Decreased Neural Stem/Progenitor Cell Proliferation in Mice with Chronic/Nonremitting Experimental Autoimmune Encephalomyelitis

Jun Guo a, d Hongzeng Li a Caiyong Yu d Fangfang Liu d Yanling Meng c Weidong Gong b Hao Yang d Xuefeng Shen d Gong Ju d Zhuyi Li a, d Jian Wang d

Departments of a Neurology, and b Interventional Radiology, Tangdu Hospital, and c Department of Immunology and d Institute of Neurosciences, Fourth Military Medical University, Xi’an, China

Key Words
Multiple sclerosis · Experimental autoimmune encephalomyelitis · Neural stem/progenitor cell · 5-Bromo-2′-deoxyuridine

Abstract
It has been reported that autoimmune inflammatory processes in human multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), may induce an alteration in neurogenesis. Studies with transgenic EAE mice have demonstrated an enhancement of neurogenesis in the subventricular zone (SVZ). In contrast, a reduction of stem cell proliferation in the same region has been observed by Pluchino et al. [Brain 2008; 131: 2564–2578] in myelin oligodendrocyte glycoprotein (MOG)-induced EAE mice. We immunized female C57BL/6 mice with MOG 35–55 peptide and successfully developed chronic/nonremitting EAE, which is believed to be analogous to the progressive form of MS. On day 21 postimmunization, coronal brain sections were collected and stained with anti-5-bromo-2′-deoxyuridine (BrdU) antibody. By counting the number of BrdU-labeled cells, we demonstrated that the neural stem/progenitor cell (NSC/NPC) proliferation decreased in the SVZ, which basically confirms the study of Pluchino et al. on the changes in the SVZ. A reduction of NSC/NPC proliferation also occurred in the hippocampal subgranular zone of the dentate gyrus. The hippocampus is well known to be an important region involved in learning and memory; thus, our finding may offer a possible explanation for the cognitive impairment in human chronic MS.

Introduction
It has been well established that neurogenesis occurs throughout adulthood in the brains of various mammals ranging from rodents to human beings [1, 2]. Neurogenesis primarily occurs in two discrete regions, namely, the subgranular zone (SGZ) of dentate gyrus (DG) of the hippocampus, which is involved in learning and memory, and the subventricular zone (SVZ) [3]. Several lines of evidence have demonstrated that adult neurogenesis can be altered by multiple pathological conditions, including autoimmunity and inflammation of the brain [4, 5].

Jun Guo and Hongzeng Li contributed equally to this work. Zhuyi Li and Jian Wang are corresponding authors.
Multiple sclerosis (MS) is an autoimmune condition in which the immune system attacks myelin proteins of the central nervous system (CNS) and leads to inflammatory demyelination and oligodendrocyte loss [6, 7]. However, most of the evidence on the pathogenesis of MS derives from animal studies. A classical animal model that mimics the main features of MS is myelin protein-induced experimental autoimmune encephalomyelitis (EAE). In EAE, myelin protein-specific CD4+ T helper (Th) 1 cells mediate inflammatory infiltration in the CNS, resulting in demyelination and axonal degeneration, which lead to clinical progressive paralysis [8]. Several studies have demonstrated in transgenic mice that EAE leads to an increase in the neural stem cell/neural progenitor cell (NSC/NPC) population of SVZ, which may be a mechanism of CNS repair in demyelinated lesions [9, 10]. In contrast, a recent study reported a reduction of stem cell proliferation in the SVZ of chronic-progressive EAE during persistent brain inflammation [11]. Here we confirm that myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide-induced chronic/nonremitting EAE (CNR EAE) leads to an apparent decrease in NSC/NPC proliferation in SVZ. However, we have discovered a reduction in NSC/NPC proliferation in the SGZ of DG as well. Since cognitive impairment occurs in more than 50% of MS patients [12], our finding may offer a possible explanation for the cognitive dysfunction in human chronic MS.

Materials and Methods

Animals

In the present study, female C57BL/6 mice (age: 6–8 weeks; Experimental Animal Center, Fourth Military Medical University, China) were used. All the mice were housed in plastic chambers, provided with ad libitum access to food and water, and treated according to protocols approved by the local ethics committee.

Induction of EAE

In order to induce EAE, each mouse was subcutaneously immunized with 200 μg of MOG 35–55 peptide (CL Bio-Scientific Co., China) at two sites in the back or the flank; MOG was emulsified in 0.1 ml phosphate-buffered saline (PBS; pH = 7.4) and 0.1 ml complete Freund’s adjuvant (CFA; Sigma-Aldrich, USA) containing 4 mg/ml killed Mycobacterium tuberculosis (H37Ra; Difco Laboratories, USA). A fixed amount (200 ng) of pertussis toxin (Sigma-Aldrich) was intraperitoneally (i.p.) administered at 0 and 2 days postimmunization (dpi). The control mice underwent a similar procedure, i.e., subcutaneous immunization with CFA and 2 i.p. injections of pertussis toxin. The mice were monitored daily, and the clinical signs of EAE were evaluated according to a 5-point scale [13]: 0 = no clinical signs; 1 = limp tail or waddling gait with tail tonicity; 2 = waddling gait with limp tail (ataxia); 2.5 = ataxia with partial limb paralysis; 3 = total paralysis of one limb; 3.5 = total paralysis of one limb with partial paralysis of the second limb; 4 = total paralysis of two limbs; 4.5 = moribund; 5 = death.

Administration of 5-Bromo-2′-Deoxyuridine and Tissue Preparation

On 21 dpi, the immunized mice and the control mice (n = 6/group) received 2 i.p. injections of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU; 150 mg/kg; Sigma-Aldrich; dissolved in PBS), with an interval of 2 h between the injections. At 24 h after the first BrdU injection, all the mice were deeply anesthetized with ethyl ether inhalation and intracardially perfused with 10 ml of 0.9% saline through the left ventricle, which was followed by perfusion with 50 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4). The brains and spinal cords of the mice were removed, postfixed for 4 h, and immersed in a phosphate-buffered 25% sucrose solution at 4 °C overnight. Free-floating, 30-μm-thick, coronal brain sections were prepared by using a freezing microtome (CM 3050S; Leica, Germany) and stored at 4 °C for immunofluorescent staining. The inflammatory infiltration was evaluated by obtaining 12-μm-thick sections of the spinal cords and brains, and staining the sections with hematoxylin and eosin (H&E).

Immunofluorescence Studies

For DNA denaturation, free-floating sections were immersed in 2 N HCl at room temperature (RT) for 30 min. Nonspecific binding was blocked by incubating the sections in PBS with 1% bovine serum albumin and 0.3% Triton X-100 at RT for 30 min. Then, the sections were incubated at 4 °C for 20 h in the primary antibody, i.e., rat anti-BrdU (ICR1; 1:600; Abcam, UK). The sections were rinsed 3 times with PBS and incubated with Texas Red-conjugated donkey anti-rat IgG (1:1,000; Jackson Immuno-research, USA) at RT for 2 h. Finally, the sections were washed 3 times in PBS, and mounted and coverslipped using SlowFade® Gold antifade reagent (Molecular Probes, USA).

In order to avoid the misidentification of the proliferating CD4+ T cells infiltrating into the CNS, the brain sections from 3 EAE mice were double-stained with the anti-BrdU and anti-CD4 antibodies. Briefly, anti-BrdU staining was performed using the above mentioned technique and selected brain sections were blocked with 3% normal rat serum at RT for 30 min. Then, the sections were incubated at 4 °C for 2 h in the primary antibody, i.e., rat anti-CD4 (ICR1; 1:600; Abcam, UK). The sections were rinsed 3 times with PBS and incubated with Texas Red-conjugated donkey anti-rat IgG (1:1,000; Jackson Immuno-research, USA) at RT for 2 h. Meanwhile, splenic sections from EAE mice were double-stained for CD4 and BrdU, and these sections were used as positive controls to determine the phenotypes of BrdU+/CD4+ cells.

Microscopic Analysis and Quantification

We used a laser-scanning confocal microscope (FV1000; Olympus, Japan) to identify the CD4+/BrdU+ cells infiltrating into the CNS, and the brain sections from 3 EAE mice were double-stained with the anti-BrdU and anti-CD4 antibodies. Briefly, anti-BrdU staining was performed using the above mentioned technique and selected brain sections were blocked with 3% normal rat serum at RT for 30 min. Then, the sections were incubated with FITC-conjugated anti-CD4 (RM4-5; 1:100; ebioscience, USA) at RT for 20 h. Meanwhile, splenic sections from EAE mice were double-stained for CD4 and BrdU, and these sections were used as positive controls to determine the phenotypes of BrdU+/CD4+ cells.
of each mouse was multiplied by the volume index, which was the ratio of the volume of the DG to the combined volume of the selected sections, to obtain the estimated number of newly generated cells per DG. For SVZ, 4 coronal sections (150 μm apart) spanning from +0.38 mm anterior to –0.34 mm of the bregma were analyzed for each mouse, and the BrdU+ cells in the lateral walls of both lateral ventricles were counted. The obtained number was multiplied by the volume index to obtain an estimate of the total number of newly generated cells per lateral ventricle wall.

**Statistical Analysis**

Data on the clinical signs of EAE mice are presented as means ± SD and those on the BrdU+ cells are presented as means ± SEM (standard error of mean). The statistical differences between the numbers of BrdU+ cells were determined by 2-sided Student’s t test, and the level of significance was preset at p < 0.05.

**Results**

**MOG 35–55 Immunization Leads to CNR EAE**

A total of 15 C57BL/6 mice underwent subcutaneous immunization with MOG 35–55 peptide and received two i.p. injections of pertussis toxin to induce EAE, and all the mice developed EAE on 14.6 ± 2.0 dpi (mean ± SD). In order to detect NSC/NPC proliferation, we randomly chose 6 mice from the above cohort and injected them with BrdU at 21 dpi. The remaining EAE mice were observed daily until they were killed at 60 dpi. The animals reached the peak of disease at 5.1 ± 0.3 days after onset, followed by a brief remission and then a stable chronic phase with the mean scores not exceeding 2 grades (fig. 1). H&E staining showed that inflammatory infiltration was present in the brain and spinal cord, and these infiltrations were mainly distributed at the meninges, parenchyma, and perivascular areas, including the ventricular choroid plexus (fig. 2).

**Impaired NSC/NPC Proliferation in CNR EAE**

The splenic sections from EAE mice were double-stained with anti-CD4 and anti-BrdU antibodies and the BrdU+/CD4+ T cells were identified as positive controls (fig. 3a). EAE is a CD4+ T cell-dependent CNS disease; therefore, to avoid the misidentification of proliferating inflammatory cells, especially the CD4+ T cells (BrdU+/CD4+) infiltrating at SGZ or SVZ, as the cells formed by endogenous neurogenesis, we double-stained sections from the brains of EAE mice by using anti-CD4 and anti-BrdU antibodies. We were able to detect both CD4+/BrdU+ T cells and CD4+/BrdU– T cells in the brains of EAE mice, and these cells were mainly located at the perivascular areas of parenchyma (fig. 3b), cerebral ventricles (fig. 3c) and their choroid plexus (fig. 3d), and the parenchyma contiguous with vessels such as fimbria hippocampus (fig. 3e). No proliferating CD4+ T cells were observed in the SGZ of DG. However, a few BrdU+/CD4+ T cells can be identified in the SVZ (fig. 3f, g), and the ratio of these cells to all BrdU+ cells was around 0.3% (data from 3 EAE mice).

BrdU+/CD4+ cells only occupied a small part of total BrdU+ cells in the SVZ. In the following study, we counted all BrdU+ cells as NSC/NPC proliferation in the SVZ and the SGZ of DG in the brain sections selected from CNR EAE and control mice. The number of BrdU+ cells in DG of EAE mice was significantly less than that in the
control mice (reduction of 15.7 ± 4.0%, mean ± SEM; p < 0.05; fig. 4a, b, e). The quantification of BrdU+ cells in SVZ showed similar results (reduction of 10.0 ± 2.9%; p < 0.05; fig. 4c, d, f). These results suggest that CNR EAE leads to an impaired NSC/NPC proliferation, manifested as a prominent decrease in the number of BrdU+ cells.

**Discussion**

MS is an autoimmune disease with a complex genetic background, and the disease is characterized by inflammation and demyelination of the CNS. EAE can reproduce many of the clinical, neuropathological, and immunological aspects of MS [17]. Since the first description in the 1950s, a number of EAE subtypes, including acute progressive EAE, CNR EAE, remitting/relapsing EAE, and monophasic remitting/nonrelapsing EAE, have been developed in several strains by using various myelin antigens. In the present study, MOG-immunized C57BL/6 mice successfully developed CNR EAE, an EAE subtype that is believed to be analogous to the progressive forms of MS [18].

Similar to MS, EAE is also a CD4+ T cell-mediated disease of CNS, characterized by mononuclear cell infiltration and demyelination resulting in paralysis [19]. It is known that activated CD4+ Th1 cells can infiltrate into brain and some of them possess the capacity of proliferation. While quantifying the number of the neural stem cells by using BrdU as a marker, the BrdU-positive CD4+ T cells, if they happen to be present in the same area of interest may interfere with the statistic conclusion. Therefore, it is requisite to distinguish the proliferating CD4+ T cells (BrdU+/CD4+) from the BrdU+ cells formed by endogenous neurogenesis in the EAE CNS. Indeed, we found that there were BrdU+/CD4+ T cells distributed among the stem cells in the SVZ, although these cells occupied only 0.3% of all dividing cells in this region and were thus negligible in our statistical analysis of the stem cell numbers in the region.

In EAE, myelin-reactive CD4+ Th1 cells, which produce proinflammatory Th1 cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), are known to play a prominent role in the disease process [20]. It has been suggested that a deviant T cell response to a Th2 phenotype is responsible for the resistance to EAE, and injection of CNS-reactive Th2 cells (which produce IL-4 and IL-10) can prevent EAE induction [21]. Begolka et al. [8] reported that the expression of IFN-γ and TNF-α in the CNS of SJL/J mice with PLP 139–151 peptide-induced relapsing/remitting EAE displayed a biphasic pattern, with the expression peaking at the height of the acute phase and relapse(s). In contrast, IL-4 is undetectable until remission(s) of the disease, demonstrating...
Fig. 3. Double staining of spleen and brain sections with anti-CD4 and anti-BrdU antibodies, showing proliferating CD4+ T cells. 

a. Representative confocal micrographs of the spleen of EAE mice; the spleen samples were double-labeled with CD4 (green) and BrdU (red), and the insets show the typical phenotypes at higher magnifications.

b–g. Confocal images of CD4+/BrdU+ T cells (arrows) in EAE brain. These cells were mainly located in the perivascular areas (b), cerebral ventricles (c), choroid plexus (d), and the parenchyma adjacent to the ventricles (e). f, g. A few CD4+/BrdU+ T cells can be identified in the SVZ. V = Vessel; D3V = dorsal 3rd ventricle; fi = fimbria hippocampus; Cp = choroid plexus; LV = lateral ventricle. Scale bar: 20 μm. Black and white drawings indicate where the sections (c–g) were taken.
its potential role in the intrinsic regulation of ongoing EAE [8]. In the present study, the MOG 35–55 peptide-immunized mice displayed a chronic and progressive clinical course (CNR EAE), which suggests that persistent CNR EAE might manifest as a continuous Th1 response.

Calzà’s group [9] is the first to demonstrate in Lewis rats that there was an enhancement of SVZ cell proliferation, which may be attributed to the high concentration of nerve growth factor during the EAE disease course. Similarly, a study conducted on a chronic EAE model in MOG transgenic mice revealed that the disease could trigger increased proliferation and mobilization of NSC/NPC from the SVZ [10]. Similar findings were reported in studies conducted on B10.PL TMBP- transgenic mice with EAE [14]. The CNS-specific autoimmune T cells, which provide neurotrophic factors such as brain-derived neurotrophic factor, affect adult neurogenesis in the DG and SVZ primarily by means of their effects on progenitor cell proliferation [14].

However, several studies have reported that proinflammatory cytokines inhibit NSC/NPC proliferation and neurogenesis. Lindvall and his colleagues [22] used a miniosmotic pump to provide sustained lipopolysaccharide infusion to rat brain, and found that the brain inflammation strongly impairs basal NSC/NPC proliferation and neurogenesis in the hippocampus of rats. Palmer’s group [23] has proved, both in vitro and in vivo, that TNF-α and IL-6 are the principal cytokines involved in the lipopolysaccharide-induced neuroinflammation that...
decreases hippocampal neurogenesis. TNF-α and IFN-γ have been proven to be the key factors in inhibiting NPC proliferation and inducing NPC migration during EAE [24]. Recently, Pluchino et al. [11] analyzed the gene expression of C57BL/6 mice with chronic MOG-induced EAE and found a significant upregulation of mRNA levels of the proinflammatory (Th1) cytokines, such as TNF-α and IFN-γ, but not of IL-1β, at 20 and 30 dpi in the SVZ from EAE mice. In parallel, they observed a reduction of brain stem cell proliferation in the SVZ. While confirming the reduction of brain stem cell proliferation in the SVZ of C57BL/6 mice with MOG 35–55 peptide-induced CNR EAE, we have found that the NSC/NPC proliferation is also reduced in the DG, another zone well known to host neural stem cells.

Taken together, these findings suggest that the discussions of NSC/NPC proliferation in EAE models should be preceded by a description of the protocol used for induction of the EAE model. Since MS is a complex disease with heterogeneous clinical, pathological, and immunological phenotypes, no EAE animal model can reproduce all the clinical aspects of MS [17]. Furthermore, although inflammation and immune responses are observed in the brains of all the EAE models, inflammation is not always synonymous with immune response. In myelin-transgenic EAE models, the CNS-specific autoimmune T cells can directly provide neurotrophic factors such as brain-derived neurotrophic factor and nerve growth factor to promote NSC/NPC proliferation and neurogenesis [10,17]. In contrast, in C57BL/6 mice with MOG-induced EAE, Th1 cytokines such as TNF-α and IFN-γ may act as the dominant factors and decrease NSC/NPC proliferation [11].

It has been reported that many MS patients have extensive cognitive impairment [25–27]. It is well known that the hippocampus is a crucial structure involved in cognitive activities such as learning and memory formation [28, 29] as well as mood regulation [30]. The finding of reduction in NSC/NPC proliferation in DG observed in the present study may suggest an explanation for the impairment of clinical MS.

In summary, the present study has reported a significantly decreased NSC/NPC proliferation in the SVZ of C57BL/6 mice with MOG 35–55 peptide-induced CNR EAE. Moreover, the SGZ of the DG of the hippocampus reveals a reduction of proliferation of the stem cells as well, which may offer a possible explanation for the cognitive impairment in human chronic MS.

Acknowledgments

We thank Jianyong Qiu for cryostat section and tissue staining, and Lingling Fei, Na Luo and Rui Xia for EAE induction. This work was partially supported by grants from the Division of Scientific Research Administration in the Fourth Military Medical University, National Natural Science Foundation of China (No. 30570641) and the PLA Military Research Fund (06G089 and 2006192005).

References


20 Pedotti R, De Voss JJ, Steinman L, Galli SJ: Involvement of both ′allergic′ and ′autoimmune′ mechanisms in EAE, MS and other autoimmune disease. Trends Immunol 2003;24:479–484.


