Chapter 2.1

Meningeal inflammation is not associated with cortical demyelination in chronic multiple sclerosis

Original Article


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Abstract
Cortical demyelination can be extensive in chronic multiple sclerosis (MS) patients. Cortical lesions are not associated with lymphocyte infiltration, blood-brain barrier disruption or complement deposition; therefore, their pathogenesis is unclear. We analysed the extent and cellular composition of leptomeningeal inflammatory infiltrates and their possible relationship with subpial cortical demyelinated lesions in brain autopsy samples from 28 chronic MS patients; samples from 6 nonneurological control patients were also studied. Immunohistochemistry was used to detect meningeal T-cells, B-cells, macrophages, mature and immature dendritic cells, T-helper cells, (activated) cytotoxic T-cells and plasma cells. Quantitative analysis revealed significant meningeal inflammation in chronic MS patients; T-cells were the predominant inflammatory cells. Morphometric analysis was performed on coronal hemisphere sections of the MS cases to assess subpial demyelination; no correlation between the extent of subpial demyelination and extent of meningeal inflammation was identified. Moreover, no differences were observed in the degree or cellular composition of meningeal infiltrates comparing areas directly adjacent to subpial lesions compared with areas adjacent to normal-appearing grey matter in the MS cases. In addition, no follicle-like structures were found in the MS samples. Our data suggest that the occurrence of cortical lesions is not related to the presence of meningeal inflammation in a large number of chronic MS patients.
Meningeal inflammation in chronic MS

Introduction

The distinct neuropathologic hallmarks of multiple sclerosis (MS) include multifocal areas of demyelination and axonal loss throughout the brain and spinal cord. Although MS has long been regarded as predominantly a white matter (WM) disease, grey matter (GM) involvement has recently received increased attention. Based on their topological distribution, Bø et al. designated 4 distinct types of cortical MS lesions; Lesion types III and IV are referred to as subpial lesions. The type III cortical lesion is the most common cortical lesion type and often affects multiple gyri. Studies on its pathogenesis suggest that its underlying disease mechanisms differ between GM and WM lesions. For example, in contrast to WM lesions, cortical GM lesions are not associated with significant lymphocyte infiltration, do not show evidence of blood-brain barrier disruption or astrogliosis, and they are usually not associated with complement deposition. Based on a large post-mortem study, Kutzelnigg et al. suggested that focal active WM lesions are mainly characteristic in the acute or relapsing-remitting phases of MS and that cortical demyelination and diffuse injury of the so-called normal-appearing white matter (NAWM) are more prominent in progressive MS. They additionally found that cortical demyelination and diffuse inflammation throughout the NAWM in these progressive cases coincided with perivascular, parenchymal, and meningeal inflammation.

Several other studies have also demonstrated meningeal inflammation in MS patients. Recently, ectopic B-cell follicles with germinal center formation in the meninges of a significant proportion of secondary progressive MS (SP-MS) patients were demonstrated. The presence of these meningeal follicles was correlated with early disease onset, shorter disease duration, high expanded disability status scale scores, and extensive cortical subpial demyelination. These observations suggest that leptomeningeal inflammation may play a pathogenic role in the development of subpial demyelination perhaps by the elaboration of soluble (i.e. myelinotoxic) substances that diffuse into the cortex and mediate damage.

Thus, there is considerable recent interest in a possible relationship between meningeal inflammation and the development of cortical lesions in chronic MS, but a systematic investigation of the type and composition of meningeal inflammation and their relationship to subpial cortical demyelination has not been undertaken. Our aim was to characterise meningeal inflammation in a large and unselected sample of chronic MS patient autopsy samples and to investigate possible regional and global correlations between meningeal inflammation and subpial demyelination.

Materials and methods

Human post-mortem brain tissue

Brain tissue samples were obtained from the Netherlands Brain Bank (Amsterdam, the Netherlands). A total of 93 paraffin-embedded tissue blocks from 28 MS patients and 14 tissue blocks from 6 donors without neurological disease were selected based on the presence of leptomeninges in the sections. In addition, for a subset of 21 MS patients, 47 coronal cut full hemispheric sections were selected for morphometric analysis of the extent of subpial demyelination. For 7 MS patients, no coronal slices were available, and 21 standard-size tissue blocks were used for assessment of subpial demyelination. Clinical data of the MS and nonneurological patients are summarised in Table 1.
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The age of MS patients at time of death ranged from 41 to 84 years (mean ± SD, 63 ± 13 years) with a mean post-mortem interval of 8 hours and 42 minutes (SD, ±3 hours 39 minutes). The ages of the controls ranged from 45 to 99 years (mean ± SD, 73 ± 21 years) with a mean post-mortem interval of 29 hours 0 minutes (SD, ± 16 hours 17 minutes). The study was approved by the local institutional ethics review board, and all donors or their next of kin provided written informed consent for brain autopsy and use of material and clinical information for research purposes.

Immunohistochemistry

Paraffin sections (5 μm thick) were collected on Superfrost Plus glass slides (VWR international, Leuven, Belgium) and dried overnight at 37°C. Sections were deparaffinised in a series of xylene (3 x 5 minutes), 100% ethanol, 96% ethanol, 70% ethanol, and water. Endogenous peroxidase activity was blocked by incubating the sections in methanol with

<table>
<thead>
<tr>
<th>Case</th>
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<th>Sex</th>
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<th>Disease duration (years)</th>
<th>Cause of death</th>
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<td>M</td>
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<td>26</td>
<td>Stroke</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>PP</td>
<td>F</td>
<td>4:50</td>
<td>25</td>
<td>Euthanasia</td>
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<td>3</td>
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<td>73</td>
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<td>63</td>
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<td>65</td>
<td>PR</td>
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<td>49</td>
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<td>F</td>
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<td>Metastasized mamma carcinoma</td>
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<td>10</td>
<td>81</td>
<td>ND</td>
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<td>8:50</td>
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<td>F</td>
<td>5:50</td>
<td>21</td>
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<tr>
<td>23</td>
<td>59</td>
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<td>M</td>
<td>22:15</td>
<td>32</td>
<td>Cardiac arrest</td>
</tr>
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<td>24</td>
<td>49</td>
<td>SP</td>
<td>F</td>
<td>7:50</td>
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<td>25</td>
<td>41</td>
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<td>F</td>
<td>8:25</td>
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<td>General deterioration due to MS</td>
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<td>11:00</td>
<td>48</td>
<td>Pneumonia</td>
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<td>Pneumonia</td>
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<td>69</td>
<td>SP</td>
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<td>7:30</td>
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<td>F</td>
<td>≥24:00</td>
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<tr>
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<tr>
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<td>-</td>
<td>M</td>
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<td>-</td>
<td>F</td>
<td>≥24:00</td>
<td>-</td>
<td>Cardiac arrhythmia</td>
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</table>

F = female, M = male; MS = multiple sclerosis; ND = MS subtype not determined; PP = primary progressive; PR = progressive relapsing; SP = secondary progressive

Table 1. Demographic and clinical data of multiple sclerosis and control cases

The age of MS patients at time of death ranged from 41 to 84 years (mean ± SD, 63 ± 13 years) with a mean post-mortem interval of 8 hours and 42 minutes (SD, ±3 hours 39 minutes). The ages of the controls ranged from 45 to 99 years (mean ± SD, 73 ± 21 years) with a mean post-mortem interval of 29 hours 0 minutes (SD, ± 16 hours 17 minutes). The study was approved by the local institutional ethics review board, and all donors or their next of kin provided written informed consent for brain autopsy and use of material and clinical information for research purposes.

F = female, M = male; MS = multiple sclerosis; ND = MS subtype not determined; PP = primary progressive; PR = progressive relapsing; SP = secondary progressive
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0.3% H₂O₂. Sources and antigen retrieval protocols used for the antibodies are listed in Table 2.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Isotype</th>
<th>Source</th>
<th>Dilution and incubation</th>
<th>Antigen retrieval</th>
</tr>
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<tr>
<td>PLP (proteolipid protein)</td>
<td>Mouse IgG2a</td>
<td>Chemicon International, (Temecula, CA, USA)</td>
<td>1:3000; 1h, RT</td>
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<tr>
<td>MBP (myelin basic protein)</td>
<td>Mouse IgG2b</td>
<td>Chemicon International</td>
<td>1:50; 1h, RT</td>
<td>None</td>
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<td>CD3 (T-cells)</td>
<td>Rabbit polyclonal</td>
<td>DAKO (Glostrup, Denmark)</td>
<td>1:500; O/N, RT</td>
<td>Microwave 10 min at 900 W and 10 min at 360 W in citrate buffer pH 6.0 followed by Proteinase K pre-treatment 5 min at 37°C</td>
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<td>CD20 (B-cells)</td>
<td>Mouse IgG2a</td>
<td>DAKO</td>
<td>1:100; 1h, RT</td>
<td>Microwave 10 min at 900 W and 10 min at 360 W in citrate buffer pH 6.0</td>
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<tr>
<td>CD68 (macrophages)</td>
<td>Mouse IgG1</td>
<td>DAKO</td>
<td>1:3200; 1h, RT</td>
<td>Microwave 10 min at 900 W and 10 min at 360 W in citrate buffer pH 6.0</td>
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<td>Granzyme B</td>
<td>Mouse IgG2a</td>
<td>Sanquin, Amsterdam, The Netherlands</td>
<td>1:300; 1h, RT</td>
<td>Microwave 10 min at 900 W and 10 min at 360 W in citrate buffer pH 6.0</td>
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<tr>
<td>DC-SIGN (dendritic cell specific ICAM3-grabbing non-integrin; (im)mature dendritic cells)</td>
<td>Rabbit polyclonal</td>
<td>Kindly provided by Prof. Y. van Kooyk</td>
<td>1:4000; O/N, 4°C</td>
<td>Microwave 10 min at 900 W and 10 min at 360 W in citrate buffer pH 6.0</td>
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<td>CD4 (T-helper cells)</td>
<td>Mouse IgG1</td>
<td>Novocastra Laboratories, New Castle upon Tyne, UK</td>
<td>1:100</td>
<td>ER2 (pH 9.0); Bond Max; Vision Biosystems, Mount Waverley, Victoria, Australia</td>
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<td>CD8 (cytotoxic T-cells)</td>
<td>Mouse IgG1</td>
<td>DAKO</td>
<td>1:200</td>
<td>ER1 (pH 6.0); Bond Max; Vision Biosystems</td>
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<td>CD138 (plasma cells)</td>
<td>Mouse IgG1</td>
<td>DAKO</td>
<td>1:250</td>
<td>ER2 (pH 9.0); Bond Max; Vision Biosystems</td>
</tr>
</tbody>
</table>

RT = room temperature; O/N = overnight; ER = epitope retrieval

Tissue sections were stained with anti-myelin proteolipid protein (PLP) or anti-myelin basic protein (MBP) for the detection of demyelination. Serial sections of these blocks were also stained for T-cells (CD3), B-cells (CD20), macrophages (CD68), mature and immature dendritic cells (dendritic cell-specific ICAM3-grabbing nonintegrin [DC-SIGN]) and activated cytotoxic T-cells (granzyme B). All primary antibodies were diluted in 0.01 mol/L phosphate buffered saline, pH 7.4 (PBS) containing 1% bovine serum albumin (Roche Diagnostics, Mannheim, Germany). For primary staining with anti-PLP, anti-MBP, anti-CD3, anti-CD20, anti-CD68 or anti-granzyme B, the sections
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were rinsed in PBS (3 x 10 minutes), incubated with EnVision horseradish peroxidase complex (DAKO, Glostrup, Denmark); 3,3'diaminobenzidine-tetrahydrochloridedihydrate (DAKO) was used as a chromogen. For staining with anti-DC-SIGN, the sections were rinsed in PBS (3 x 10 minutes) and incubated with biotin-labeled swine anti-rabbit immunoglobulins F(ab')2 (1:500) (DAKO), diluted in 10% normal swine serum (DAKO), 10% normal human serum, and PBS with 1% bovine serum albumin for 30 minutes. The sections were then rinsed for 3 x 10 minutes and incubated for 1 hour at room temperature with streptavidin-biotin-peroxidase complexes (streptABComplex; DAKO). Sections were rinsed for 3 x 10 minutes and incubated with 3,3'diaminobenzidine-tetrahydrochloridedihydrate. After a short rinse in tap water, all sections were counterstained with hematoxylin for 1 minute and intensely washed with tap water for 5 minutes. Tonsil tissue was used as a positive control for the leukocyte markers; negative controls included omission of either primary or secondary antibodies. Sections of a subset of the material serial sections were also stained for T-helper cells (CD4), cytotoxic T-cells (CD8) and plasma cells (CD138). Immunostainings for CD4, CD8 and CD138 were performed automatically using the Bond Max (Vision Biosystems, Mount Waverley, Victoria, Australia).

Morphometry and quantification

The coronal hemisphere and standard sections immunostained with anti-MBP or anti-PLP were scanned using an Agfa Duoscan T2000XL scanner for preparation of digital images and prints. The cortical demyelinated areas in the plane of the section were analysed using light microscopy, and the same orientations were applied for the tissue on prints. The areas of subpial demyelination were measured on the digital images using ImageJ software (freely downloadable from: U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/index.html).

In MS and control tissues, random areas in the meninges were chosen and photographed at 50x original magnification. Cells were counted in corresponding areas on serial sections that were stained for the leukocyte markers. Cell counts were done in a total of 50 meningeal areas in the control tissue samples. For MS tissue samples, meningeal areas that were adjacent to subpial lesions were counted separately from those that were adjacent to normal-appearing GM (NAGM). Cell counts were made in a total of 432 meningeal areas, of which 297 areas were adjacent to NAGM and 135 areas were adjacent to subpial lesions. On a subset of the meningeal areas investigated semiquantitative analysis was performed in order to discriminate between the amount of T-helper cells and cytotoxic T-cells in the MS meninges on the basis of anti-CD4 and anti-CD8 immunostaining. The presence of CD4+ cells and CD8+ cells was determined as follows: 0, no immunopositive cells; +, 1-10 immunopositive cells; ++, 10-20 immunopositive cells; ++++, >20 immunopositive cells. The meningeal lengths of each area investigated were measured using ImageJ software. Calibration of the software program was performed using a standard millimeter scale.

Statistical methods

Data were analysed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA); data distribution was tested for normality. Because all variables were not normally distributed, the nonparametric Mann Whitney U test was used for comparing MS meningeal leukocyte infiltrates with those in the controls. Within the MS group,
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Meningeal infiltrates adjacent to subpial lesions and adjacent to NAGM were also compared using Mann Whitney U statistics. Possible correlations between the degree of meningeal inflammation and the extent of subpial demyelination were investigated with the nonparametric Spearman rank correlation. Differences were considered to be significant at $P < 0.05$.

Results

Extent and composition of meningeal inflammatory infiltrates

Meninges from chronic MS patients contained significantly more leukocyte infiltrates than meninges of controls ($P < 0.05$; Table 3). Most of the meningeal infiltrates in the MS
patients consisted of CD3⁺ T-cells, CD68⁺ macrophages and DC-SIGN⁺ dendritic cells (Table 3). By contrast, CD20⁺ B-cells and granzyme B⁺ activated cytotoxic T cells were far less common (Table 3). CD138⁺ plasma cells were only occasionally observed in the meninges of the chronic MS patients (Fig. 1H). Semiquantitative analysis revealed that most T-cells found in the meninges of MS patients were CD8⁺ cytotoxic T-cells and to a lesser extent CD4⁺ T-helper cells (Fig. 1I,J).

**Table 3.** Immunohistochemical analysis of meningeal inflammatory cells in brain samples

<table>
<thead>
<tr>
<th>Marker</th>
<th>Controls</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>3.70 ± SD 2.81</td>
<td>13.28 ± SD 17.39*</td>
</tr>
<tr>
<td>CD20</td>
<td>0.02 ± SD 0.12</td>
<td>1.13 ± SD 3.58*</td>
</tr>
<tr>
<td>CD68</td>
<td>3.39 ± SD 3.82</td>
<td>7.58 ± SD 8.49*</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>0.31 ± SD 0.53</td>
<td>0.79 ± SD 1.40*</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>2.40 ± SD 2.45</td>
<td>4.06 ± SD 4.84*</td>
</tr>
</tbody>
</table>

Mean cells per millimeter ±SD of meningeal infiltrates of T-cells (CD3), B-cells (CD20), macrophages (CD68), activated cytotoxic T cells (GranzymeB) and (im)mature dendritic cells (DC-SIGN) in nonneurological control and chronic multiple sclerosis (MS) brain tissue samples.

* A significant difference exists between MS and controls (P < 0.05).

**Relationship between meningeal infiltrates and cortical demyelination: global associations**

To determine whether meningeal inflammation was globally associated with subpial demyelination, the percentage of type III and type IV cortical lesions of each MS patient (Fig. 1A) were compared with the extent of meningeal inflammation. The percentage areas of subpial demyelination did not correlate with the extent of meningeal inflammation (mean total cells per millimeter) (Fig. 1B; P = 0.73). Not every chronic MS patient had leukocyte infiltrates in their meninges; therefore, the results were heterogeneous. Hence, extensive subpial demyelination (Fig. 1A) could be observed in the absence of meningeal infiltrates (Fig. 1C-G). There was also no correlation between the extent of meningeal inflammation and disease duration (P = 0.54) (data not shown); finally, no differences in the extent of meningeal inflammation were found between primary progressive and SP-MS cases (P = 0.60).

**Relationship between meningeal infiltrates and cortical demyelination: regional associations**

Because meningeal inflammation did not correlate with overall subpial demyelination areas, further comparison was made between leptomeningeal inflammatory cells counts adjacent to subpial lesions and counts in areas adjacent to NAGM. No differences were identified between infiltrates of T-cells, macrophages, activated cytotoxic T-cells, or dendritic cells in the meninges adjacent to subpial lesions (Fig. 2A) and those adjacent to NAGM (Fig. 2B) (Table 4; Fig. 2C-L). Moreover, no follicle-like structures were observed in
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Discussion

In agreement with previous findings, we demonstrated extensive subpial demyelination in a substantial proportion of chronic MS patients. We also provided a detailed characterisation of meningeal inflammation in a large unselected cohort of chronic MS autopsy material and showed that meningeal leukocyte infiltrates are predominantly composed of \((CD8^+)\) T-cells, macrophages, and dendritic cells. Remarkably, we found no correlation between the extent of meningeal inflammation and the extent of overall subpial demyelination; regional analysis demonstrated that subpial demyelination was also not associated with adjacent meningeal inflammation. MS cortical lesions were previously found not to be associated with lymphocyte infiltration. Our study extends these findings in that subpial demyelination was also not associated with T-cell infiltration.
infiltration in the MS meninges.

It has recently been shown that T-cells accumulate early in the disease course in mice with experimental autoimmune encephalomyelitis (EAE) in the meninges;\textsuperscript{27} by analogy, the T-cell mediated meningeal inflammation observed in MS material might reflect overall white matter disease activity. This could explain T-cell infiltrates in the meninges of the MS patients, but further analysis should confirm this. Although the numbers of B-cells in meningeal areas directly adjacent to subpial were slightly increased compared to areas directly adjacent to NAGM, B-cells in the MS meninges were relatively sparse overall. Therefore, their direct pathogenic roles are unclear.

The formation of leptomeningeal ectopic B-cell follicles has been associated with severe cortical pathology and more aggressive clinical disease in a subset of SP-MS patients.\textsuperscript{19,28} In these studies, it was further shown that type III cortical lesions extending over large cortical areas were predominantly found in SP-MS patients exhibiting ectopic B-cell follicles,\textsuperscript{19} whereas SP-MS patients lacking ectopic B-cell follicles showed a more heterogeneous cortical pathology.\textsuperscript{19} In relation to this, it was suggested that myelinotoxic substances diffusing from the subarachnoid and perivascular spaces might cause subpial demyelination. SP-MS patients harbouring ectopic B-cell follicles in the meninges had several clinico-pathological characteristics, including a shorter disease duration, earlier onset of the disease and more extensive subpial demyelination.\textsuperscript{19} In the present study, however, we found extensive subpial demyelination in the absence of substantial B-cell infiltration or ectopic B-cell follicles. It is possible that subpial cortical lesions might been induced by antecedent inflammatory infiltrates earlier in the disease course, but the lack of correlation between the extent of meningeal inflammation and disease duration argues against this possibility. We cannot, however, rule out the possibility that differences between previous and the current findings might be caused by differences in disease onset or other parameters in the MS cases studied.

Interest in B-cells in MS has received impetus following new treatment options of relapsing-remitting MS (RR-MS) patients. Rituximab, an anti-CD20 monoclonal antibody, was shown to deplete CD20\textsuperscript{+} B-cells that correlated with a significant and sustained reduction of gadolinium-enhancing lesions and of relapses in RR-MS patients.\textsuperscript{29} Because plasma cells do not express CD20, treatment of RR-MS patients with rituximab did not result in the reduction of total antibody levels;\textsuperscript{29} anti-myelin antibodies might not relate to MS disease progression.\textsuperscript{30} It is possible, however, that a negative effect on the antigen-presenting capacities of B-cells\textsuperscript{31-33} might have played a role in effects of rituximab on WM lesion development.\textsuperscript{34} Whether rituximab treatment exerts an effect on the progression of RR-MS patients and on the development of subpial cortical GM lesions, remains elusive and warrants further investigation.

In EAE mice, T-cell recognition of their cognate antigen by dendritic cells and macrophages in the meninges and perivascular spaces is sufficient for inducing autoimmune-driven inflammation in the brain and resulting in neurological dysfunction.\textsuperscript{27,35} We recently reported the presence of large amounts of extracellular myelin in the meninges of MS patients and proposed that this might lead to the induction or continuation of neuroinflammation.\textsuperscript{36} Alternatively, the presence of brain-derived antigens inside and outside the central nervous system could suppress immune responses leading to tolerogenic effects or even neuroprotection.\textsuperscript{37-45}

Because immune cells produce neurotrophic factors,\textsuperscript{46-48} meningeal inflammation
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may also exert beneficial effects on the underlying cortex. Indeed, extensive cortical remyelination has recently also been described in the cerebral cortex of chronic MS patients.49 Because in the present study no discrimination was made between NAGM and remyelinated cortex, the areas designated as NAGM might have included remyelinated areas. Since no electron microscopy in this study was performed, we felt unable to reproducibly assess remyelination in the cortex on the basis of PLP immunostainings. Future studies are warranted to investigate whether the extent of cortical remyelination is correlated with meningeal inflammation in chronic MS.

Although GM pathology is present in the earliest stages of MS,50-54 it only becomes prominent in the progressive phase of the disease.55-57 Interestingly, GM atrophy greatly accelerates after secondary disease progression to a greater extent than WM atrophy.58 As for possible pathogenetic mechanisms contributing to GM damage, several pathogenetic mechanisms have been proposed; these include mitochondrial dysfunction,59,60 the exhaustion of compensatory mechanisms for adaptive cortical reorganisation,61,62 remyelination,63 redistribution of sodium channels on demyelinated axons,64 and the expression of neurotrophic factors by immune and central nervous system resident cells.65,66 Also, it was hypothesised that meningeal inflammation and the production of myelinotoxic substances could be causally related to cortical GM demyelination. Whether and to what extent these different pathogenetic mechanisms play a role in the development of subpial lesion formation remains elusive, but the present findings indicate that at least in a subset of chronic MS patients, the development of subpial lesions is not related to the presence of meningeal inflammation.

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