Neuroscience 156 (2008) 586–596

GLIAL REACTIONS AND DEGENERATION OF MYELINATED PROCESSES IN SPINAL CORD GRAY MATTER IN CHRONIC EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

J. WU, M. OHLSSON, E. A. WARNER, K. K. LOO, T. X. HOANG, R. R. VOSKUHL AND L. A. HAVTON*

Department of Neurology, David Geffen School of Medicine at University of California, Los Angeles, Charles Young Drive South, Los Angeles, CA 90095, USA

Abstract—Multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) result in inflammatory white matter lesions in the CNS. However, information is sparse with regard to the effects of autoimmune demyelinating disease on gray matter regions. Therefore, we studied the late effects of chronic EAE in C57BL/6 mice on the spinal cord gray matter using immunohistochemistry. Here, EAE induced marked astrocytic, microglial, and macrophage activation in the ventral horn gray matter, without any motoneuron loss. Activated caspase-3 was also increased in the ventral horn gray matter. Furthermore, activated poly (ADP-ribose) polymerase (PARP), another apoptotic marker, co-localized with myelin basic protein (MBP) of oligodendrocyte processes, but not with the oligodendroglial cell body marker, adenomatous polyposis coli gene clone CC1 (APC-CC1), or with neurofilament marker (RT-97) or synaptophysin of axonal arbors. However, there was no associated increase in the number of terminal deoxynucleotidyl transferase (TdT) mediated-dUTP nick end labeling positive nuclei in the spinal cord gray matter of EAE mice. In addition, co-localization of MBP and the low-affinity neurotrophin receptor, p75, was demonstrated, further supporting the notion of apoptotic oligodendrocyte process degeneration in the gray matter of EAE mice.

Key words: multiple sclerosis, inflammation, mouse, degeneration, demyelination.

Multiple sclerosis (MS) is an immune-mediated disorder, which affects the CNS with inflammatory lesions and demyelination (Lucchinetti et al., 1998). In addition to the focal destruction of myelin in the white matter tracts of the brain and spinal cord, MS lesions may also exhibit transected axons (Trapp et al., 1998). The latter findings are of particular clinical importance, as neurological disability has been correlated with axonal loss in the spinal cord of chronic MS patients (Bjartmar et al., 2000). Interestingly, in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, a similar axonal loss in spinal cord white matter is correlated with permanent neurological disability (Wujek et al., 2002). Therefore, from a pathological perspective, the axonal transections encountered in MS and EAE may result in partial or complete neurological disconnection syndromes, which are similar to those encountered following traumatic brain and spinal cord injuries.

In recent years, an emerging literature from the neuroimaging field has demonstrated pathologic changes remote from the inflammatory white matter lesions of MS and EAE. For instance, human imaging studies have demonstrated gray matter atrophy in patients with MS (Chard et al., 2002; Dalton et al., 2004). Gray matter atrophy has been similarly documented in the cerebellar cortical gray matter of mice with EAE (MacKenzie-Graham et al., 2006). However, pathological effects within gray matter structures remain unclear.

The goal of this study was to investigate long-term effects of EAE on spinal cord gray matter beyond the sites of inflammatory white matter lesions. During the chronic phase of EAE in C57BL/6 mice, we investigated late glial and inflammatory changes in the ventral horn gray matter as well as in the dorsal corticospinal tract. In addition, the ventral horn gray matter was studied for signs of neuronal and myelin degeneration. We report that EAE induces a marked astrocytic, microglial, and macrophage activation in the ventral horn gray matter in the absence of motoneuron death, but in the presence of apoptotic degeneration of oligodendrocyte processes. This degeneration of oligodendrocyte processes was associated with the expression of the low affinity neurotrophin receptor, p75.

EXPERIMENTAL PROCEDURES

Animal procedures

All procedures were performed according to the standards established by the National Institutes of Health (NIH) Guide for the care and use of Laboratory Animals (NIH publications No. 80-23, revised 1996) and were approved by the Chancellor's Animal Research Committee at UCLA. All efforts were made to minimize the number of animals used and their suffering. Two-month-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were used for the study ($n=14$). To induce active EAE, rodent myelin oligodendrocyte glycoprotein (MOG) peptide, amino acids 35–55 (200 μg/mouse), mixed with mycobacterium tuberculosis (200 μg/mouse) in 0.2 ml complete Freund's adjuvant (CFA) was administered by s.c. flank injection on day 0 and day 7, and the pertussis toxin (500 ng/mouse in 200 μl PBS) was administered

*Corresponding author. Tel: +1-310-206-5757; fax: +1-310-206-5353.
E-mail address: LHavton@mednet.ucla.edu (L. A. Havton).

Abbreviations: AIF1, allograft inflammatory factor; APC-CC1, adenomatous polyposis coli gene clone CC1; CFA, complete Freund's adjuvant; ChAT, choline acetyl transferase; DAB, diaminobenzidine; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NIH, National Institutes of Health; PARP, poly (ADP-ribose) polymerase; ROI, region of interest; RT-97, neurofilament marker; TUNEL, terminal deoxynucleotidyl transferase (TdT) mediated-dUTP nick end labeling.

0306-4522/08
doi:10.1016/j.neuroscience.2008.07.037

on day 0 and day 2 (Hjelmstrom et al., 1998). Controls consisted of gender, age and strain matched mice. The mice were observed daily, and neurological deficits were ranked and recorded on a five-point scale as previously described: 0, unaffected; 1, tail limpness; 2, failure to resist on attempt to roll over; 3, partial paralysis; 4, complete paralysis; 5, moribund (Suen et al., 1997).

Spinal cord tissue fixation and processing

All mice were deeply anesthetized (sodium pentobarbital, Abbott Laboratories, North Chicago, IL, USA) and perfused transcardially with phosphate-buffered 4% paraformaldehyde (pH 7.4) at 42 days post-immunization. The spinal cord was removed and post-fixed at room temperature with phosphate-buffered 4% paraformaldehyde (pH 7.4) for 1–2 h. Then, the spinal cord was immersed in 30% sucrose at 4 °C overnight prior to being embedded in O.C.T cryostat mounting compound (Sakura, Torrence, CA, USA). The L4 and L5 spinal cord segments were cryosectioned serially in the transverse plane (14 μm thickness). Subsequently, the spinal cord sections were mounted on glass slides for histological and immunohistochemical studies.

Histology

Spinal cord sections of the L4 and L5 segments were stained with 0.1% Cresyl Violet in 1% acetic acid for 15 min at room temperature. After a brief rinse with distilled water, the sections were dehydrated through increasing concentrations of ethanol and coverslipped in Permount (Fisher Scientific, Pittsburgh, PA, USA).

Immunohistochemistry

For light stable immunohistochemistry, L4/L5 spinal cord sections on slides were first treated with 0.3% H_2O_2 for 10 min to quench endogenous peroxidase activity. Then, the sections were blocked with 5% BSA/0.3% Triton-X-100 in PBS (pH 7.4) at room temperature for 1 h followed by a brief rinse with PBS. Next, the sections were incubated with primary antibodies (Table 1), which were diluted in the blocking buffer overnight at 4 °C. After extensive washing in PBS, the tissue sections were incubated with biotinylated secondary antibodies of appropriate species (1:200 in PBS, Vector Laboratories, Burlingame, CA) at room temperature for 1 h, followed by incubation with avidin-biotin complex (1:200 in PBS, Vectastain ABC Elite kit; Vector Laboratories Inc., Burlingame, CA, USA) at room temperature for 1 h. Finally, immunoreactivity (IR) was visualized with diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) and the sections were coverslipped in Permount. For fluorescent immunohistochemical staining, the tissue sections were blocked in 5% BSA/0.3% Triton-X-100 in PBS (pH 7.4) at room temperature for 1 h, followed by incubation with primary antibodies overnight at 4 °C. After extensive washing, the sections were incubated with secondary antibodies conjugated to Rhodamine Red™ (1:100 in PBS; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), or Alexa Fluor 488 (green) or 594 (red) of appropriate species (1:500 in PBS; Invitrogen, Carlsbad, CA, USA) at room temperature for 1 h. The fluorescence labeled sections were dried and coverslipped in Vectashield (Vector Laboratories Inc.). For colocalization studies, the sections were coverslipped in Vectashield with DAPI (Vector Laboratories Inc.).

Terminal deoxynucleotidyl transferase (TdT) mediated-dUTP nick end labeling (TUNEL) staining

Apoptotic cells were detected with an *in situ* TUNEL kit (Chemicon, Temecula, CA, USA). L4/L5 spinal cord sections on slides were treated with pre-cooled ethanol:acetic acid (2:1) for 5 min at −20 °C for permeabilization. Then, the manufacturer's protocol was followed in labeling DNA fragments with digoxigenin-conjugated nucleotides and subsequently with anti-digoxigenin antibody that is conjugated to peroxidase. The apoptotic cells were visualized by DAB (Sigma). The tissue sections were counterstained with 0.5% (w:v) Methyl Green. The slides were mounted in Permount.

Light microscopy and quantitative analysis

Four non-overlapping light stable or fluorescent microscopic images of the L4-L5 ventral horn from all animals were captured (Objective lens 40×) with a Micropublisher five megapixel digital camera (Q Imaging, Burnaby, BC) attached to a Nikon E600 microscope (Nikon Inc., Melville, NY, USA) and analyzed using C-imaging software (Compix Inc., Sewickley, PA, USA). Two regions of interest (ROI) were selected for quantitative analysis. One ROI was within the ventral horn gray matter, which contains spinal motoneurons innervating hind limb muscles, and the other ROI was within the ventral portion of the dorsal funiculus (Fig. 2). The quantitative data were presented as mean labeled area as a percentage of the ROI. The motoneurons from eight hemi-sections per mouse were counted using the Abercrombie method (Coggeshall and Lekan, 1996). Sections labeled with fluorescent markers for colocalization studies were photographed using a confocal microscope (TCP-SP; Leica, Mannheim, Germany).

Statistical analysis

All quantitative data were presented as mean±S.E.M. Statistical analysis was performed by using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test (Sigmastat 3.1, Systat Software, Inc., Point Richmond, CA, USA), and

Table 1. Listing of primary antibodies used

Antibody	Species and type	Dilution	Source	Reference
AIF1	Goat polyclonal	1:100	Abcam #ab5076	(Ramprasad et al., 1996)
APC-CC1	Mouse monoclonal	1:500	Calbiochem #OP80	(McTigue et al., 2001)
Caspase3	Rabbit polyclonal	1:100	Promega #G748	(Guseva et al., 2002)
CD68	Rat monoclonal	1:50	Cell Science #HM1070	(Autieri and Agrawal, 1998)
ChAT	Goat polyclonal	1:200	Chemicon #AB144P	(Shiromani et al., 1987)
GFAP	Rabbit polyclonal	1:1000	Chemicon #AB5804	(Hammerle et al., 2003)
MBP	Rat monoclonal	1:200	Chemicon #MAB386	(Glynn et al., 1987)
p75	Rabbit polyclonal	1:100	Promega #G323A	(Chao and Hempstead, 1995)
PARP	Rabbit polyclonal	1:100	Cell signaling #9544	(Oliver et al., 1998)
RT97	Mouse monoclonal	1:2000	Hybridoma bank, NICHD	(Young and Black, 2004)
Synaptophysin	Mouse monoclonal	1:2000	Chemicon #MAB5258	(Masliah et al., 2001)