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A new EAE model of brain demyelination induced by intracerebroventricular pertussis toxin

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is a primary animal model of multiple sclerosis (MS). MS predominantly presents with evidence of lesions in the subcortical periventricular white matter regions of the brain. Research into the pathogenesis of the demyelinating lesions in the brain has been hampered by the fact that conventional models of EAE present with progressive ascending paralysis which recapitulates mainly the spinal cord lesions of multiple sclerosis. There is little evidence of brain involvement. Systemic administration of pertussis toxin (PTx) has been shown to induce the proinflammatory cascade of TGF-β, IL-6, and Th17 in the central nervous system, which recently has been identified as essential in the development of EAE. To determine whether intracerebroventricular (icv) administration of PTx would result in subcortical periventricular demyelinating lesions in the brain, we examined the effect in a MOG induced EAE model. We found that icv PTx induced subcortical periventricular brain lesions that resemble the pathologic demyelinating lesions of MS. Moreover, icv PTx induced Th17 infiltration and increased expression of cytokines IL-6 and TGF-β. We thus generated a highly reproducible model with remarkable histological similarities to the predominant demyelinating brain lesions seen in MS.

Introduction

Multiple sclerosis (MS) is often characterized by early evidence of demyelinating lesions in the periventricular white matter regions of the brain. Experimental autoimmune encephalomyelitis (EAE) is considered an animal model of MS. The inflammatory reaction in the central nervous system (CNS) is predominantly driven by autoreactive T lymphocytes and is attributed to complex interactions between immunogenic and cellular elements, ultimately leading to demyelination [1–3]. EAE usually manifests clinically as an ascending progressive paralysis in the caudal to rostral direction. The clinical signs of most conventional EAE models correlate strongly with infiltration of inflammatory cells in the spinal cord with limited brainstem and little brain periventricular white matter involvement. The lack of brain involvement in the EAE model has been a source of controversy regarding its validity as a model for MS [4,5].

Pertussis toxin (PTx) is an immunoadjuvant utilized to effectively promote an inflammatory response in multiple autoimmune disease models and is critically involved in the pathogenesis of EAE [6–8]. PTx is used to enhance the severity of EAE in permissive strains, and render resistant strains susceptible to disease and dose has been positively correlated with disease activity [7,9]. Systemic administration of PTx has been shown to induce the proinflammatory cascade of IL-6, TGF-β, and Th17, in the CNS, which recently have been identified as essential in the development of EAE [10–12]. Furthermore, studies targeting focal demyelination, indicated that the immunopathology of EAE lesions was strongly dependent upon the site of lesion initiation [13].

We hypothesized that intracerebroventricular (icv) administration of PTx after MOG induction would localize infiltration of autoreactive T lymphocytes and demyelinating lesions to the periventricular white matter regions of the brain. We used the MOG35–55 induced EAE model in C57BL/6 mice. In the present study, we report that icv PTx in the course of EAE, results in T lymphocytes infiltration and demyelination in the white matter of the brain. The infiltrating cells are identified as Th-17 cells and elevated levels of IL-6 and TGF-β are measured in the periventricular subcortical regions of the brain recapitulating the classic early pathogenic brain lesions seen in MS. Our results suggest that icv PTx induces a novel EAE model with prominent MS-like pathology in the brain.

Materials and methods

Animals. The experiments were carried out in 6–8 weeks old C57BL/6 female mice obtained from Taconic (New York, USA). The animals were kept in groups on a 12:12 h light/dark cycle with food and water ad libitum. Experiments were

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approved by the Institutional Animal Care and Use Committee of the Barrow Neurological Institute and performed according to the Revised Guide for the Care and Use of Laboratory Animals.

Induction of EAE. Mice were injected subcutaneously with 0.1 ml emulsion, composed of 200 μg MOG 35–55 peptide (M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K) (Bio-synthesis Inc., Lewisville, TX) in 50 μl of PBS and 50 μl of Complete Freund's Adjuvant (CFA) containing 0.5 mg of heat killed *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI). This was followed by two intravenous injections with 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) on day 0 and 2 [14,15]. Clinical signs and weight were monitored daily in these mice. Five point standardized rating scale was used to evaluate motor deficit: 0, no deficit; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund state or death.

Stereotactic intracerebroventricular injection. To induce cerebral EAE lesions, sensitized mice were anaesthetized by injection of a ketamine/xylamine cocktail on day 7 after MOG immunization (day 0). A guarded, 27-gauge 0.5-in. needle was stereotactically inserted, targeting the lateral ventricle, 0.7 mm caudal to bregma and 1.0 mm lateral to the sagittal suture at depth of 3.5 mm. A 10.0-μl Hamilton 1700 series gastight syringe was used to inject 5 μl of normal saline or pertussis toxin (200 μg/ml dissolved in normal saline) into lateral ventricle over a three minute period. Post-surgical recovery was uneventful in all cases, with no overt clinical signs.

Histochemistry. Mice were euthanized on day 14 or 22 following EAE induction. They were perfused with PBS followed by 4% paraformaldehyde. Fixed brain tissues were embedded in paraffin. Paraffin-embedded sections were cut at 6 μm thickness and mounted on Silane-coated standard glass microscope slides (Sigma, St. Louis, MO). Histological evaluation was performed by staining with hematoxylin and eosin (H&E), Luxol fast blue/periodic acid Schiff agent (LFB/PAS), and Bielschowsky silver impregnation to assess inflammation, demyelination, and axonal pathology, respectively.

The tissue samples were examined using digital Axoplan microscope (Zeiss, Thornwood, NY), and the degree of inflammation was assessed by counting the inflammatory foci throughout the brain and the spinal cord. Inflammatory focus was defined as presence of more than 20 mononuclear cells in the perivascular space of a given blood vessel [16].

Immunohistochemistry. After paraformaldehyde perfusion, the brains and spinal cords were harvested, post-fixed for 1 h with 4% paraformaldehyde, prepared as frozen blocks and sectioned at a thickness of 6 μm. Immunohistochemistry was performed with antibodies against astrocytes (GFAP, 1:1600, Chemicon, Temecula, CA), amyloid precursor protein (1:1600, Abcam Inc., Cambridge, MA), CD4 T cell (1:1600), CD8 T cell (1:1600, Chemicon, Temecula, CA), CD 68 (1:800, Abcam Inc., Cambridge, MA), and MOG (1:800, R&D Systems Inc. Minneapolis, MN). Immunolabeling was detected by applying the peroxidase–antiperoxidase procedure with 3,3′-diaminobenzidine (DAB) as cosubstrate [17].

For double fluorescent staining, tissues were harvested and snap frozen in liquid nitrogen, embedded in OCT compound in cryomolds. The embedded sections were cut at 8 μm and mounted on Silane-coated standard glass microscope slides. After being washed, the sections were incubated in normal serum blocking solution and then incubated in the mixture of two primary antibodies for 1 h at room temperature, followed by incubation with two fluorescent conjugated secondary antibodies (FITC conjugated and Texas Red conjugated) in PBS for 30 min at room temperature. After washing, the sections were cover slipped with anti-fade fluorescent mounting medium.

Adjacent sections were used to detect colocalization. Respective negative controls that omit primary antibodies and positive controls were applied for each case.

Western blot protein analysis. Aliquots of 5 μg proteins were prepared in loading buffer and spotted onto polyacrylamide gels (12% total acrylamide (T), 2% acrylamide/bisacrylamide (C) for the resolving gel and 6.3% T, 4% C for the stacking gel) in a Mini-protein® II cell (Bio-Rad, Hercules, CA) using the method of Laemmli. Loading buffer and respective proteins from mouse brain/spinal cord were applied as negative and positive controls, respectively. After SDS–polyacrylamide gel electrophoresis, gels were transferred electrically to nitrocellulose membrane (Bio-Rad, Hercules, CA). Blots were incubated first with blocking buffer to minimize nonspecific binding and then with primary antibodies (anti-IL-6, 1:1000 and anti- IL-17, 1:3000 Abcam Inc., Cambridge, MA; anti-TGF-b 1:1000 Cell signaling technology, Davers, MA) in blocking buffer. Finally blots were exposed to the secondary antibody (horseradish peroxidase-labeled anti-rabbit, 1:10,000, Cell signaling technology, Danvers, MA) in blocking buffer. After being washed, air-dried blots were treated with chemiluminescent reagents (Amersham Corp.) for 1 min and exposed to X-ray film for 5–20 min. The film was analyzed by an imaging densitometer (Model GS-670, Bio-Rad, Hercules, CA).

Statistical analysis. Data were analyzed with SPSS version 10 for windows. The two-way analysis of variance was applied to determine the significance of the difference among the experimental groups. Kruskal–Wallis nonparametric analysis was used for data presented as percentage. The Mann–Whitney *U* test was used when Kruskal–Wallis showed significance among groups. $p < 0.05$ was considered significant.

Results

EAE+ PTx icv mice developed focal periventricular leukocyte infiltration and demyelination in the brain, whereas mice with EAE alone developed only mild inflammation around meninges

The brains of EAE+ PTx icv mice showed massive perivascular infiltration of inflammatory T cells in para-meningeal area (Fig. 1B) and the parenchyma (Fig. 1C). There was extensive gli-

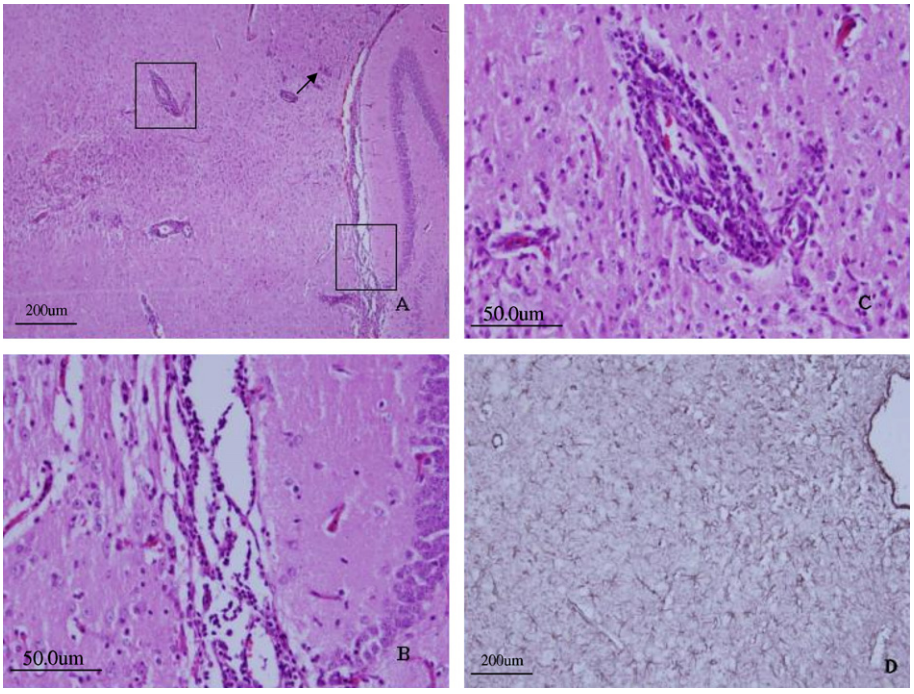


Fig. 1. EAE+ PTx icv mice ($n = 6$) showed extensive inflammation in the periventricular parenchyma (arrow in A), the meninges and subpial regions (B), and surrounding the intra-parenchymal micro-vessels (C) of the brain. Extensive gliosis was also identified (D, GFAP staining). (B,C) Inserts in (A). Original magnification 40× in (A,D); 200× in (B,C).

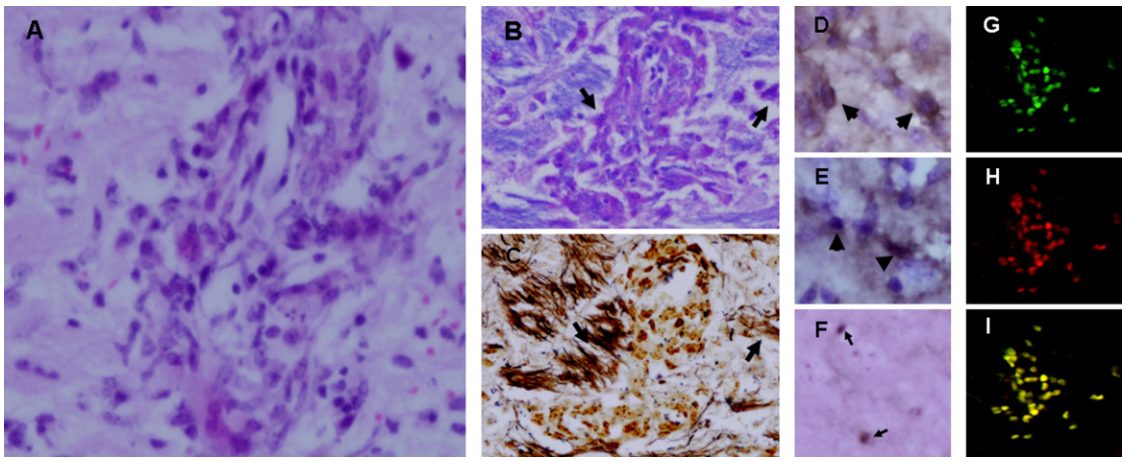


Fig. 2. In the EAE+ PTx icv mice, extensive inflammation was shown around micro-vessels in the parenchyma (A, H&E staining). LFB/PAS (B) and Bielschowsky silver staining (C) revealed extensive myelin loss with relatively spared axons (arrows). (B,C) Same sections. Demyelination (PAS positive granules), as well as infiltration of CD4+ (D) and CD8+ (E) cells were identified. APP positive axons were also displayed in the same region (F). CD68+ cells (H) and MOG (G) were detected and co-localized (I). (A–F) Original magnification 400×; (G–I) 200×.

osis extending to the lateral ventricles (Fig. 1D). By contrast, the brains of EAE alone mice showed very mild inflammation adjacent to sub-pial areas and no evidence of overt parenchymal infiltration (Supplementary Fig. 1A) or gliosis (Supplementary Fig. 1E). All other control groups manifest no parenchymal pathology except mild meningeal inflammation (Supplementary Fig. 1B–D and F–H).

Brain parenchymal lesions in EAE+ PTx icv mice resembled MS lesions

The EAE+ PTx icv mice manifested multiple inflammatory foci around the small vessels in brain parenchyma (Fig. 2A); these resembled the "lymphocyte cuff" seen in MS pathologically. Infiltrating T cells in the brains of the EAE+ PTx icv mice, stained positive for CD4 (Fig. 2D) and CD8 (Fig. 2E). APP positive axons, indicating axonal injury were identified but minimally represented (Fig. 2F). The colocalization of CD68+ and MOG with immunofluorescence staining indicated the active uptake of myelin breakdown products by macrophage (Fig. 2G–I). Demyelination, the pathonogmonic pathologic feature of MS was characterized here by myelin loss or breakdown with relative axon preservation. Demonstration of demyelination was accomplished by LFB/PAS staining of myelin (Fig. 2B). The density of intact myelin fibers was decreased. Silver staining showed relatively preserved axons (Fig. 2C). Many macrophages were PAS positive secondary to the uptake and metabolism of myelin breakdown fragments (Fig. 2B, arrow). PTx icv administered in the course of EAE induced lesions that closely resembled the brain lesions in MS.

Cytokine expression in the brain and spinal cord of the EAE+ PTx icv versus EAE alone mice

The role of PTx in cytokine expression has been attributed to the generation of IL-6 dependent IL-17 CD4+ cells, i.e., Th17 cells. To determine the expression of these inflammatory mediators, we assessed the cytokine profile after EAE induction.

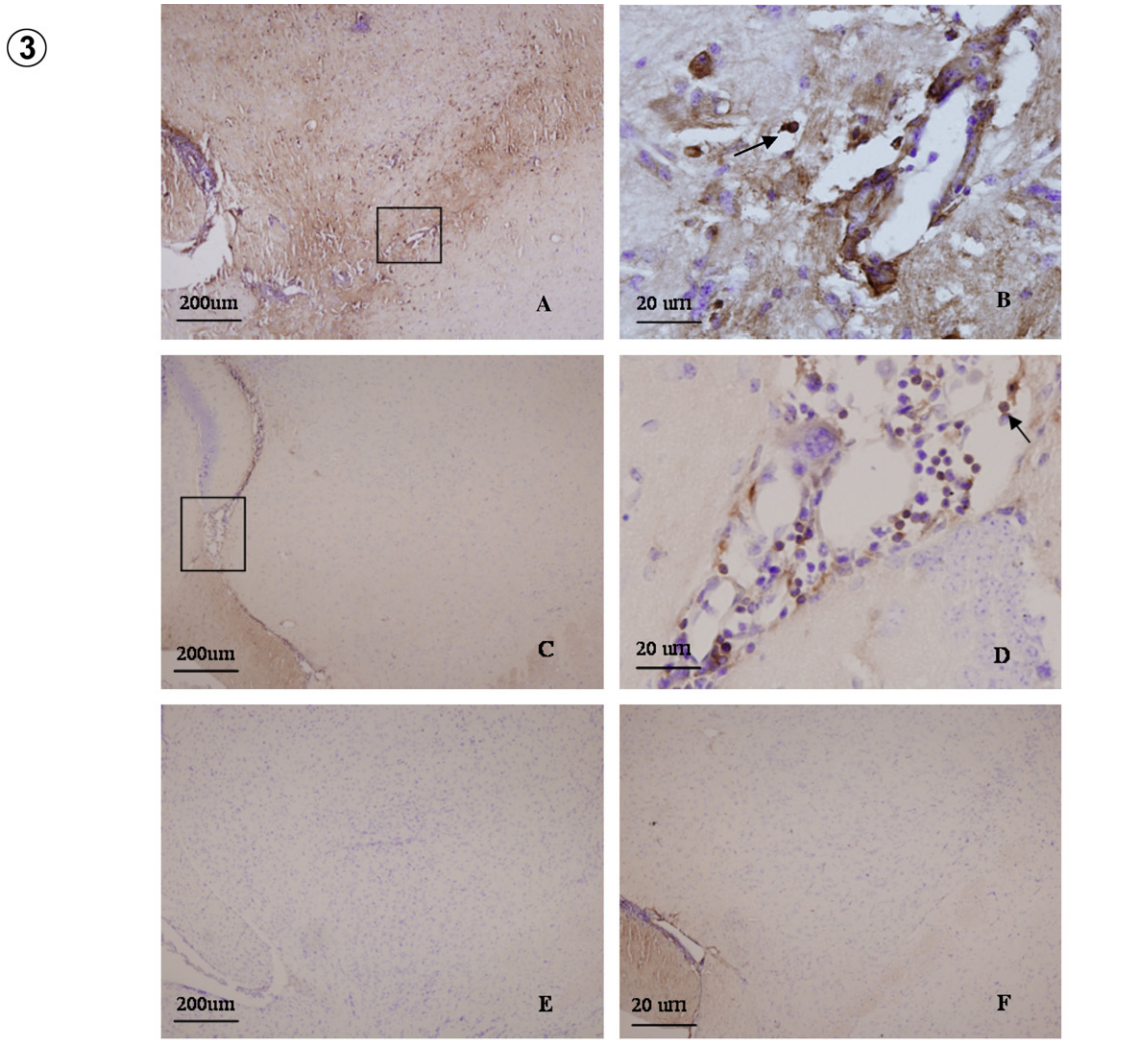
In the brain, the EAE+ PTx mice exhibited infiltrating leukocytes which stained positive for CD4 and IL-17. The majority of these colocalized cells were in the periventricular white matter (Fig. 3A–B, double staining Fig. 4G–I), confirming the infiltration of proinflammatory of Th-17 cells induced by PTx icv. Whereas, in the EAE alone mice, the presence of Th-17 cells in the brain was limited to the meninges (Fig. 3C and D, double staining Fig. 4J–L). The protein level of IL-17 was significantly elevated in the brain of EAE+ PTx icv mice, relative to the controls and the EAE alone mice (Supplementary Fig. 2A) ($p < 0.05$). IL-6 expression was also elevated in the brains of EAE+ PTx icv mice relative to controls and EAE alone mice (Supplementary Fig. 2B) ($p < 0.05$). Similarly, TGF-β was elevated in the brains of EAE+ PTx icv mice (Supplementary Fig. 2C) ($p < 0.05$). In normal control and CFA+ PTx icv groups, no IL-17+ cells were detected in brain (Fig. 3E and F).

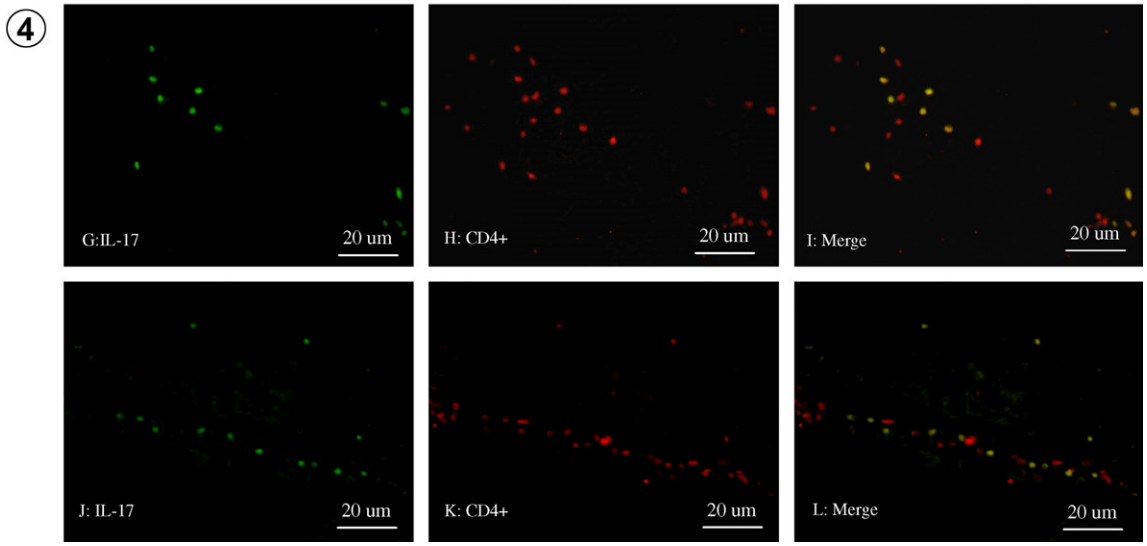
Discussion

In this report, we describe a novel EAE model induced by PTx icv. It replicates the key features of the most common pathonogmonic lesions seen in the human demyelinating disease MS. We further localize the infiltrating T cells and demyelinating lesions to the subcortical white matter regions of the brain.

The present study made two salient observations. First, it has shown that icv PTx primes the CNS tissue for a deleterious inflammatory reaction in the subcortical periventricular regions of the brain. This finding may be of interest given that human neuroinflammatory disorders such as MS predominantly present in the white matter of the brain rather than spinal cord [18]. Second, the critical role of PTx in the induction of proinflammatory cytokines, TGF-β and IL-6 as well as the infiltration of Th-17 cells has been demonstrated for the first time in the periventricular white matter of the brain in this EAE+ PTx icv model. Although it remains plausible that other factors, i.e., MOG can mediate early aspects of neuroinflammation [19], the dominant role in the leukoencephalopathic infiltration and demyelination clearly is played by PTx. These observations indicate that PTx plays a critical role at the interface of innate and adaptive immunity, facilitating the development distinct clinical outcomes.

The mechanisms whereby PTx mediates proinflammatory effects are multifactorial. Early proposals suggested that PTx through vasoactive measures compromised the blood–brain barrier and thereby provided the primed T cells access [20]. Recent studies indicate that PTx increases the expression of adhesion molecules (P-selectin) by binding to TLR4, thereby facilitating leukocyte infiltration/migration into the brain [21]. This migration is abrogated with the blockade of adhesion molecules discounting the vasoactive permeability theory of as the primary mechanism of entry





Figs. 3 and 4. IL-17+ cells were detected in the brain parenchyma of the EAE+ PTx icv mice (A,B), while these cells were limited to the meningeal and subpial area in the EAE alone mice (C,D). IL-17+ cells co-localized with CD4+ cells (G–I) EAE+ PTx icv group; (J–L) EAE alone group. No IL-17+ cells were detected in either normal control (E) or CFA+ PTx icv group (F). (B,D) Inserts in (A,C), respectively. (A, C, E, and F) Original magnification 40×; (B, D, G–L) 400×.

[22]. Furthermore, PTx also induced the maturation of dendritic cells resulting in the expansion of T effector cell and the differentiation of both Th1 and Th2 cells [23,24]. Finally, in vivo studies evidenced that PTx reduces the number of mouse CD4+CD25+ FoxP3+ T regulatory cells (Treg) and impairs the immunosuppressive functions of Treg [25,26].

Interferon gamma (IFN-γ) primary to the development of distinct clinical forms of EAE also has been shown in recent studies to promote PTx induced T cell entry into the CNS. IFN-γ induces a characteristic glial chemokine response that by itself is insufficient to promote inflammation as is PTx alone. However, IFN-γ-induced CNS chemoattractant signals can synergize with PTx to drive T cell entry into the CNS [27].

A recent study investigated the time course of infiltration and pathologic changes in EAE following MOG injection. They determined that disease onset occurred at day 14 and peaked at day 21 and the most severe pathologic changes in the spinal cord, i.e., T-cell infiltration, demyelination, and microglial activation correlated with the clinical severity [19]. However, early in the course on day 7, recruitment of effector T cells were identified and distributed relatively evenly in the meninges, periventricular, and superficial white matter structures of the brain as well as the spinal cord [19]. Later preferential accumulation and infiltration of the spinal cord occurred; the residual T cell presence in the brain was limited to the meninges.

Prevailing theories indicate that T cells have access to ventricular and meningeal spaces, partially through the choroid plexis, in the process of normal immune surveillance [28]. T cell activation is stimulated after exposure to the target antigen (MOG) in adjacent (to meninges) white matter [29]. Activated T cells secrete cytokines for microglial recruitment and T cell-mediated inflammation, which along with antibody/complement and activated microglia mediate destruction of myelin sheaths and distal axons. This distal axonal damage initiates further vascular T cell recruitment partially through the upregulation of VCAM-1 [19,30].

The predilection for the spinal and cranial nerve structures (i.e., optic and auditory nerves) in EAE was attributed the intersection of several factors, proximity of myelinated structures to meninges and CSF outlets, adjacency to portals of T cell entry into CSF, as well as cytokine gradient [31]. However, other structures (anterior commissure, posterior corpus callosum, etc.) within the brain with similar exposure, which did evidence early inflammatory changes, did not progress to neurodegeneration or further infiltration. There is evidence that trafficking events in the spinal cord differ from those that are observed more rostrally in the brain. Intra vita microscopy studies in the spinal cord, revealed that encephalitogenic T cell blasts undergo a potentially variant form of tethering at vascular branch points, resulting in the sudden arrest and no rolling of the cells under flow conditions before entry. This process depends upon unique adhesions molecules [32] and thus may be differentially affected by pertussis toxin.

In conclusion, PTx icv administration during the course of EAE resulted in inflammatory T cell infiltration and demyelination in the periventricular white matter regions of the brain that resembles brain lesions in MS. The current data suggest that the instructive role of icv PTx in adaptive immunity is dependent on the specific context of the inflammatory reaction. In summary, the icv-PTx EAE model provides a platform for studying periventricular brain T cell infiltrating forms of demyelinating disease and the symptoms of associated encephalopathy and may uncover novel strategies for ameliorating the impact of clinical neuroinflammatory disorders.

Disclosures

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.02.161.

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