclusive diagnosis of RE can only be made if at least two out of three of the second set of criteria are present. These criteria are the clinical presence of EPC or progressive unilateral cortical deficit(s), progressive unihemispheric focal cortical atrophy demonstrated via MRI imaging, and T-cell dominated encephalitis with activated microglial cells (typically but not necessarily forming nodules) and reactive astrogliosis. RE diagnosis was excluded if there were numerous parenchymal macrophages, B-cells, plasma cells, or viral inclusion bodies present (Bien et al., 2005; Farrell et al., 2007).

Sampling of tissue and areas for analysis

Depending on the availability of resected tissue from each patient and the presence of representative pathology within the specimens, tissue slides from 1-4 resected areas from each patient were chosen for quantitative analysis. The size of each specimen analyzed was determined by measuring the area of the entire Iba1 labeled tissue section applying a method similar to what is described below for quantification of microglial reactivity using Adobe Photoshop (Adobe Systems Inc., San Jose, CA) and ImageJ (NIH, Bethesda, MD). The average areas of sections analyzed from RE patients were $276 \pm 57 \text{ mm}^2$ and from controls $280 \pm 59 \text{ mm}^2$ (mean $\pm$ standard deviation). In tissue from RE patients, 1-7 areas and in tissue from

control patients 2-3 areas from each slide were photographed and the severity of microglial reactivity was analyzed and determined as described below.

Iba1 immunohistochemistry

Microglia were visualized in tissue sections by immunohistochemical labeling of the Iba1 antigen (Ito et al., 1998). Sections of 6 μ m thickness were cut from paraffin-embedded specimens. For immunohistochemistry (IHC) all slides were initially heated at 60°C for 30 min. and then deparaffinized and rehydrated in a series of xylene and graded ethanol. Following rinsing in ddH₂O and PBS and blocking of endogenous peroxidase (3% H₂O₂ in PBS for 10 min.), heat induced epitope retrieval was applied. Sections were heated in TEG buffer (10 mM Trizma base, 0.5 mM EGTA, pH 9.0) in a microwave oven at 100% power (700 W) for 7 min., then for 15 min. at 70% power and finally left to cool down at room temperature for 15 min. After rinsing in ddH₂O and PBS sections were blocked in 2.5% normal horse serum (NHS) and incubated with polyclonal rabbit anti-Iba1 antibody (Wako Chemicals, Richmond, VA) diluted to 1mg/L in antibody diluent (DakoCytomation, Carpinteria, CA) overnight at 4°C. The sections were then rinsed in PBS and incubated with ImmPRESS anti-rabbit Ig (peroxidase) solution (Vector Labs, Burlingame, CA) for 1 hr. at 37°C. After rinsing again the immunohistochemical reaction was visualized using 3,3'-diaminobenzidine (DAB) and the sections were dehydrated through graded ethanol, cleared in xylene, and coverslipped in Cytoseal 60 mounting medium.

Double immunohistochemical labeling of Iba1-CD8 and Iba1-NeuN

For double labeling with Iba1 and CD8 the tissue slides were initially processed as described above for Iba1 labeling. After visualizing the reaction with DAB, sections were blocked again with NHS and incubated overnight with monoclonal mouse anti-CD8 antibody in (Dako, Glostrup, Denmark) in 2 mg/L dilution in antibody diluent (DakoCytomation, Carpinteria, CA) overnight at 4°C. After rinsing in PBS and incubation with ImmPRESS anti-mouse Ig (peroxidase) solution (Vector Labs) for 1 hr. at 37°C, the immunohistochemical reaction was visualized using Vector VIP substrate kit (Vector Labs). Sections were then dehydrated, cleared, and mounted as described above.

For Iba1 and NeuN co-labeling Iba1 was visualized as detailed above. For NeuN labeling slides were incubated with monoclonal mouse anti-NeuN antibody (Chemicon International, Temecula, CA) in 10 mg/L dilution and otherwise processed as described previously for CD8 labeling.

Double immunofluorescence labeling of Iba1-Ki-67

For immunofluorescence co-labeling slides were heated, deparaffinized, rehydrated, and heat induced epitope retrieval was applied as previously described. After rinsing in ddH₂O and PBS sections were blocked in 10% normal goat serum (NGS) in PBS and incubated with polyclonal rabbit anti-Iba1 antibody (Wako Chemicals, Richmond, VA) diluted to 1mg/L and monoclonal mouse anti-Ki-67 antibody (Dako, Glostrup, Denmark) diluted to 1.1 mg/L in antibody diluent (DakoCytomation, Carpinteria, CA) overnight at 4°C. Sections were then rinsed in PBS and incubated with Alexa Fluor 488 conjugated goat anti-rabbit and Alexa Fluor 568 conjugated goat anti-mouse antibodies (Invitrogen, Eugene, OR) diluted to 10 mg/L in 10% NGS. Finally sections were rinsed in PBS, stained with TO-PRO3 counterstain, and mounted in aqueous mounting medium.

Quantification of microglial reactivity

The severity of reactive microgliosis in each case was determined by comparing microglial activity in areas with extensive microgliosis to areas with minimal or no microgliosis both

within the neocortex in RE cases and in control cases. This was carried out by acquiring digital photomicrographs using an Olympus BX41 microscope and a DP10 camera through a 10X objective (Olympus America Inc., Center Valley, PA), opening the images in Adobe Photoshop CS2 (Adobe Systems Inc.), auto adjusting contrast and applying the “Magic Wand Tool” for auto selecting areas of microglial staining. Images were converted to black and white and opened in ImageJ (NIH) for analysis. The area of microglial immunostaining was determined by ImageJ, which provided an absolute area of microglial immunolabeling within each image. These numbers were analyzed and compared using one-way ANOVA and Bonferroni correction in Prism 5 (GraphPad Software, San Diego, CA). Numbers from areas of severe and minimal microgliosis in RE cases were analyzed unpaired.

Results

Description of the Cohort

The clinical characteristics of the patients used in this study are shown in Table 1; tissue was studied from 8 patients with RE, 6 cases of cortical dysplasia (CD), and 6 individuals with tuberous sclerosis complex (TSC). There were 8 (40%) females, and 9 (45%) left sided resections. There were 11 (55%) cases of hemispherectomy, 3 (15%) of multilobar resections, and 6 (30%) lobar/focal resections. More cases of hemispherectomy occurred in RE cases compared with patients with CD and TSC (Chi-square; $p=0.020$). Before surgery, all patients were having multiple daily seizures. After surgery, 15 (75%) patients were seizure free, and there were no differences between patients with RE, CD, or TSC ($p=0.087$). For the entire cohort, mean ($\pm$ SD) age at seizure onset was 3.3 ± 4.1 years, age at surgery was 6.5 ± 4.5 years and calculated duration of epilepsy was 3.2 ± 3.3 years. By etiology and as expected from previous clinical studies, age at seizure onset was older for RE cases (6.4 ± 4.9 years) compared with patients with CD (1.9 ± 1.9 years) and TSC (0.5 ± 0.7 years; ANOVA, $p=0.011$) (Harvey et al., 2008). However, age at surgery ($p=0.149$) and seizure duration ($p=0.670$) were not statistically different between patients with RE, CD, and TSC in this cohort.

Microglial morphology

Areas of reactive microgliosis in the brains of children diagnosed with RE were evident, however, often unevenly distributed throughout the parenchyma, with interposed areas of little or no microglial activation. In areas of activation various levels of reactive microgliosis could be observed. The degree of activation often varied among areas within the brain from the same patient. Some regions displayed pronounced microglial reactivity (Fig. 1D-F) while in other areas microglial activation was minimal (Fig. 1A-C). Activated microglia on occasion presented a rod cell morphology displaying a thin and elongated shape (Fig. 1F). In other reactive areas a more bushy and hyper-ramified microglial morphology could be observed (Fig. 1E).

Microglia-neuronal association and microglial rod cell formation in cerebral cortex in RE

Elongated microglial processes in RE line up along neuronal apical dendrites (Fig. 2B, long arrows) in areas of cerebral cortex with strong microglial activation. This gives rise to formation of microglial rod cells as illustrated in Fig. 2A and B (arrowheads). Close association between neuronal cell soma and microglia was also observed, with microglia enveloping single neuronal cell bodies (Fig. 2B, short arrows).

Microglial reactivity

The severity of microglial reactivity in this study was quantified by measuring the area occupied by microglia determined by Iba1 immunolabeling. Fig. 3 illustrates two images of the cerebral cortex from the same patient with RE. Fig. 3A shows an area of minimal microglial reactivity

compared to Fig. 3C from the same patient showing a region of more pronounced microglia activation. Microglia in their resting state as observed in Fig. 3A typically displayed small slightly oval or polymorphic cell somas with a few fine, elongated, and ramified processes extending from them. Reactive microglia as demonstrated in Fig. 3C clearly present the hyper-ramified, dense, and bushy morphology characteristic of the reactive state of these cells. Figures 3B and 3D illustrate the transformation of Fig. 3A and C respectively into black and white for quantification of the area of microglial immunolabeling by ImageJ (NIH).

Statistical analysis was applied to the average areas of microglial immunolabeling in histological sections in the four categories of neocortex chosen for investigation in this study, i.e. severely affected areas with highly reactive microglia in sections from RE cases, minimally affected areas with low grade or no microglial reactivity from the same patients, and areas from control cases of TSC and CD as presented in Fig. 4. The average of the selected areas of severe microglial reactivity in the brains from RE patients (0.21mm^2 , 29%) was significantly increased compared to the selected minimally affected areas (0.11mm^2 , 15%) within the brains from the same patients and compared to the selected neocortical areas in control cases of TSC (0.10mm^2 , 14%) and CD (0.09mm^2 , 12%) (ANOVA, $P < 0.001$).

Of interest were three patients who were examined and thought clinically to have RE, but in whom characteristic neuropathologic changes could not be found.

Microglial reactivity by Iba1 labeling compared to CD68 labeling

An often employed immunohistochemical microglial marker for pathological diagnostics is antiCD68. CD68 is a predominantly intracellular molecule associated with the endosomal compartment and is a panmacrophage marker also expressed in microglia (Ramprasad et al., 1996; Gough et al., 2001; Parwaresch et al., 1986). The calcium binding protein Iba1 is also expressed in microglia (Ito et al., 1998). Microglia immunolabeled with anti-Iba1 display a distinctively different part of their cellular structure and morphology (Fig. 5A,C) when compared to cells labeled with anti-CD68 (Fig. 5B,D). The immunohistochemical labeling of the Iba1 protein in microglia reveals a cellular morphology with dense coherent staining of the cell soma and cellular processes. This reveals great morphological detail in individual microglia. Fig. 5A and C illustrates microglial rod cell formation where the thin, extended, and parallel shape of multiple microglial rod cells can be readily appreciated. A different component of microglial cellular morphology is revealed in immunohistochemical labeling of CD68. Microglia rod cell morphology is still apparent, but individual microglial cells are depicted with a granular appearance and rod cell processes present an interrupted morphology (Fig. 5B, D).

Proliferative activity of microglia

An indicator of microglial activity, other than change in morphology and antigen expression, is proliferation. Iba1⁺Ki67⁺ proliferating microglia were counted in serial sections (gray and white matter included) corresponding to the ones used for Iba1 quantification in tissue from RE patients and control tissue from TSC and CD patients. Comparison of the number of Iba1⁺Ki67⁺ microglia between areas of severe and minimal microglial activation in the RE cases was not possible because the quantification of Iba1⁺ microglial reactivity in these areas was done in small microscope frames so the two regions were combined together for this analysis. The median number of Iba1⁺Ki67⁺ proliferating microglia in sections from RE patients was 0.056 cells/mm². In the sections from TSC and CD control patients the median numbers of Iba1⁺Ki67⁺ proliferating microglia were 0.093 and 0.0088 cells/mm², respectively. Statistical analysis (ANOVA) of the RE, CD, and TSC patient groups revealed no significant differences.

Figure 6 illustrates the morphology of two Iba1⁺Ki67⁺ microglia, with Iba1 demonstrated in green (A), Ki-67 displayed in red (B), and the TO-PRO-3 nuclear stain illustrated in blue (C). The overlay (D) shows co-labeling of Iba1 and Ki67. During the quantification process qualitative features of Iba1⁺Ki67⁺ microglia were apparent. For example, Iba1⁺Ki67⁺ microglia often occurred together in distinct foci and, in some cases, in tighter clusters as previously observed in proliferating microglia (Wirenfeldt et al., 2007). Still, individual Iba1⁺Ki67⁺ microglia were observed scattered in the parenchyma. However, we also noted considerable variability of Iba1⁺Ki67⁺ co-labeling within different sections within the same RE patient. Hence, there appears to be considerable variability of microglia proliferation in RE brain tissue.

Microglial reactivity and T-cell infiltration in RE

In order to investigate and determine any morphological correlations between cytotoxic T-cell infiltration and areas of dense microglia reactivity, sections of RE brain tissues were double-labeled for CD8 and Iba1 respectively. In the cerebral parenchyma in tissue from RE patients infiltration of CD8⁺ T-cells was present in various patterns. We observed both a diffuse presence and absence of CD8⁺ T-cell infiltration in areas of severe microglial reactivity (Fig. 7B and D, resp.). Also in areas of more localized microglial reactivity both the presence and absence of CD8⁺ T-cell infiltration was observed. Therefore no overall association between microglial activation and CD8⁺ T-cell infiltration can be inferred.

Correlations with Iba1 staining and clinical variables

Clinical variables were compared with the percent staining for Iba1 for the different patients groups (Table 1). Two statistical analyses were performed. In the first analysis, the more severe areas from RE cases were compared with Iba1 staining for CD and TSC cases. A second analysis used the data for Iba1 labeling from the least involved areas of RE cases. Results of the two statistical analyses showed similar results. Controlling for patient group (RE, CD, and TSC; ANCOVA), the percent staining for Iba1 did not correlate with gender ($P>0.35$), age at seizure onset ($P>0.44$), age at surgery ($P>0.16$), type of surgery ($P>0.38$), seizure outcome ($P>0.12$), side of surgery ($P>0.09$), or seizure duration ($P>0.08$).

Discussion

A disease presenting neuropathologic features as varied as RE, and for which an etiology has not been established, presents a challenge in diagnosis and treatment. In a recent survey of 20 pediatric epilepsy surgery centers, RE was the etiology in 2.7% of pediatric epilepsy surgery patients (Harvey et al., 2008). As rare as the disease may be, the debilitating nature of the symptoms encourages every effort to elucidate pathogenetic processes.

The disease initially presents with the active stages followed by a more chronic phase. Seizures may persist even though the other neurological symptoms stabilize (Larner et al., 1995; Antel et al., 1996). To date, the only lasting effective treatment is hemispherectomy of the involved cerebral hemisphere, with inevitable contralateral hemiparesis ensuing (Freeman, 2005). Albeit not routinely applied, visualization of the inflammatory changes in the brain present in RE is possible by PET scanning using the ¹¹C(R)-PK11195 ligand, which binds to activated microglia. In patients with RE PET-scans have shown diffuse increase in ¹¹C(R)-PK11195 binding throughout the affected hemisphere (Banati et al., 1999). Regardless of its underlying etiology, reactive microgliosis is an essential hallmark of RE. Whether microglial activation is secondary to a yet unknown primary cause or perhaps even the primary pathological process is still to be determined.

Interestingly the severity of microglial reactivity quantified by counting the number of microglia observed in focal cortical dysplasia has been found to correlate with the duration of epilepsy and seizure frequency (Boer et al., 2006). This could have relevance to RE as well, where seizures and reactive microgliosis are both hallmarks of the disease. Microgliosis was significantly greater in RE than in brain specimens from patients with non-RE seizure etiologies (in spite of no statistically significant difference in duration of epilepsy) and greater than areas of least involved pathology in RE cases, and there were no correlations of Iba1 staining with seizure duration. These data from our study indicate that the microglial activation is not simply an effect of seizure duration or frequency. This study focused on determining the range of microglial reactivity in RE. As demonstrated here, the severity of reactive microgliosis, as determined by the area of Iba1 immunohistochemical labeling, was increased in RE, both when compared to lesser or nonreactive areas within the same RE cases and to control cases. This does not provide any direct clue towards understanding the etiology of the disease. It does, however, establish quantitatively that extensive microglial activation does take place, and that the nature of this microglial reactivity is diverse as demonstrated by the various cellular morphologies observed. Interestingly, as demonstrated in Fig. 2, a close association between pyramidal neurons and microglia was also observed. The significance of this is unknown. It could be speculated that the effect of reactive microglial on neurons could be destructive or on the contrary maybe even trophic, a potential area for future research. The fact that no correlation between clinical parameters and microglial reactivity was found indicates that reactive microgliosis could be a separate event from the epileptogenic process in the course of RE.

No apparent correlation between microglial activation (either local or diffuse) and infiltration of CD8⁺ T-cells could be found. Infiltration of CD8⁺ T-cells (a hallmark of RE) was, however, observed in some areas with local as well as diffuse microglial reactivity, but not in others. These two inflammatory events could have independent etiological mechanisms and be initiated by different signals, possibly reflecting diversity in the inflammatory process both in terms of acute and chronic inflammatory activity. To further examine the inflammatory process the number of Iba1⁺Ki-67⁺ proliferating microglia was counted in and compared between the RE, CD, and TSC cases. Even though no statistically significant differences were found in the fraction of Iba1⁺Ki-67⁺ proliferating microglia between the RE, CD, and TSC groups, within each of these groups individual sections showed a qualitatively distinct variation in the number of these cells.

Microglia react almost immediately to disturbances in the CNS parenchyma (Nimmerjahn et al., 2005). Virtually any type of lesion in the CNS initiates reactive microgliosis (Kreutzberg, 1996; Streit et al., 1999). Factors contributing to recruitment of microglia to a specific site upon activation are probably diverse and not known in RE. Chemokine receptor CXCR3 is, however, identified as having a crucial role in microglial migration and recruitment to zones of axonal degeneration. Therefore it could be speculated that CXCR3 might potentially also have a role in recruiting microglia in RE as well (Rappert et al., 2004). Purines have additionally been shown to induce directional migration of microglia towards injured pyramidal neurons in slices of neonatal rat hippocampus (Kurpius et al., 2007). A similar mechanism of chemotaxis could perhaps also apply to recruitment of microglia in RE.

The method of analysis of microglial reactivity applied here should be regarded as semi-quantitative, as there is no systematic random sampling of the brain, and areas quantified are selected based on an initial visual judgment of the level of probable microglial reactivity, an important caveat in interpreting the results. Another potential source of bias in the analysis is the way Photoshop (Adobe Systems Inc.) converts the image to black and white and selects labeled areas when applying the 'Magic Wand Tool', as this feature tends to select larger areas in darker regions of the image. The methods of analysis applied are, however, quantitative and applicable for the kind of material available in this study.

When employing a quantification method such as the one used here, it is preferable that individual cells are labeled in their entirety in a uniform manner with low background staining, to ensure that the image software recognizes any change in cellular morphology. CD68 is a protein associated with the endosomal compartment. Anti-CD68 IHC often reveals microglia with a granular morphology (Fig. 5B,D). This is not optimal for the method of quantification applied here, as changes in microglial reactivity are determined by measuring changes in the area of microglial immunolabeling. Iba1 immunolabeling was assessed based on the morphology of the labeled microglia to be better suited for that purpose, as individual microglial morphology appeared consistent with strong Iba1 immunolabeling. Counterstaining was omitted to avoid recognition of cell nuclei by the image software.

Currently the therapeutic options for RE are limited, and non-surgical treatments seem only to provide temporary relief for the neurological symptoms (Bien et al., 2005; Vining, 2006). As no etiology has been established, targeting therapies against RE is difficult. In this study our goal was to determine the extent and severity of reactive microgliosis based on morphological examination of brain resection material obtained during surgical treatment of patients suffering from RE. We were able to illustrate the patchy distribution of microglial activation and show variable microglial reactivity in RE. Although this does not provide further clues to the etiology of RE, it does help to describe the reactive microgliosis observed in RE, which almost certainly plays a role in the disease progression, whether it be a primary or secondary process. It has been a long-debated issue if reactive microgliosis should be perceived as a beneficial or destructive process in the brain parenchyma (Hailer, 2008). This is highly relevant in RE, since the activation of microglia could be a potential target for therapeutic intervention. The invasive nature and severe side effects of the current surgical treatment options encourage generation of new knowledge of the pathological process underlying RE to promote development of future treatment with better outcome for these patients.

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Figure 1.

Section demonstrating the different patterns of microglial activation as observed in this study labeled with anti-Iba1 immunohistochemistry in neocortical sections from patients with RE. A-C: Microglia displaying various levels of minimally activated morphology with relatively small cell somata and processes. D-F: Pronounced microglial reactivity demonstrated by activated microglial morphologies including hyperramified cells (E) and rod cell formation (F). Scale bar: 50µm.

Figure 2.

Co-labeling of NeuN⁺ neurons (purple) and Iba1⁺ microglia (brown) in a section of cerebral cortex from a patient with RE. A: Low magnification of part of the cerebral cortex demonstrating the relationship between neurons and microglia. B: Illustrated at higher magnification is the close association between pyramidal neurons and microglia, and the alignment of microglial rod cells along apical dendrites. ml: molecular layer, pm: pia mater, long arrows: apical dendrites, short arrows: microglial cell bodies, arrowheads: microglial rod cells. Scale bars: 100 μ m (A), 25 μ m (B).

Figure 3.

Images prepared for quantification of anti-Iba1 immunolabeled microglia in neocortex from a patient with RE. A: Microglia displaying minimally reactive morphology. B: Image A after selection of labeled area with the “Magic Wand Tool” and conversion to b/w in Adobe Photoshop (Adobe Systems Inc.). C: Microglia in a different neocortical area from the same case displaying a more prominently reactive morphology. D: Image C after selection and conversion to b/w as in B. Scale bar: 50 μ m.

Figure 4.

Severity of microglial reactivity measured by area of Iba1 immunolabeling. Microglial Iba1 immunolabeling in areas of severe microglial reactivity in RE cases is significantly increased compared to areas of minimal microglial reactivity within the same cases, and in control cases of tuberous sclerosis and cortical dysplasia. *** $P < 0.001$, bars: standard error of the mean.

Figure 5. Comparison of Iba1 and CD68 immunohistochemical labeling of reactive microglia. A, C: Rod cell formation observed in reactive microglia immunolabeled with anti-Iba1 in two separate cases of RE. B, D: Microglial rod cell formation in slides from cases displayed in A, D resp. visualized by immunolabeling with anti-CD68. Scale bar: 100 μ m.

Figure 6. Immunofluorescent co-labeling of Iba1⁺ (green) and nuclear antigen Ki-67⁺ (red) proliferating microglia demonstrated in a confocal microscopic image. A: Iba1⁺ microglia, B: Ki-67⁺ nuclei, C: TO-PRO-3 nuclear staining and D: A, B, and C images combined to illustrate co-expression of Iba1 and Ki-67 in microglia. Scale bar: 25 μ m

Figure 7.

CD8⁺ T-cell infiltration in areas of pronounced microglia activation. Co-labeling of tissue sections with anti-Iba1 (brown) and anti-CD8 (purple). A, C: In areas of marked localized microglia reactivity there is both a corresponding localized presence (A) and absence (C) of CD8⁺ T-cell infiltration. B, D: Different areas of diffuse severe microglia reactivity have varying levels of CD8⁺ T-cell infiltration. B: This section shows a high density of CD8⁺ T-cell infiltration corresponding with the dense microglia reactivity, whereas image D illustrates the absence of infiltration in an area of pronounced microglial activation. Scale bar: 100 μ m.

Table 1

Clinical Data on Patient Cohorts Used in this Study

Rasmussen encephalitis cases									
Patient	Gender	Age at seizure onset (years)	Age at surgery (years)	Seizure duration (years)	Daily/Weekly seizures	Surgery type	Side	Area of sample	Seizure outcome
1	F	7.5	8.5	1	Daily	Hemispherectomy	Left	Central Operculum	Seizure free
2	M	5.3	6.3	1	Daily	Hemispherectomy	Right	Temporal and Neocortex	Seizure free
3	M	2	14.3	12.3	Daily	Hemispherectomy	Left	Frontal Lobe	Seizure free
4	F	1	3.5	2.5	Daily	Hemispherectomy	Right	Central Operculum	Seizure free
5	M	4	4.7	0.7	Daily	Hemispherectomy	Right	Central Operculum	Seizure free
6	F	7.5	7.6	0.1	Daily	Hemispherectomy	Right	Lateral Temporal Neocortex	Seizure free
7	F	6.7	7.5	0.8	Daily	Hemispherectomy	Right	Central Operculum	Seizure free
8	M	17	19	1.8	Daily	Hemispherectomy	Right	Frontal Lobe	Seizure free
Cortical dysplasia controls									
Patient	Gender	Age at seizure onset (years)	Age at surgery (years)	Seizure duration (years)	Daily/Weekly seizures	Surgery type	Side	Area of sample	Seizure outcome
1	M	0.8	3.5	2.7	Daily	Hemispherectomy	Right	Central Operculum	Seizure free
2	F	4	10.8	6.8	Daily	Frontal focal resection	Right	Inferior Frontal, Middle Frontal Gyrus	No follow up
3	M	0.33	2.4	2.1	Daily	Hemispherectomy	Left	Central Operculum	Not seizure free
4	M	0.84	4.5	3.7	Daily	Frontal lobar resection	Right	Frontal Lobe	Seizure free
5	M	4.8	7.4	2.6	Daily	Frontal focal resection	Left	Frontal Lesion	Seizure free
6	F	0.75	3.2	2.4	Daily	Multilobar resection	Left	Fronto-Parietal Lobe	Seizure free

Rasmussen encephalitis cases									
Patient	Gender	Age at seizure onset (years)	Age at surgery (years)	Seizure duration (years)	Daily/Weekly seizures	Surgery type	Side	Area of sample	Seizure outcome
Tuberous sclerosis complex controls									
Patient	Gender	Age at seizure onset (years)	Age at surgery (years)	Seizure duration (years)	Daily/Weekly seizures	Surgery type	Side	Area of sample	Seizure outcome
1	F	0	4	4	Daily	Multilobar resection	Left	Parietal Tuber	Seizure free
2	M	0.4	3.2	2.8	Daily	Frontal tuber resection	Left	Frontal Cortex	Not seizure free
3	M	0.25	5.5	5.2	Daily	Frontal lobar resection	Left	Frontal Lobe	Not seizure free
4	M	0.25	1.7	1.4	Daily	Frontal tuber resection	Right	Frontal Tuber	Seizure free
5	F	0.2	11.5	9.5	Daily	Hemispherectomy	Right	Central Operculum	Not seizure free
6	M	0	1.25	1.25	Daily	Multilobar resection	Left	Parietal Occipital Lobe	Seizure free