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Time-course expression of CNS inflammatory, neurodegenerative, tissue repair markers and metallothioneins during experimental autoimmune encephalomyelitis

Expresión de marcadores de inflamación, neurodegeneración, reparación tisular y metalotioneínas en el SNC durante el curso clínico de la encefalomielitis autoinmune experimental

En este trabajo se describe el patrón de expresión de un grupo de marcadores de inflamación, neurodegeneración y reparación tisular, así como de la expresión de MT durante el curso clínico de la EAE inducida con PLP. Se inmunizaron ratones SJL con el péptido 139-151 de la PLP para inducirles una EAE remitente-recurrente. Se sacrificaron grupos de cinco ratones en diferentes momentos del brote clínico, tanto durante la exacerbación como durante la remisión de los signos clínicos. Mediante inmunohistoquímica se analizaron una serie de marcadores relacionados con el proceso inflamatorio, neurodegenerativo y de reparación tisular que acontecen durante la EAE y se relacionaron con la expresión de MT-I y MT-II. Curiosamente, pudimos distinguir dos patrones de expresión: en el primer patrón (patrón 1), la expresión de los marcadores aumentaba a medida que los signos clínicos empeoraban y volvían a los niveles basales con la remisión de la enfermedad. En este patrón se incluían los marcadores de los procesos neurodegenerativos como la infiltración celular, expresión de citocinas proinflamatorias, estrés oxidativo, daño axonal y apoptosis. En el segundo (patrón 2), la expresión de los marcadores se iniciaba más tarde que en el patrón 1, alcanzaba su máximo cuando la gravedad de la enfermedad era máxima y se mantenía elevada tras la remisión de los signos clínicos. En este patrón se englobaban los marcadores relacionados con la reparación tisular como factores de crecimiento, proliferación de oligodendrocitos y regeneración neuronal. El patrón de expresión de MT se correspondía con el patrón 2 indicando su posible implicación en los mecanismos de remisión de la EAE y su posible potencial terapéutico en la EAE/EM.
TIME-COURSE EXPRESSION OF CNS INFLAMMATORY, NEURODEGENERATIVE TISSUE REPAIR MARKERS AND METALLOTHIONEINS DURING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Abstract—Experimental autoimmune encephalomyelitis (EAE) is an animal model for multiple sclerosis (MS). EAE and MS are characterized by CNS inflammation, demyelination and neurodegeneration. The inflammatory response occurring within the CNS leads to glial activation, dysfunction and death, as well as axonal damage and neurological deficit. Although the pathogenic mechanisms involved in EAE/MS are not well understood, accumulating data suggest that oxidative stress plays a major role in lesion development, and contributes to axonal dysfunction and degeneration. Metallothionein-I and -II are anti-inflammatory, neuroprotective, antioxidant proteins expressed during EAE and MS, in which they might play a protective role. The present study aimed to describe the expression profile of a group of inflammatory, neurodegenerative and tissue repair markers as well as metallothioneins during proteolipid protein-induced EAE, and to establish the time-relationships these molecules had during EAE. Interestingly, we found two marker expression profiles. In the first, marker expression increased as clinical signs worsened and reverted to baseline expression during recovery; in the second, marker expression increased at a later point during relapse, peaked at highest clinical score, and remained elevated throughout recovery. Of note, metallothionein expression was found to be related to the second profile, which would suggest that metallothionein proteins are implicated in the clinical recovery of EAE and perhaps these antioxidant proteins may provide therapeutic benefits in MS. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

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Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease, regarded as an animal model of the human immune-mediated disease multiple sclerosis (MS). EAE is characterized by the generation of autoreactive T cells which accumulate in the CNS and recognize myelin antigens. Its exacerbations are mainly determined by immunological responses resulting in the breakdown of the blood–brain barrier (BBB) and perivascular inflammatory mononuclear cell infiltration within the CNS. Inflammatory infiltrates consist mainly of activated T lymphocytes, blood- and microglia-derived macrophages and reactive astrocytes (Swanborg, 1995). During EAE/MS, these cells are main sources of a number of detrimental factors that can contribute to the ongoing inflammation and CNS damage. In this sense, it has been described that production of proinflammatory cytokines such as interleukin (IL)-1, IL-6, or tumor necrosis factor (TNF)-α correlates with inflammation and tissue destruction during EAE (Renno et al., 1995; Eng et al., 1996; Taupin et al., 1997). Inflammation is also associated with other proinflammatory molecules such as reactive oxygen species (ROS) that contribute to brain tissue destruction (Cassarino and Bennett, 1999; Halliwell, 2001). In this regard, there is evidence pointing to oxidative stress as a major player in EAE/MS lesion development (Cross et al., 1996, 1998; Vladimirova et al., 1998) thus contributing to axonal dysfunction and degeneration (Redford et al., 1997; Smith et al., 2001). Recent research has stressed the relevance of axonal injury/degeneration in the pathogenesis of EAE/MS. Axonal damage occurs in the early stages of the acute EAE/MS lesion, beginning at disease onset and then correlating with the degree of inflammatory demyelination (Ferguson et al., 1997; Trapp et al., 1998; Kuhlmann et al., 2002; Bjartmar et al., 2003). On the other hand, studies on the pathogenesis of EAE have demonstrated that Th2-type cytokines, including IL-4, IL-10 and transforming growth factor (TGF)-β, are expressed in the CNS of animals recovering from EAE (Kennedy et al., 1992; Olsson, 1995).

Metallothionein (MT)-I and MT-II are heavy metal-binding, low-molecular weight, cysteine-rich proteins induced during EAE (Penkowa and Hidalgo, 2000a; Espejo et al., 2001) in which they play a beneficial role (Penkowa and Hidalgo, 2000a,b). Nevertheless, the mechanisms underlying these processes are not yet fully understood.
For the present study, we planned to describe the expression profile of a group of CNS inflammatory, neurodegenerative and tissue repair markers as well as MT-I and -II during proteolipid protein (PLP)-induced EAE, with the aim to establish the time-relationship of these pathogenic processes during EAE. Our results allowed us to establish two different profiles of marker expression. Markers implicated in CNS inflammation and neurodegeneration were found in the first profile, while the second profile involved anti-inflammatory molecules and markers of neuronal regeneration. Interestingly, MT expression was related to the second profile, supporting that the neuroprotective role previously described for MT-I and -II in other neuropathological conditions such as trauma, ischemia, epilepsy and neuroglial degeneration (van Lookeren Campagne et al., 1999; Penkova et al., 1999, 2000b; Trendelenburg et al., 2002) is also relevant in EAE/MS.

**EXPERIMENTAL PROCEDURES**

**Mice**

Female SJL/J Rj mice 8–10 weeks of age were obtained from Janvier (Le Genest-St. Isle, France). They were fed with standard chow and had access to water *ad libitum*. Anesthesia was induced by i.p. injection of 37 mg/kg of ketamine (Ketolar; Parke & Davis, Morris Plains, NJ, USA) and 5.5 mg/kg of xylazine (Rompun; Bayer, Leverkusen, Germany). Animal welfare was observed in full compliance with the European Community regulations on this subject and experiments were approved by the ethics committee for animal research of Hospital Universitari Vall d’Hebron (Spain). Efforts were made to minimise the number of animals used and their suffering.

**Immunization**

Mice were immunized by s.c. injection of 0.9% saline containing 50 µg of 139–151 PLP peptide (PLP,139–151) emulsified in Freund’s adjuvant (Sigma Chemical CO, St. Louis, MO, USA; 9:1 v/v) containing 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA). PLP,139–151 was synthesized by Dr. D. Andreu (Secció de Proteòmica; Serveis Científics, Universitat Pompeu Fabra, Barcelona, Spain). Mice received 0.05 ml s.c. of emulsion in the four limb flanks. On days 0 and 2 post-immunization (pi) each mouse received 0.2 ml (2 IU/ml) of inactivated *Bordetella pertussis* (Vaxicoq; Pasteur Merieux, Lyon, France) i.v. As controls, mice were immunized in the same way using PBS instead of the PLP,139–151. Non-immunized mice were also included.

**Clinical evaluation**

All animals were weighed and examined daily for neurological signs using the following six-point scale (Espejo et al., 2001): 0 = no clinical signs; 0.5 = partial loss of tail tonus for 2 consecutive days; 1 = paralysis of whole tail; 2 = mild paraparesis of one or both hind limbs; 3 = paraplegia; 4 = tetraparesis; 5 = tetraplegia; 6 = death.

**CNS samples**

We obtained CNS samples from five mice at different time points of clinical score in order to monitor MT-I and -II expression, alongside with different markers involved in EAE-clinical ongoing or recovery. Animals were killed and the brain and spinal cord removed at score 0 (day 7 pi), score 1 (day 13 pi), score 2 (days 15–19 pi), scores 3–4 (days 22–24 pi), score 2 during clinical recovery (days 26–29 pi), and score 0 upon full recovery (days 34–37 pi). Control mice were killed on day 7 pi. Tissue samples from non-immunized (control group 1), and PBS immunized (control group 2) mice were also obtained.

**Tissue processing**

All specimens were fixed in 10% buffered formalin and embedded in paraffin. The tissue was cut in 4–8 μm sections that were preincubated and treated as described elsewhere (Penkova and Hidalgo, 2000a; Penkova et al., 2000a).

**Immunohistochemistry**

Sections were incubated with rat anti-mouse F4/80 1:15 (S erotec, UK, code MCA 497); rat anti-mouse MOMA-2 (a marker for perivascular monocytes/macrophages) 1:50 (S erotec, UK, code MCA519GQ); mouse anti-rat CD3 1:50 (S erotec, UK, code KD MCA 772); mouse anti-rat CD4 1:50 (S erotec, UK, code MCA 55G); rabbit anti-cow gliarial fibrillary acidic protein (GFAP) 1:250 (Dakopatts, DK, code Z 334); rabbit anti-rat CD4 1:50 (S erotec, UK, code MCA 55G); rabbit anti-cow glial fibrillary acidic protein (GFAP) 1:250 (Dakopatts, DK, code Z 334); rabbit anti-rat-MT-I and -II (Penkova and Hidalgo, 2000a, Penkova et al., 2000b); mouse anti-human IL-1β 1:50 (B iogenesis, Kingston, NH, USA, code 5375-4329); rat anti-mouse IL-6 1:10 (Harlan Sera Lab, Sussex, UK, code MAS 584); rabbit anti-mouse TNF-α 1:100 (Biosource Int., Camarillo, CA, USA, code AMC 3012); rabbit anti-human basic fibroblast growth factor (bFGF) 1:100 (Santa Cruz, USA, code sc-79); rabbit anti-human transforming growth factor (TGF)-β 1:200 (Santa Cruz, USA, code sc-146); goat anti-mouse IL-10 1:50 (Santa Cruz, USA, code sc-1783); goat anti-rat nerve growth factor (NGF) 1:100 (RD Systems, USA, code N-56-NA); rabbit anti-human myelin basic protein (MBP) 1:100 (Dakopatts, DK, code A0623); mouse anti-nonphosphorylated neurofilaments (SMI-32) 1:50 (Stemberger Monoclonals Inc., USA, code SMI-32) (detecting axonal transection and demyelination); goat anti-human amyloid precursor protein (APP, frame shift mutant) 1:50 (Chemicon, Int., USA, code AB5342) (a marker for acute axonal injury/degeneration); rabbit anti-nitrotyrosine (NITT) 1:100 (Alpha Diagnostic Int., San Antonio, TX, USA, code NITT 12-A) (as a marker for oxidative stress); rabbit anti-malondialdehyde (MDA) 1:100 (Alpha Diagnostic Int., San Antonio, TX, USA, code MDA 11-S) (as a marker for oxidative stress); rabbit anti-human platelet derived growth factor receptor (PDGF) 1:100 (Santa Cruz, USA, code sc-338) (a marker of oligodendrocyte progenitor cells); rabbit anti-rat NG2 (NG2 Chondroitin sulfate Protein) 1:100 (Chemicon, USA, code AB5320) (a marker of oligodendrocyte progenitor cells); mouse anti-rat GAP-43 1:100 (C a biochem, USA, code CP098) (a marker for neuronal growth cones); mouse anti-human P-40 1:100 (S erotec, UK, code MCA1712) (a marker for CNS growth cones); mouse anti-human Cu,Zn-superoxide dismutase (Cu,Zn-SOD) 1:100 (Sigma-Aldrich, USA, code S2147); sheep anti-bovine Mn-SOD 1:100 (Biogenesis, UK, code 8474-9550); sheep anti-human catalase 1:100 (The Binding Site, UK, code PC 136).

The primary antibodies were detected by using biotinylated anti-mouse IgG 1:200 (Sigma; code B8774), or biotinylated anti-mouse IgM (λ chain specific) 1:50 (Jackson Immunoresearch Laboratory Inc., USA; code 115-065-020) or biotinylated anti-rabbit IgG 1:400 (Sigma; code B2375), or biotinylated donkey anti-goat/sheep IgG 1:20 (Amersham, UK; code RPN 1025), or biotinylated goat anti-rat IgG 1:1500 (Amersham; code RPN 1005) followed by streptavidin–biotin–peroxidase complex (StreptABComplex/HRP; Dakopatts; code K377) prepared at the manufacturer’s recommended dilution. Afterward, sections were incubated with biotinylated tyramide and streptavidin–peroxidase complex (tyramide signal amplification; TSA indirect; NEN, Life Science Products, USA; code NE600A) prepared following the manufacturer’s recommendations. The immunoreaction was visualized using 0.015% H2O2 in DAB/TBS for 10 min at room temperature. Control sections were processed in parallel with antibodies preabsorbed with their antigenic proteins.
Immunofluorescence

To determine which cells expressed MT-I and -II, we performed double immunofluorescence by incubating sections overnight at 4 °C with mouse anti-porcine vimentin 1:50 (DakoCytomation; code M0725) simultaneously with rabbit anti-rat MT-I and -II (as described above). The primary antibodies were detected using goat anti-mouse IgG linked with Texas Red 1:40 (Jackson Immunoresearch Laboratory Inc.; code 111-075-144) and goat anti-rabbit IgG linked with aminomethylcoumarin 1:20 (Jackson Immunoresearch Laboratory Inc.; code 111-075-144).

In situ detection of DNA fragmentation

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labeling (TUNEL) staining was performed as described elsewhere (Penkowa et al., 1999).

Cell counts

Counts of positively stained cells (defined as those cells with cytoplasmic staining or, in the case of TUNEL, cells with nuclear staining) were carried out in five animals of each score point analyzed. Cell counts were done in matched brainstem areas containing EAE infiltrates, which were identified assuming a perivascular location. Then a 0.5 mm² perivascular area was selected for countings in a blinded manner. Results are mean ± standard deviation (SD) of cell counts.

Statistical analysis

Statistical analysis was performed comparing the data from cell counts of the same clinical score during EAE, during both clinical ongoing and recovery. Due to the sample size and the number of comparisons made, the Welch test was used and Bonferroni correction applied (0.05/18). A significant difference was considered when P value < 0.0026.

RESULTS

Histopathological characteristics of EAE

Cell infiltrates and reactive astrogliosis. A very high number of perivascular infiltrates was observed by examining H&E-stained sections of EAE-sensitized mice, in which recruitment of activated F4/80+ macrophages, CD4+ T cells, and reactive astrogliosis were seen (Table 1; Fig. 1).

EAE-sensitized mice with score 0 showed some round F4/80+ macrophages and a few CD4+ T lymphocytes. The recruitment of macrophages and T cells, and reactive astrogliosis was more pronounced in score 1, and increased with the degree of EAE severity (Table 1; Fig. 1). Reactive astrogliosis were observed surrounding the EAE-demyelinated areas, which contained only few GFAP positive astrocytes. During recovery from EAE, the numbers of recruited macrophages and T lymphocytes were reduced significantly, while GFAP+ astrocytes remained high both during and after clinical recovery (Table 1, Fig. 1G, H). These results were confirmed by also using MOMA-2 and CD3 immunostainings to identify macrophages and T cells respectively (data not shown). Mice from the control groups did not present brainstem lesions (data not shown). Although some dispersed GFAP+ astrocytes were found, significant number of F4/80+ macrophages or CD4+ T cells in the brainstem parenchyma of the control mice was not detected (Table 1, Fig. 1).

Demyelination. Demyelination was examined by detecting MBP immunoreactivity and disappearance during EAE. In control mice, no demyelination was observed in the brainstem, which showed intact MBP staining (Fig. 2A, B).

Table 1. Time-course of inflammatory, neurodegenerative, tissue repair markers and metallothioneins in brainstem during EAE*

<table>
<thead>
<tr>
<th></th>
<th>Control 1</th>
<th>Control 2</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3–4</th>
<th>Score 2 recovery</th>
<th>Score 0 recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>pi Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>F4/80 (macrophages)</td>
<td>7.2 ± 0.4</td>
<td>7.4 ± 0.4</td>
<td>37.2 ± 4.0</td>
<td>52.2 ± 2.9</td>
<td>112.2 ± 8.0</td>
<td>146.4 ± 6.7</td>
<td>55.2 ± 7.2</td>
<td>36.2 ± 3.1</td>
</tr>
<tr>
<td>CD4 + T-lymphocytes</td>
<td>1.6 ± 0.9</td>
<td>1.2 ± 0.8</td>
<td>17.2 ± 4.8</td>
<td>48.0 ± 5.7</td>
<td>83.6 ± 7.6</td>
<td>106.8 ± 6.9</td>
<td>34.0 ± 2.3</td>
<td>17.4 ± 3.4</td>
</tr>
<tr>
<td>GFAP (astrocytes)</td>
<td>15.0 ± 4.6</td>
<td>12.4 ± 1.8</td>
<td>39.8 ± 4.9</td>
<td>56.6 ± 3.1</td>
<td>130.2 ± 8.1</td>
<td>197.4 ± 6.5</td>
<td>125.4 ± 13.5</td>
<td>97.2 ± 16.1</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.6 ± 0.5</td>
<td>0.6 ± 0.9</td>
<td>65.8 ± 4.3</td>
<td>116.6 ± 6.6</td>
<td>122.4 ± 8.8</td>
<td>120.4 ± 6.2</td>
<td>39.0 ± 4.3</td>
<td>104 ± 1.8</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.2 ± 1.5</td>
<td>1.8 ± 1.3</td>
<td>13.0 ± 2.5</td>
<td>64.8 ± 3.6</td>
<td>111.0 ± 6.2</td>
<td>140.2 ± 4.9</td>
<td>54.4 ± 3.2</td>
<td>15.2 ± 3.1</td>
</tr>
<tr>
<td>NIT</td>
<td>0.8 ± 0.8</td>
<td>1.2 ± 1.3</td>
<td>24.8 ± 4.7</td>
<td>76.2 ± 7.2</td>
<td>135.0 ± 13.6</td>
<td>175.2 ± 8.6</td>
<td>60.8 ± 10.1</td>
<td>13.8 ± 3.1</td>
</tr>
<tr>
<td>MDA</td>
<td>1.0 ± 1.4</td>
<td>0.8 ± 0.8</td>
<td>25.2 ± 5.2</td>
<td>75.4 ± 6.7</td>
<td>136.0 ± 14.1</td>
<td>176.2 ± 8.9</td>
<td>59.2 ± 9.1</td>
<td>10.8 ± 2.3</td>
</tr>
<tr>
<td>APP</td>
<td>0.8 ± 1.3</td>
<td>0.6 ± 0.9</td>
<td>17.6 ± 3.2</td>
<td>52.8 ± 6.0</td>
<td>114.6 ± 7.6</td>
<td>155.4 ± 8.1</td>
<td>33.0 ± 5.7</td>
<td>8.8 ± 1.9</td>
</tr>
<tr>
<td>TUNEL (apoptosis)</td>
<td>1.2 ± 0.8</td>
<td>1.4 ± 1.7</td>
<td>26.8 ± 5.4</td>
<td>77.4 ± 6.9</td>
<td>137.6 ± 13.0</td>
<td>183.2 ± 6.8</td>
<td>55.4 ± 4.0</td>
<td>9.2 ± 1.3</td>
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<tr>
<td>PDGFα-receptor</td>
<td>0.8 ± 0.4</td>
<td>1.2 ± 0.8</td>
<td>16.0 ± 2.0</td>
<td>24.8 ± 5.8</td>
<td>37.2 ± 5.1</td>
<td>66.5 ± 6.5</td>
<td>70.0 ± 6.0</td>
<td>59.4 ± 3.0</td>
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<td>NG2</td>
<td>1.0 ± 1.2</td>
<td>1.2 ± 1.1</td>
<td>19.4 ± 2.1</td>
<td>27.6 ± 6.1</td>
<td>41.6 ± 5.0</td>
<td>72.4 ± 4.2</td>
<td>75.4 ± 5.4</td>
<td>64.0 ± 6.2</td>
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<tr>
<td>IL-10</td>
<td>8.4 ± 2.6</td>
<td>10.6 ± 2.0</td>
<td>15.8 ± 4.4</td>
<td>22.2 ± 2.1</td>
<td>63.4 ± 7.6</td>
<td>69.4 ± 6.1</td>
<td>61.6 ± 5.2</td>
<td>51.4 ± 2.7</td>
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<tr>
<td>TGF-β</td>
<td>4.2 ± 1.3</td>
<td>5.8 ± 2.6</td>
<td>15.8 ± 3.7</td>
<td>20.4 ± 2.3</td>
<td>70.2 ± 7.8</td>
<td>85.8 ± 8.3</td>
<td>72.0 ± 6.0</td>
<td>63.2 ± 7.0</td>
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<tr>
<td>bFGF</td>
<td>5.6 ± 2.9</td>
<td>4.8 ± 1.5</td>
<td>18.6 ± 3.6</td>
<td>25.6 ± 3.2</td>
<td>80.0 ± 3.4</td>
<td>94.8 ± 4.3</td>
<td>82.8 ± 5.3</td>
<td>71.6 ± 4.0</td>
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<tr>
<td>Cu, Zn-SOD</td>
<td>5.2 ± 1.6</td>
<td>5.0 ± 1.6</td>
<td>23.8 ± 6.3</td>
<td>28.6 ± 5.1</td>
<td>82.6 ± 13.8</td>
<td>90.2 ± 15.7</td>
<td>97.2 ± 10.1</td>
<td>81.2 ± 9.1</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>2.6 ± 1.5</td>
<td>2.4 ± 1.1</td>
<td>13.6 ± 3.0</td>
<td>19.6 ± 3.6</td>
<td>42.6 ± 8.7</td>
<td>48.6 ± 9.6</td>
<td>31.4 ± 6.2</td>
<td>29.0 ± 3.7</td>
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<tr>
<td>Catalase</td>
<td>2.2 ± 0.8</td>
<td>2.2 ± 1.3</td>
<td>8.8 ± 2.6</td>
<td>15.4 ± 4.2</td>
<td>23.8 ± 5.7</td>
<td>31.6 ± 4.0</td>
<td>26.8 ± 6.2</td>
<td>11.8 ± 3.7</td>
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<tr>
<td>MT-I and -II</td>
<td>4.8 ± 1.8</td>
<td>4.2 ± 1.6</td>
<td>29.2 ± 4.2</td>
<td>34.4 ± 3.4</td>
<td>116.6 ± 6.3</td>
<td>152.2 ± 5.3</td>
<td>139.2 ± 7.7</td>
<td>125.2 ± 7.1</td>
</tr>
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</table>

*Counts of positively stained cells (defined as those cells with cytoplasmatic staining or in the case of TUNEL cell with nuclear staining) were carried out in five animals of each score point analyzed. Cell counts were done in matched brainstem areas containing EAE infiltrates. EAE infiltrates were identified assuming perivascular location. Then a 0.5 mm² perivascular area was selected for countings in a blinded manner. Results are mean ± standard deviation (SD) of cell counts.
MBP immunoreactivity was also relatively unaffected in EAE-sensitized mice with score 0, whereas demyelinated MBP negative areas appeared in EAE-sensitized mice with score 1 (Fig. 2C, D), and increased even further as EAE developed. Especially in scores 3–4, mice displayed a pronounced deterioration of myelin and extensive myelin damage and demyelination. Demyelination and myelin corrosion were still obvious in mice recovering from EAE, mainly in mice with score 2 (Fig. 2G), whereas MBP immunoreactivity and myelin structure were considerably restored in recovering mice with score 0 (Fig. 2H).

Axonal damage. We also studied SMI-32 immunostaining, which marks axonal degeneration and transection due to demyelination (Trapp et al., 1998; Mancardi et al., 2001). As expected, SMI-32 immunostaining was practically absent in control mice (Fig. 3A, B). The EAE-sensitized mice with score 0 showed a slightly increased SMI-32 immunoreactivity, which was further increased in EAE-sensitized mice with scores 1 and 2 (Fig. 3C–E). The mice with maximum disease score (scores 3–4) showed the most increased SMI-32 immunoreactivity (Fig. 3F). Thus, EAE-sensitized mice at scores 3–4 displayed several axons with axonal swellings and ter-
minal spheroids as well as discontinuous immunostaining, which indicated axonal degeneration and transection due to demyelination. SMI-32 immunostaining was still manifest in mice with score 2 (Fig. 3G), while SMI-32 levels were clearly decreased in recovering mice with score 0 (Fig. 3H).

Neurodegeneration was also examined by using immunoreactivity to APP, which is an early and sensitive marker for axonal pathology (Ferguson et al., 1997; Kornek et al., 2000; Kuhlmann et al., 2002). APP immunoreactivity was not present in control mice (Table 1). During EAE, the increase of APP+ cells correlated to clinical signs, whereas APP levels peaked in mice with EAE at scores 3–4 (Table 1). In the clinical recovery phase, the number of APP+ degenerating cells decreased drastically in all mice (Table 1).

Proinflammatory cytokines

Proinflammatory cytokines such as IL-1β, IL-6 and TNF-α were very low or absent in the brainstem of control mice (Fig. 4; Table 1). In contrast, during active EAE, all of the mice increased rapidly and significantly IL-1β (not shown), IL-6 (Fig. 4; Table 1), and TNF-α (Table 1). Thus, EAE-sensitized mice with scores 0 and 1 showed increasing levels of IL-1β, IL-6 and TNF-α. These were all further increased with disease severity. Interestingly, IL-6 levels were very high at an early stage (score 0) and peaked already at score 2, while TNF-α increased more slowly and peaked during scores 3–4 (Table 1). During recovery from EAE, these proinflammatory cytokines decreased rapidly (Fig. 4; Table 1). Their expression profile was parallel to the clinical course and both demyelination and axonal damage markers (Fig. 5A).

Fig. 2. MBP immunoreactivity showing demyelination in brainstem. (A, B) MBP immunolocalization of non-immunized mice (control 1; A) and PBS immunized mice (control 2; B). (C) Showing MBP immunoreactivity of EAE-sensitized mice with score 0. (D–F) EAE-sensitized mice with score 1 (D) and with score 2 (E) and with scores 3–4 (F) showed increasing demyelination. Thus, a few demyelinated areas are seen in mice with score 2, and in mice with scores 3–4, extensive myelin damage and demyelination were observed. (G, H) Demyelination was still prominent in mice recovering from EAE clinical signs as shown in mice with score 2 (G) and with score 0 (H). Scale bars ~48 μm A–H.
Oxidative stress and apoptosis

Oxidative stress was determined by using NITT and MDA immunoreactivity. We observed that control mice showed no significant signs of oxidative stress (Table 1). However, in EAE-sensitized mice with scores 0 and 1 the numbers of both NITT/H11001 and MDA/H11001 cells increased relatively to control mice, and a further increase in oxidative stress was even more pronounced and peaking in mice with scores 3–4 (Table 1). During the clinical recovery phase, the number of cells suffering oxidative stress decreased radically in all mice (Table 1). Thus, the levels of oxidative stress during EAE followed the pattern of demyelination and axonal damage markers (Fig. 5A).

Apoptotic cell death was judged by TUNEL, and TUNEL/H11001 cells had the same profile expression observed for proinflammatory cytokines (Table 1; Fig. 5A).

Fig. 3. SMI-32 immunostainings indicating axonal degeneration and transection in brainstem. (A, B) SMI-32 immunostaining is practically absent in non-immunized mice (control 1; A) and PBS-immunized mice (control 2; B). (C) SMI-32 immunoreactivity was slightly increased in EAE-sensitized mice with score 0. (D–F) EAE-sensitized mice with score 1 (D) score 2 (E) and scores 3–4 (F) showed increasing SMI-32 immunoreactivity, which suggests increasing axonal degeneration and transection due to demyelination in these mice. Mice with EAE scores 3–4 showed the most increased SMI-32 immunostaining. (G) SMI-32 immunostaining was still prominent in mice recovering from clinical EAE signs with score 2. (H) SMI-32 levels were clearly decreased in recovering mice with score 0. Scale bars = 42 μm A–H.

Oxidative stress and apoptosis

Oxidative stress was determined by using NITT and MDA immunoreactivity. We observed that control mice showed no significant signs of oxidative stress (Table 1). However, in EAE-sensitized mice with scores 0 and 1 the numbers of both NITT+ and MDA+ cells increased relatively to control mice, and a further increase in oxidative stress was even more pronounced and peaking in mice with scores 3–4 (Table 1). During the clinical recovery phase, the number of cells suffering oxidative stress decreased radically in all mice (Table 1). Thus, the levels of oxidative stress during EAE followed the pattern of demyelination and axonal damage markers (Fig. 5A).

Markers of neuroregeneration

Oligodendroglial regeneration was studied by using immunostainings for PDGFα-rec and NG2, which stained oligodendrocyte progenitor cells (De Nicola et al., 2003; Penkowa et al., 2003). As shown in Table 1, the number of oligodendrocyte progenitor cells slowly and gradually increased during EAE. During the clinical recovery, the numbers of oligodendrocyte progenitor cells remained high.

To analyze neoroglial regeneration, sprouting and regrowth during EAE we used antibodies against GAP-43 and P-40 (Penkowa et al., 2003). Both GAP-43 (Fig. 6) and P-40 (not shown) were almost absent from the CNS of control mice. During EAE, only few sprouting cells showing GAP-43 (Fig. 6C, D) were found in the brainstem. However, a slight increase in regrowing cells was observed...
during disease progression (Fig. 6E, F). During the clinical recovery phase, the expression of GAP-43 was positive throughout the brainstem. Thus, mice with both scores 2 and 0 during recovery from clinical EAE signs showed several GAP-43+/H11001 growth processes (Fig. 6 G, H). The staining pattern of P-40 positive cells (not shown) paralleled to that of GAP-43.

All these markers increased slowly during the EAE progression and remained high during the clinical recovery phase.

Anti-inflammatory cytokines and growth factors

Similar to the markers of neuroregeneration, anti-inflammatory cytokines like IL-10 (Table 1), TGF-α (Table 1) and the growth factors analyzed, bFGF (Fig. 7; Table 1) and NGF (not shown), followed the same expression profile of oligodendroglial progenitor and neuroglial regeneration markers (Fig. 5B).

MT-I and -II expression

We monitored the expression of the non-enzymatic antioxidant proteins MT-I and -II during EAE. In control mice MT-I and -II expression was low in intraparenchymal CNS cells and MT were only seen in the ependyma, choroid plexus, and meninges. In EAE-sensitized mice MT-I and -II levels increased slowly (Fig. 8; Table 1). Thus, only a small increase was seen in EAE-sensitized mice with score 0, while the levels were further increased in mice with score
MT-I and -II immunoreactivity peaked at scores 3–4 (Fig. 8F; Table 1). However, mice recovering from EAE clinical signs also showed high levels of MT-I and -II, at either score 2 or score 0 (Fig. 8G, H). Thereby, MT-I and -II were not only induced during active disease but also during clinical recovery. Cells expressing MT-I and -II were mainly vimentin/reactive astrocytes and monocyte/macrophages (Fig. 9).

In parallel to MT expression, we analyzed the expression of some enzymatic proteins that have a role in the detoxification of oxidant molecules such as Cu,Zn-SOD, Mn-SOD or catalase (Table 1). While catalase and Mn-SOD were weakly induced, Cu,Zn-SOD expression followed a similar pattern to MT-I and -II.

Overall, we could differentiate two different profiles of marker expression. In the first profile (pattern 1), marker expression increased as clinical signs worsened and reverted to baseline expression during clinical recovery. In the second, marker expression increased at a later point during relapse, peaked at highest clinical score, and remained elevated during recovery (Fig. 5). Markers implicated in EAE-clinical ongoing as proinflammatory cytokines, oxidative stress, demyelination, and neuronal damage markers were found in the first profile (Fig. 5A), while the second profile (pattern 2) included markers related to EAE-clinical recovery as anti-inflammatory cytokines, growth factors and oligodendroglial progenitor and neuroglial regeneration markers (Fig. 5B). Interestingly, MT-I and -II were induced not only during the active phase of EAE but also during the clinical recovery phase, suggesting that these antioxidant proteins may participate in the anti-inflammatory and neuroregenerative mechanisms triggered during the relapse resolution phase.

There is much evidence supporting a role of oxidative stress in the pathogenesis of neurodegenerative diseases. It has been shown that MS plaques have increased immunoreactivity for both nitric oxide synthase 2 and NITT (Bagasra et al., 1995) and that a high concentration of NO causes axonal degeneration and neuronal death (Dawson et al., 1993; Tamatani et al., 1998; Smith et al., 1999). This most probably occurs because neurons are highly susceptible to oxidative stress due to the fact that CNS white matter has low concentrations of antioxidant enzymatic activities (Zhang et al., 2001). Nitration has also been associated with compromised integrity of the BBB in MS (Kean et al., 1995).

**DISCUSSION**

In this study we describe the expression of a group of pro- and anti-inflammatory cytokines and markers of neurodegeneration and tissue repair as well as MT at different clinical points during EAE, in which we found two profiles of marker expression. The first profile (pattern 1), involving molecules like proinflammatory cytokines, oxidative stress, demyelination, and neuronal damage markers, in which expression increases as clinical signs worsen and then revert to baseline expression during clinical recovery. The second profile (pattern 2), in which marker expression increases at a later point during relapse, peaks at highest clinical score, and remains elevated during recovery. This second pattern includes markers related to EAE clinical recovery as anti-inflammatory cytokines, growth factors and oligodendroglial progenitor and neuroglial regeneration markers. Interestingly, MT-I and -II were induced not only during the active phase of EAE but also during the clinical recovery phase, suggesting that these antioxidant proteins may participate in the anti-inflammatory and neuroregenerative mechanisms triggered during the relapse resolution phase.
We found that markers for oxidative stress (NITIT and MDA), axonal damage and transection (APP and SMI-32) or demyelination were increased during disease relapse and decreased during recovery back almost to baseline expression. This expression profile was similar to T-lymphocyte and monocyte infiltration and coincidental to the degree of severity of the clinical course. These immune cells, and also resident reactive astrocytes and microglia, use ROS for carrying out many of their functions. ROS increase influences a number of cellular redox-sensitive signaling processes such as activation of nuclear factor-kB and activator protein-1, which up-regulate the transcription of a variety of genes involved in inflammation, including cytokines like IL-1, TNF-α, and IL-6 (Serkkola and Hurme, 1993; Delerive et al., 1999). In this context, we also found that expressions of IL-1β, IL-6, and TNF-α were increased during relapse and decreased when clinical signs remitted. Interestingly, the fact that IL-6 expression was induced earlier than IL-1β and TNF-α, supports data from other authors (Samoilova et al., 1998b) showing that IL-6 is necessary for EAE development.

It has been described that proinflammatory cytokines, as IL-6 and TNF-α (Hernandez et al., 1997; Carrasco et al., 1998; Giralt et al., 2002a) and oxidative stress (Andrews, 2000; Hidalgo et al., 2002), induce MT expression. MT-I and -II are extraordinarily efficient antioxidant proteins and scavengers of ROS that accumulate under conditions where oxidative stress has taken place (Aschner, 1996, 1998; Coyle et al., 2002). In MT knockout mice, in fact, the expression of oxidative stress markers is higher than in wild-type mice (Penkowa et al., 2001) and therapy with MT-II reduces oxidative stress and ROS formation in Lewis
rats with EAE (Penkowa and Hidalgo, 2000a). We found that MT-I and -II induction in SJL mice started after proinflammatory cytokines and oxidative stress markers were induced. In contrast, these markers started to decrease when MT-I and -II expression was at its highest (scores 3–4). All these data suggest that proinflammatory cytokines are important in the EAE model for the development of the (auto-)immune response and that they also participate in the induction of other factors, as MT-I and -II, which are implicated in disease recovery.

The time-course for the expression of anti-inflammatory IL-10 and TGF-β cytokines was also analyzed. In the same way as seen in MT, IL-10 and TGF-β remained high during recovery. This supports the role these cytokines play in EAE remission as reported elsewhere (Kennedy et al., 1992; Olsson, 1995; Bettelli et al., 1998; Samoilova et al., 1998a; Young et al., 2000). A similar pattern was observed for bFGF and NGF. The latter promotes the biosynthesis of myelin by oligodendrocytes and neuronal growth (Cohen et al., 1996; Lewin and Barde, 1996) and bFGF directly stimulates proliferation and/or regeneration of oligodendrocytes within the lesion area (Liu et al., 1998; Ruffini et al., 2001). These growth factors may contribute to the neuroglial regeneration and remyelination observed.
during EAE recovery. It is noteworthy that MT-I and -II may also induce anti-inflammatory cytokine, growth factor and neurotrophin expression (Penkowa and Hidalgo, 2000a; Giralt et al., 2002b; Youn et al., 2002). All these data suggest that MT-I and -II are important in the remission of EAE.

Besides the nonenzymatic MT-I and -II antioxidant proteins, we also quantified the expression of some enzymatic molecules implicated in free-radical detoxification such as SOD and catalase. We found that catalase and Mn-SOD were only slightly induced during EAE compared with the cytoplasmic SOD isoform (Cu,Zn-SOD), which had an expression profile similar to that of MT. These results contrast with those observed by Qi et al., 1997, who reported an increase in Mn-SOD positive cells but no changes in Cu,Zn-SOD positive cells in optic nerve of guinea-pigs with EAE. As for catalase, it has been described that exogenous administration reverses BBB disruption and demyelination of the optic nerve in EAE (Guy et al., 1989, 1994), thus suppressing experimental optic neuritis (Guy et al., 1999).

Fig. 8. MT-I and -II expression in the brainstem. (A, B) MT-I and -II expressing cells were scarcely observed in the brainstem parenchyma of non-immunized mice (control 1; A) and PBS-immunized mice (control 2; B). (C, D) In EAE-sensitized mice with score 0 (C) and with score 1 (D), some stellate glial cells were showing MT-I and -II. (E, F) MT-I and -II immunoreactivity was significantly increased in mice with score 2 (E), while the MT-I and -II levels peaked in scores 3–4 (F). (G, H) During clinical recovery, the number of cells expressing MT-I and -II was still very high. Accordingly, mice in clinical EAE recovery with score 2 showed significantly increased MT-I and -II levels (G), which were almost as high as during the peak expression. The mice in recovery with score 0 also showed very high levels of MT-I and -II (H). However, the number of MT-I and -II containing cells decreased. Scale bars = 39 μm.
Several studies have reported on the role of non-enzymatic antioxidants, such as uric acid and vitamins, in the pathogenesis of EAE/MS. Uric acid, a natural peroxynitrite scavenger, has been shown to have a protective role during EAE (Hooper et al., 2000; Scott et al., 2002). Furthermore, levels of uric acid in MS patients are lower than in healthy controls (Sotgiu et al., 2002; Toncev et al., 2002), although it has not been proven whether it is primarily deficient or secondarily reduced because of its scavenger properties. In this regard, the regulation of the CNS inflammatory response by antioxidant molecules might significantly affect both the neuroregenerative response and the functional outcome seen in the CNS after an EAE/MS relapse.

### Table 2. Statistical data of inflammatory, neurodegenerative, tissue repair markers and metallothioneins in brain stem during EAEa

<table>
<thead>
<tr>
<th></th>
<th>Score 2 recovery, score 2</th>
<th>P-value</th>
<th>Score 0 recovery, score 0</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean difference (95% CI)</td>
<td>Mean difference (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4/80</td>
<td>-57.00 (-66.61, -47.39)</td>
<td>0.00000</td>
<td>-1.00 (-6.25, 4.25)</td>
<td>0.67273</td>
</tr>
<tr>
<td>CD4+</td>
<td>-49.60 (-59.17, -40.03)</td>
<td>0.00006</td>
<td>.20 (-5.81, 6.21)</td>
<td>0.94096</td>
</tr>
<tr>
<td>GFAP</td>
<td>-4.80 (-21.06, 11.46)</td>
<td>0.51937</td>
<td>57.40 (37.67, 77.13)</td>
<td>0.00079</td>
</tr>
<tr>
<td>IL-6</td>
<td>-83.40 (-93.47, -73.33)</td>
<td>0.00000</td>
<td>-55.40 (-60.24, -50.56)</td>
<td>0.00000</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-56.60 (-63.80, -49.40)</td>
<td>0.00000</td>
<td>2.20 (-1.95, 6.35)</td>
<td>0.25772</td>
</tr>
<tr>
<td>NITT</td>
<td>-74.20 (-81.43, -67.97)</td>
<td>0.00002</td>
<td>-11.00 (-16.78, -5.22)</td>
<td>0.00322</td>
</tr>
<tr>
<td>MDA</td>
<td>-76.80 (-94.06, -59.54)</td>
<td>0.00002</td>
<td>-14.40 (-20.22, -8.58)</td>
<td>0.00169</td>
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<tr>
<td>APP</td>
<td>-81.60 (-91.43, -71.77)</td>
<td>0.00000</td>
<td>-8.80 (-12.66, -4.94)</td>
<td>0.00145</td>
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<tr>
<td>TUNEL</td>
<td>-82.20 (-98.03, -66.37)</td>
<td>0.00005</td>
<td>-17.60 (-23.33, -11.87)</td>
<td>0.00138</td>
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<tr>
<td>PDFG-α rec</td>
<td>32.80 (24.73, 40.87)</td>
<td>0.00002</td>
<td>43.40 (39.64, 47.16)</td>
<td>0.00000</td>
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<tr>
<td>NG2</td>
<td>33.80 (26.18, 41.42)</td>
<td>0.00001</td>
<td>44.60 (37.85, 51.35)</td>
<td>0.00003</td>
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<tr>
<td>IL-10</td>
<td>-1.80 (-11.26, 7.66)</td>
<td>0.67384</td>
<td>35.60 (30.24, 40.96)</td>
<td>0.00000</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1.80 (-8.34, 11.94)</td>
<td>0.69374</td>
<td>47.40 (39.19, 55.61)</td>
<td>0.00001</td>
</tr>
<tr>
<td>bFGF</td>
<td>2.80 (-3.66, 9.26)</td>
<td>0.35140</td>
<td>53.00 (47.39, 58.61)</td>
<td>0.00000</td>
</tr>
<tr>
<td>Cu, Zn-SOD</td>
<td>14.60 (-9.34, 32.24)</td>
<td>0.09601</td>
<td>57.40 (46.01, 68.79)</td>
<td>0.00001</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>-11.20 (-22.24, -0.16)</td>
<td>0.05086</td>
<td>15.40 (10.53, 20.27)</td>
<td>0.00011</td>
</tr>
<tr>
<td>Catalase</td>
<td>3.00 (-5.71, 11.71)</td>
<td>0.45032</td>
<td>3.00 (-1.66, 7.66)</td>
<td>0.18014</td>
</tr>
<tr>
<td>MT-I and -II</td>
<td>22.60 (12.39, 32.81)</td>
<td>0.00104</td>
<td>96.00 (87.50, 104.50)</td>
<td>0.00000</td>
</tr>
</tbody>
</table>

*a Statistical analysis was performed comparing the data from cell counts of the same clinical score during both EAE ongoing and EAE recovery. Due to the sample size and the number of comparisons made, Welch test was used and Bonferroni correction was applied (P-value was fixed in 0.0028). The comparison of score 2 established two profiles of marker expression: Those markers with negative difference and P-value < 0.0028 (bold type), related to inflammation and CNS degeneration (pattern 1). The rest of them (excluding endogenous antioxidant protein Mn-SOD and catalase which had low induction) were related to anti-inflammatory and neuroregenerative processes (pattern 2). The comparison of score 0 also allowed us to establish these two profiles of marker expression: Markers with positive difference and a P-value < 0.0028 (bold type) correspond to pattern 2.
In summary, we show here that induction and remission of EAE clinical signs are related to a different expression of cytokines and markers involved in processes of neurodegeneration and neuroregeneration. Markers involved in the development of EAE were first expressed early after the induction of the disease, peaked at the highest clinical score and decreased drastically at the beginning of clinical recovery. In contrast, a second group of markers were later induced, peaked at highest clinical score, and remained elevated during recovery. MT-I and –II expression followed the second expression pattern. The kinetics of the different molecules might be explained, at least in part, by their interaction with each other. In this context, linking with our previous studies, we hypothesize that the MT-I and -II proteins may have a role in the pathogenesis of EAE by mediating neuroregeneration either by down-modulating neurodegenerative processes (such as inflammation, oxidative stress, demyelination and axonal injury) or by up-modulating tissue repair processes (including induction of growth factor expression). Therefore, based on their antioxidant, anti-inflammatory and neuroprotective properties, MT-I and MT-II might be considered as potential therapeutic candidates to promote neuroregeneration in MS.

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