

# Immunohistochemical analysis of spinal cord components in mouse model of experimental autoimmune encephalomyelitis

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## Abstract

**Introduction.** Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model for studying immunopathology of multiple sclerosis (MS) because it repeats the hallmarks of the human disease, such as focal inflammation and demyelination of the central nervous system, subsequently leading to axonal and neuronal loss. The interrelationships, timing and sequence of different pathological processes that lead to histologically observed lesions in SM are still incompletely understood.

**Material and methods.** EAE was induced in female C57Bl/6 mice by active immunization with MOG35-55 antigen. Development of the neurological symptoms in the animals was monitored and on that basis spinal cords were collected in three successive phases of the disease (onset, peak, chronic). Total leukocytes, T cells, macrophages/microglia, oligodendrocytes, damaged axons and surviving neuronal cell bodies were visualized using appropriate immunohistochemical markers and their density was quantitatively assessed using image analysis software.

**Results.** The density of all studied cells except neurons was significantly higher in EAE mice than in the control mice. The density of total leukocytes, T cells, and damaged axons increased from the onset to the peak phase and decreased in the chronic phase to reach values lower than those in the peak phase. The density of macrophages/microglia increased in the peak phase and remained at the elevated level in the chronic phase. Oligodendrocytes showed the highest density in the onset phase and gradually decreased afterwards. The density of neuronal cell bodies decreased only in the chronic phase of the disease.

**Conclusions.** In mouse model of EAE, inflammatory cells predominate in the early phases of the disease. This study shows for the first time that inflammation precedes oligodendrocyte death and neuronal loss and that macrophages/microglia are the only cells persisting in large numbers in the chronic phase of the disease, probably because of the switch from proinflammatory to anti-inflammatory phenotype. (*Folia Histochemica et Cytobiologica* 2018, Vol. 56, No. 3, 151–158)

**Key words:** EAE; spinal cord; leukocytes; T cells; macrophages; microglia; oligodendrocytes; neuronal damage; immunohistochemistry

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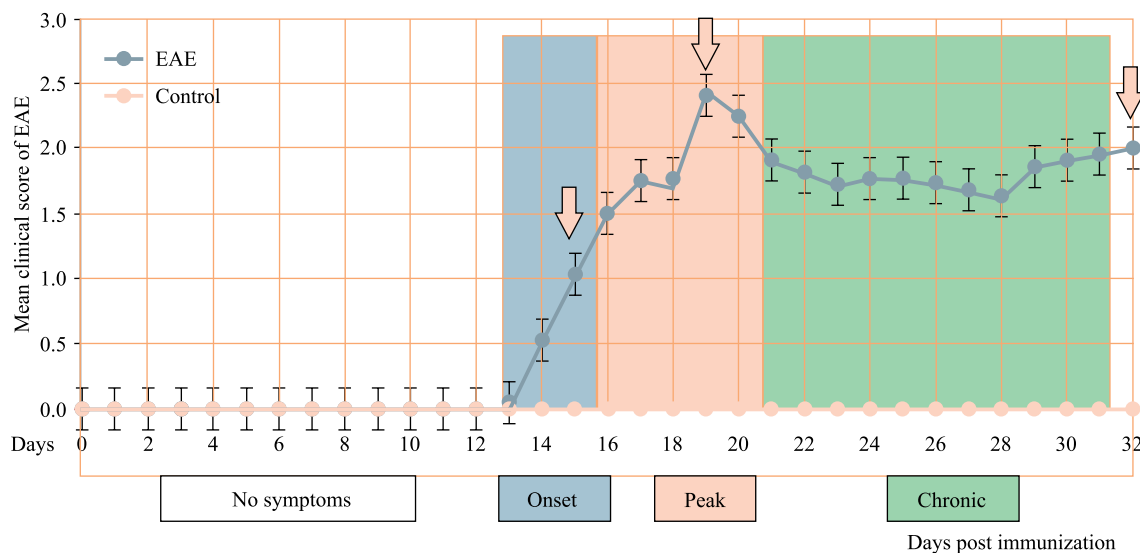
## Introduction

Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model for studying immunopathology of multiple sclerosis (MS) because it

repeats the hallmarks of the human disease, such as focal inflammation and demyelination of the central nervous system (CNS), subsequently leading to axonal and neuronal loss [1]. In C57Bl/6 mice, EAE can be induced by active immunization with MOG35-55 antigen which triggers the inflammatory demyelinating disease [2]. The clinical symptoms in EAE reflect inflammation localized predominantly in the spinal cord, although MOG-induced EAE also targets the optic nerve [3]. The neuroinflammatory response is manifested by infiltrations of T cells, B cells and

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**Figure 1.** Development of neurological symptoms in experimental autoimmune encephalitis (EAE) mice. EAE was elicited and mice were scored for EAE symptoms over 30 days using the scale as described in Methods. Day 0 was the day of immunization. Arrows show three time points (tissue collection for EAE and control groups) representing three successive phases of the disease. Values represent means  $\pm$  SEM.

macrophages, as well as by formation of focal demyelination plaques in the CNS, similar to the pathology seen in MS [4]. The interrelationships, timing and sequence of different pathological processes that lead to histologically observed lesions in MS are still incompletely understood. It is also unclear, whether neuroinflammation or demyelination are primary or secondary events during development of the disease [5]. Hence, EAE is a good model for studying MS mechanisms and for testing or developing drugs [6].

The aim of the present study was to investigate the progressive neurodegenerative process in EAE mice by analyzing the occurrence and number of the demyelination plaque components: leukocytes, macrophages/microglial cells, oligodendrocytes, as well as damaged axons and surviving nerve cells at different stages of the disease. These parameters reflect spinal cord inflammation and neurodegeneration in EAE which occur in both white and gray matter.

## Material and methods

**Animals.** Female C57Bl/6 mice (18 animals for EAE and 18 for control) aged 10–11 weeks were habituated for at least 1 week before the experiment and then were immunized. The animals were housed under controlled environmental conditions (LD 12:12 h regime in air-conditioned rooms:  $22 \pm 2^\circ\text{C}$ ,  $55 \pm 10\%$  humidity) and allowed free access to food and water throughout the experiment. All experiments were conducted in compliance with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010

on the protection of animals used for scientific purposes and approved by the First Local Ethics Committee of the Jagiellonian University Medical College, Krakow, Poland.

**Induction of EAE.** For induction of EAE, Hooke Kits™ EAE Emulsion (Hooke Laboratoires, Lawrence, Massachusetts USA) were used. Kits contained MOG35-55 antigen in an emulsion with Complete Freund's Adjuvant (CFA) including *Mycobacterium tuberculosis* (H37Ra). Emulsion was administered subcutaneously at two sites ( $100 \mu\text{l}$  between the ears and  $100 \mu\text{l}$  in hind flank). Each mouse was also injected intraperitoneally (*i.p.*) with  $340 \mu\text{l}$  of *Bordetella pertussis* pertussis toxin (PTx) dissolved in phosphate-buffered saline (PBS) (Hooke Laboratoires) 2 h after the administration of the emulsion and again 24 h later. PTx enhanced EAE development by providing additional adjuvant and facilitating entrance of autoimmune T cells into the CNS [2]. Control mice were injected with CFA and PTx only (Hooke Control Kits™, Hooke Laboratoires, USA) according to the same schedule.

**Monitoring of the course of the disease and study design.** All mice were weighed and examined daily for the neurological symptoms of EAE, scored according to the following scale: (0) no symptoms; (1) floppy tail; (2) hindlimb weakness; (3) hindlimb paralysis; (4) forelimb paralysis; (5) mouse moribund or dead (0.5 gradations represent intermediate scores) [7].

For histological analysis EAE mice were sacrificed at three different time points representing three phases of disease: onset phase (day 15;  $n = 6$ ), peak phase (day 19;  $n = 6$ ) and chronic phase (day 32;  $n = 6$ ) (Fig. 1). Control

mice were sacrificed at the same time points ( $n=6$  for each phase).

All EAE mice showed initial symptoms of the disease between 13th and 16th day after immunization and this was the onset phase in our experiment. The peak of disease almost always occurred 3–4 days later, between days 16 and 21. The maximum scores of the peak phase continued for several days and then mice partially recovered (chronic phase). During the progress of EAE, changes in body weight also reflected disease severity. Mice steadily increased their body weight until disease onset. Afterwards, mice gradually lost 5–10% of their weight and after the peak of disease they slowly gained weight again.

**Tissue collection and processing.** Animals were anaesthetized *i.p.* with 100 mg/kg ketamine and 10 mg/kg xylazine and transcardially perfused with ice-cold PBS for 10 min, followed by 4% paraformaldehyde for the next 10 min. Spinal cords were carefully removed from the vertebral canal and postfixed in the same fixative for 4 h. After overnight incubation in 5% sucrose at 4°C, tissue was embedded in OCT (Shandon Cryomatrix, Thermo Fisher Scientific, Rockford, IL USA) and snap-frozen at –80°C. The examined area of the spinal cord included the lumbar part, a region commonly and rapidly affected in EAE. Serial cryosections 10  $\mu\text{m}$  — thick were cut at 100  $\mu\text{m}$  intervals, collected on poly-L-lysine coated slides and air dried.

**Immunofluorescence staining.** Immunohistochemistry was performed using the following primary antibodies: rat anti-CD45 (1:100, Thermo Fisher Scientific, MA1-81247) for total leukocytes, rat anti-CD3 (1:100, Thermo Fisher Scientific, cat. # MA1-80783) for T cells, rabbit anti-ionized calcium binding adaptor molecule (anti-Iba1) for activated macrophages and microglial cells (1:200, Synaptic Systems, Goettingen, Germany, cat. # 234003), rabbit anti-2',3'-Cyclic nucleotide 3'-phosphodiesterase 1 (anti-CNP1) for oligodendrocytes (1:500, Synaptic Systems, cat. # 355002), rabbit anti-beta-amyloid precursor protein (beta-APP) for detection of injured axons (1:100, Synaptic Systems, cat. # 218903) and rabbit anti-NeuN for detection of surviving neurons (1:100, Thermo Fisher Scientific, cat. # PA5-37407).

In short, the sections were preincubated for 40 min in PBS containing 5% normal goat serum (Sigma-Aldrich, St. Louis, MO, USA), 0.01% sodium azide, 0.05% thimerosal, 0.1% bovine serum albumin, 0.5% Triton X-100, and 2% dry milk. They were next incubated overnight at room temperature with primary antibodies and after a rinse in PBS incubated for 90 min with the secondary goat anti-rat Alexa488-conjugated antibodies (1:100, Jackson IR, West Grove, PA, cat. # 112-545-167), goat anti-rabbit Alexa488-conjugated antibodies (1:100, Jackson IR, West Grove, PA, cat. # 111-545-144) or Cy3-conjugated goat

anti-rat antiserum (1:300, Jackson IR, West Grove, PA, 112-165-167). Sections were washed three times in PBS and mounted in glycerol/PBS solution.

**Microscopy and morphometry.** The sections were examined under Olympus BX50 brightfield/epifluorescence microscope (Olympus, Tokyo, Japan). Images of the spinal cords were recorded using Olympus DP71 digital CCD camera, stored as TIFF files and processed for quantitative analysis using CellSense Dimension Software (Olympus). A total of at least 24 slides were analyzed per experimental group ( $n = 6$ ). The density of the immunopositive cells was assessed in sections encompassing the whole area occupied by the gray and white matter and expressed in two ways: as the number of T cells and surviving neurons and as the immunoreactive profile area in case of total leukocytes, macrophages, oligodendrocytes and damaged axons. The analysis was done blind (the observer did not know whether the specimen comes from the control or EAE mouse).

**Statistical analysis.** The obtained results were analyzed by the Mann-Whitney U-test for nonparametric data using Prism 5.0 software (GraphPad, La Jolla, California, USA).  $P < 0.05$  was regarded as statistically significant.

## Results

All mice immunized with MOG developed neurological symptoms of EAE. Control mice did not develop any signs of neurological disease.

### *Histological examination of spinal cords of EAE and control mice*

Immunoreactivity for CD45 showed large accumulations of leukocytes in the white and gray matter in EAE mice and much less leukocytes in control mice. In all phases of the disease, leukocytes formed local aggregates with very high cell density, indicative of the presence of inflammatory foci.

The immunostaining of the spinal cord sections with anti-CD3 antibody revealed areas containing numerous CD3-positive T cells in white matter of EAE mice and relatively few in gray matter of EAE and control mice.

Iba1-positive activated macrophages/microglial cells showed an equal distribution in white and gray matter and were much more numerous in EAE mice, as compared to control animals.

Oligodendrocytes revealed by CNP1 immunoreactivity were abundant, mainly in the white matter of both EAE and control mice. In EAE mice they seemed to be most numerous during the onset phase and to decrease in number in the following phases of the experimental disease.

Axonal damage was observed in the white matter of EAE mice in all phases of the disease with the highest immunoreactivity at the peak of neurological symptoms. Control mice did not show immunostaining indicative of axonal damage.

NeuN-positive cells bodies of surviving neurons in gray matter seemed to be less numerous in EAE mice than in control mice only in the chronic phase of EAE.

### **Quantitative analysis of spinal cord sections of EAE and control mice**

The density of all studied cells was significantly higher in EAE mice than in the control mice. The only exception were neurons — the density of neuronal cell bodies in EAE mice was comparable to that in control mice in the onset and peak phases. The density of total leukocytes, T cells, and damaged axons generally revealed a similar phase-dependent course in EAE mice: it increased from the onset to the peak phase and decreased in the chronic phase (Figs. 2, 3, 6) to reach values lower than those in the peak phase. The density of activated macrophages/microglia increased in the peak phase and remained at the elevated level in the chronic phase (Fig. 4). Oligodendrocytes showed the highest density in the onset phase and gradually decreased afterwards. In the chronic phase, their density was even significantly lower than that in the control mice (Fig. 5). The number of neuronal cell bodies decreased only in the chronic phase and there were no significant differences between the other phases of the disease (Fig. 7). Numerical results of the measurements are presented in Table 1.

## **Discussion**

Results of this study show dynamics of cells involved in the pathogenesis of EAE and progress of the main histopathological changes, axonal damage and neuronal death during development and progression of the disease. The investigated parameters were correlated with three successive phases of EAE: onset, peak and chronic, characterized by neurological symptoms [2]. It could be expected that cells involved in the inflammatory process in the onset phase would reveal higher density as compared with the control, their density would further increase in the peak phase and decline during the chronic phase, as suggested by partial recovery of the mice in that phase.

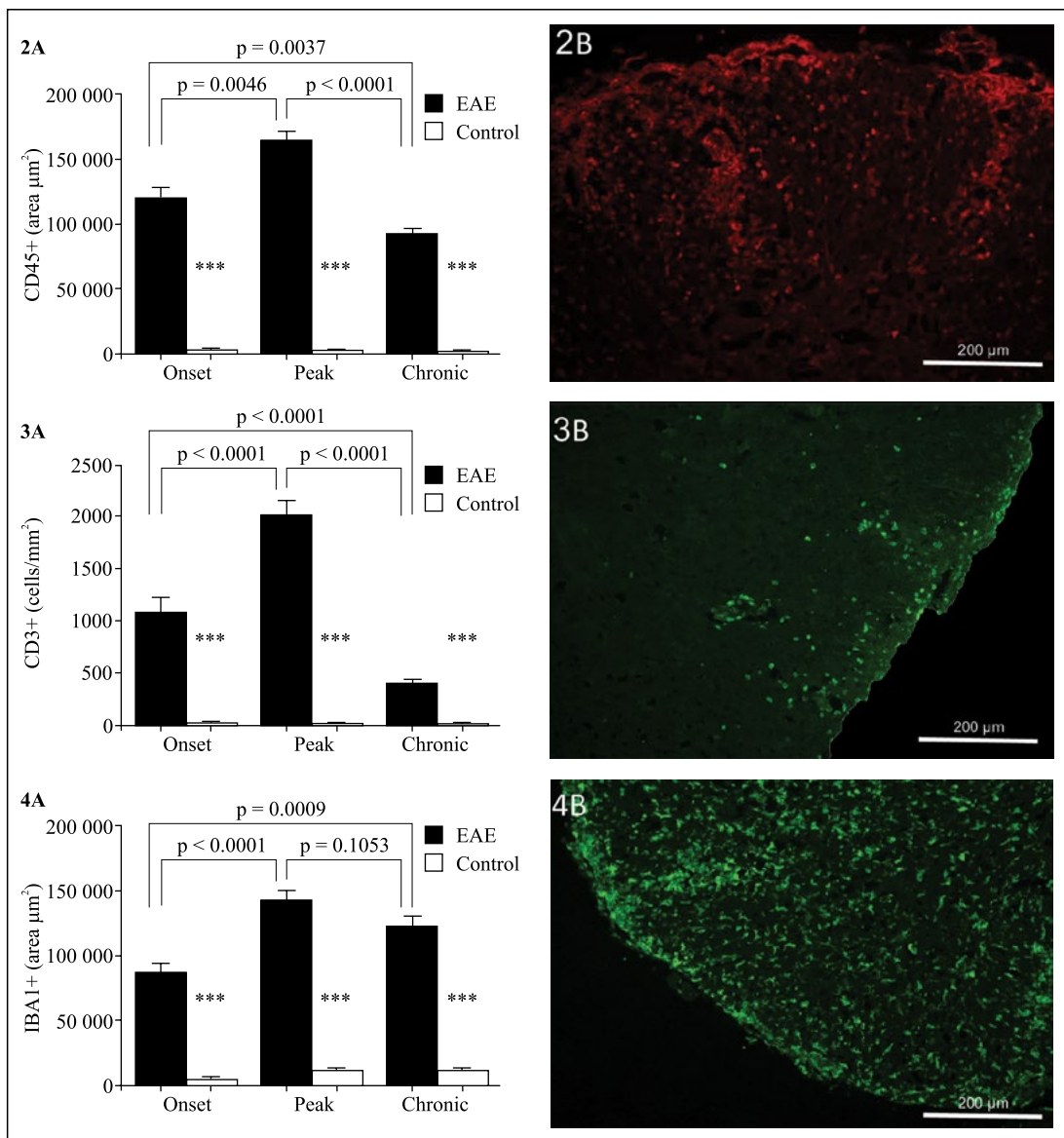
Indeed, the density of T cells, the key players in the pathology of EAE and SM follows such pattern. It is widely accepted that EAE is initiated by T helper (Th) cells which escape self-tolerance mechanisms and bind myelin proteins *via* their TCR receptors. In further phases of the disease, two subsets of T cells:

IFN $\gamma$ -secreting Th1 cells and IL-17-secreting Th17 cells, acting in cooperation with antigen presenting cells, recruit effector cells of EAE: monocytes/macrophages and microglia to the areas of the developing inflammation (mostly perivascular regions of the central nervous system and meninges) and the phagocytotic activity of the latter cells is directly responsible for demyelination [8, 9]. A decrease in the number of T cells at later stages of EAE was also reported by Herrero-Herranz *et al.* [10].

Total leukocytes showed a similar behavior. CD45 antibody detects T cell, B cells, neutrophils and monocytes/macrophages. B cells and neutrophils have been recently demonstrated to play significant roles in the development and progression of EAE. B cells amplify the autoimmune process by taking up myelin antigens and presenting them to Th cells, they also produce anti-myelin antibodies [11, 12]. It has been recently demonstrated that some, not yet identified, secretory products of B cells obtained from MS patients induce apoptosis of oligodendrocytes and neurons *in vitro* [13].

Neutrophils, which were found to infiltrate EAE lesions in large quantities, together with lymphocytes and macrophages [14], seem to contribute to the pathogenesis of EAE *via* several mechanisms. They significantly participate in the disruption of blood-brain/spinal cord barrier, thus facilitating transmigration of other cells involved in EAE [15]. By producing IL-1 $\beta$ , neutrophils promote differentiation of a Th cell subset important for the development of EAE [16], they also secrete cofactors necessary for maturation of professional antigen presenting cells in EAE lesions [17].

We observed that the density of macrophages/microglia increases during the first two phases of EAE and remains at the elevated level in the chronic phase. This behavior well corresponds with the activity of these cells during progression of the disease. The initial phase of EAE is characterized by massive infiltration of the nerve tissue by blood monocytes which are transformed into macrophages and by activation of resident microglia [18]. The vast majority of these cells show “classically activated” proinflammatory M1 phenotype [19] and they secrete cytokines promoting tissue damage, neuronal death and demyelination, the latter being additionally intensified by the phagocytotic activity of both cell types. This involvement of M1 macrophages/microglia greatly contributes to the development of the lesions in the initial and peak phases [20]. However, later in the course of EAE, M1 phenotype is gradually replaced by M2 macrophages/microglia [21] showing anti-inflammatory properties promoting tissue repair and subsequent functional

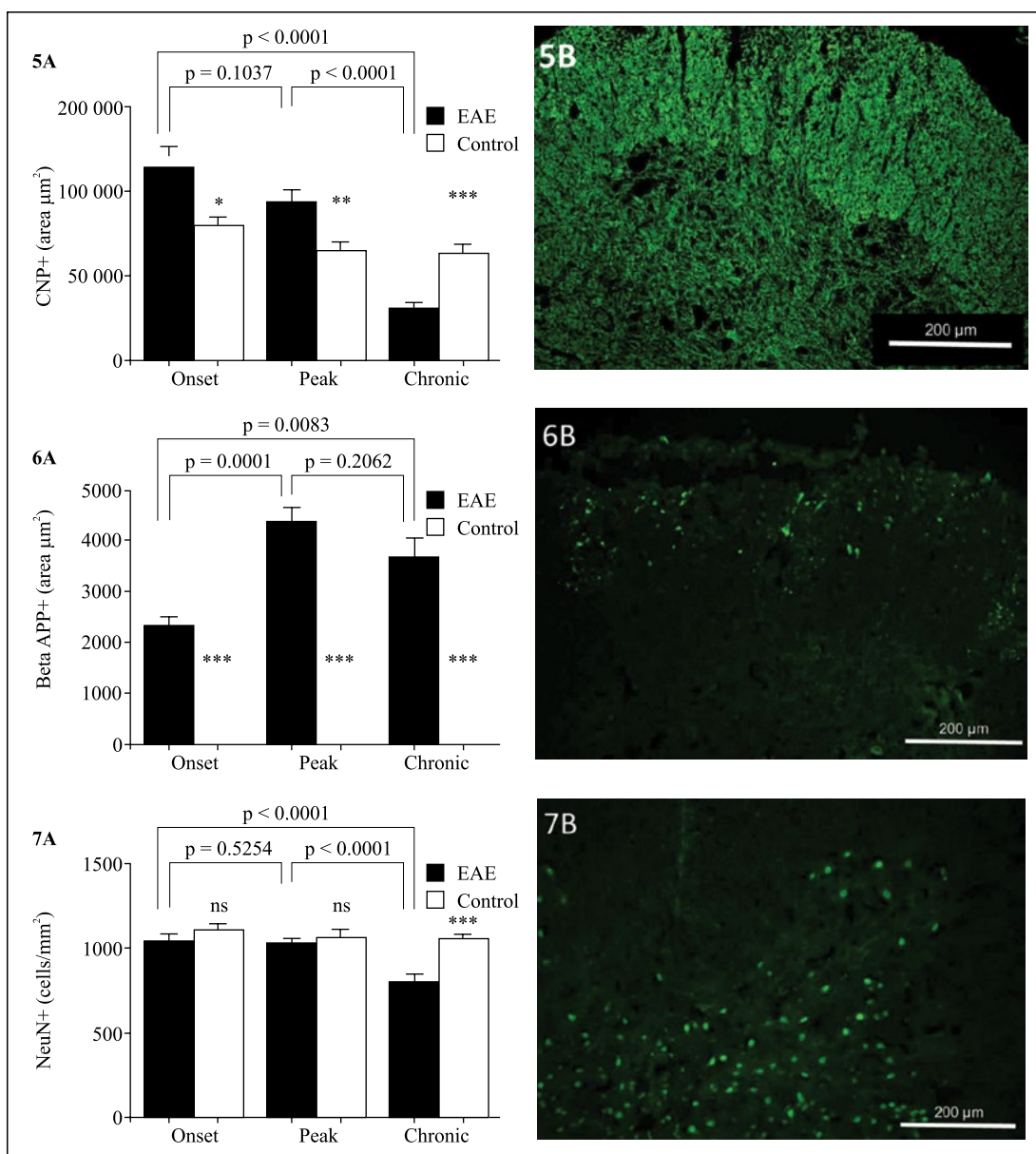


**Figures 2–4.** Cell density in spinal cords of EAE mice in three successive phases of the disease (A) with representative micrographs showing immunostained cells in the peak phase of EAE (B). **Fig. 2.** Total leukocytes immunostained for CD45. **Fig. 3.** T cells immunostained for CD3. **Fig. 4.** Macrophages/microglia immunostained for Iba1. Histomorphometry was performed as described in Methods. Values represent means  $\pm$  SEM; n = 6 per group; EAE vs control: \*\*\*p < 0.0001.

improvement observed in the chronic phase of EAE. This seems to explain our finding of the retention of macrophages/microglia in EAE lesion after the peak phase of the disease.

It has been a striking observation made in this study that already at the onset phase of EAE the number of oligodendrocytes is substantially higher than in the control mice. Under demyelinating conditions, oligodendrocyte precursor cells (OPCs) are

recruited to the lesion site and mature into myelinating oligodendrocytes [22] involved in tissue repair. Our observation indicates that the recruitment of OPC and their maturation occurs very early, shortly after the beginning of the demyelination processes. High density of oligodendrocytes persists in the peak phase; however, intense demyelination and extensive accumulation of myelin debris in the lesion results in the inhibition of OPC maturation [23] and inefficient



**Figures 5–7.** Cell and axonal damage density in spinal cords of EAE mice in three successive phases of the disease (A) with representative micrographs showing immunostained structures in the peak phase of EAE (B). **Fig. 5.** Oligodendrocytes immunostained for CNP1 (note much higher density in white matter, top). **Fig. 6.** Damaged axons immunostained for beta APP. **Fig. 7.** Surviving neurons immunostained for NeuN. Histomorphometry was performed as described in Methods. Values represent means  $\pm$  SEM.;  $n = 6$  per group; EAE vs control: \* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ .

re-myelination. The density of oligodendrocytes showed a decrease in the chronic phase, to a level even significantly lower than that in the control mice, indicating a substantial loss of these cells. Indeed, advanced EAE is characterized by a massive oligodendrocyte death [24]. The main mechanism of death seems to be caspase-mediated apoptosis [25]. It has been a matter of debate, whether oligodendrocyte death precedes local inflammation or whether inflammation induces death of these cells. Results of this

study clearly point to the latter sequence of events, at least in the model of MOG-induced EAE, since a decrease in oligodendrocyte density was noted no sooner than in the chronic phase of EAE.

According to the expectations, axonal damage shows progress in the onset and peak phases of EAE and becomes stabilized in the chronic phase, in which the demyelination and remyelination processes become balanced. This result confirms earlier ultrastructural data indicating that axonal damage in EAE



**Table 1.** Density of cells and damaged axons in the spinal cord of EAE mice in three phases of the disease

Parameter	Onset phase (EAE vs control)	Peak phase (EAE vs control)	Chronic phase (EAE vs control)	Onset vs peak phase	Peak vs chronic phase	Onset vs chronic phase
Total leukocyte density [area, $\mu\text{m}^2$ ]	90654 $\pm$ 28966 vs 2474 $\pm$ 790 p < 0.0001	124097 $\pm$ 48195 vs 1009 $\pm$ 292 p = 0.0012	68546 $\pm$ 23228 vs 982 $\pm$ 186 p = 0.0013	p = 0.0046	p < 0.0001	p = 0.0037
T cells [cells/mm <sup>2</sup> ]	1149 $\pm$ 340 vs 14 $\pm$ 5 p = 0.0002	2013 $\pm$ 693 vs 14 $\pm$ 4 p = 0.0001	415 $\pm$ 158 vs 13 $\pm$ 4 p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001
Macrophage/microglia density [area, $\mu\text{m}^2$ ]	87653 $\pm$ 28563 vs 5183 $\pm$ 1901 p < 0.0001	143202 $\pm$ 37075 vs 11828 $\pm$ 2961 p < 0.0001	123526 $\pm$ 33754 vs 11198 $\pm$ 3837 p < 0.0001	p < 0.0001	p = 0.1053	p = 0.0009
Oligodendrocyte density [area, $\mu\text{m}^2$ ]	121391 $\pm$ 35603 vs 79879 $\pm$ 9872 p = 0.024	94413 $\pm$ 29815 vs 64925 $\pm$ 19537 p = 0.0039	31532 $\pm$ 11378 vs 63479 $\pm$ 13669 p = 0.0002	p = 0.1037	p < 0.0001	p < 0.0001
Axonal damage density [area, $\mu\text{m}^2$ ]	2383 $\pm$ 494 vs 0 p < 0.0001	4442 $\pm$ 1483 vs 0 p < 0.0001	3722 $\pm$ 1253 vs 0 p < 0.0001	p = 0.0001	p = 0.2062	p = 0.0083
Surviving neurons [cells/mm <sup>2</sup> ]	1052 $\pm$ 169 vs 1109 $\pm$ 171 p = 0.8195	1037 $\pm$ 82 vs 1063 $\pm$ 205 p = 0.5407	805 $\pm$ 156 vs 1056 $\pm$ 117 p < 0.0001	p = 0.5254	p < 0.0001	p < 0.0001

Values represent mean  $\pm$  SEM, n = 6 per group. Cells were identified by immunofluorescent staining as described in Methods.

coincided with initial neurological symptoms and remained stable in the further course of the disease [26]. However, as demonstrated in the latter study, myelin pathology (myelin swelling or loss) gradually accumulates with disease progression.

Axonal damage is not concomitant with neuronal death, since significant decrease in the number of surviving neurons was observed only in the chronic phase. Although the predominant view emphasizes the principal role of demyelination in the pathogenesis of MS/EAE, there is a growing evidence indicating the significance of neuronal loss in the disease [27]. Neuronal apoptosis in EAE can be induced by ER stress [28], matrix metalloproteinase 9 released by microglia [29] and perforins secreted by Tc cells [30]. We found that neuronal loss occurred relatively late, in the chronic phase of EAE. It suggests that neurons can survive demyelination and damage of their axons and that prolonged exposition to the inflammatory milieu is necessary to induce their death. This observation does not, however, agree with the results of a study of Vogt *et al.* [27] who reported death of neurons already in the early phase of an identical model of EAE and no further neuronal loss in the later phases. The discrepancy can stem from different quantification method employed by the latter authors (high precision stereology), and the time course of neuronal loss should be verified in further studies.

Histological correlates of autoimmune disease can provide important information concerning the

underlying pathology. The analysis of cells involved and their behavior in the successive phases of EAE could also be useful in evaluating the therapeutic efficiency of anti-inflammatory, immunosuppressive and neuroprotective strategies employed in MS.

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