Pertussis toxin-induced hyperacute autoimmune encephalomyelitis in Lewis rats is correlated with increased expression of inducible nitric oxide synthase and tumor necrosis factor alpha

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This paper was published in Neuroscience Letters, 308:41~44(2001)

Abstract

The involvement of inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α), which have diverse roles in the progression of autoimmune disease models, was studied in pertussis toxin (PT)-induced hyperacute experimental autoimmune encephalomyelitis (EAE) in Lewis rats. The expression of TNF-α mRNA (increased 5 fold, $p<0.01$) and iNOS protein (3 fold, $p<0.01$) was much greater in the spinal cords with PT(+) EAE at the peak stage of EAE than in those with PT(-) EAE, as shown by competitive PCR and Western blot analysis, respectively. Immunohistochemistry showed that the majority of ED1-positive macrophages in EAE lesions contained iNOS, and that there were many more iNOS-positive cells in the CNS lesions of PT(+) rats than in those of PT(-) rats. These findings suggest that PT-induced hyperacute EAE is partly mediated by the enhanced expression of iNOS and TNF-α in the early stages of rat EAE.

Key words: experimental autoimmune encephalomyelitis, inducible nitric oxide synthase, TNF-α, pertussis toxin

Introduction

Experimental autoimmune encephalomyelitis(EAE) is an autoimmune T-cell-mediated central nervous system (CNS) disease that is used as an animal model for the human demyelinating disease multiple sclerosis (Raine *et al.*, 1984). After immunization with brain tissue antigen, including myelin basic protein, susceptible animals develop paralysis that is followed by spontaneous recovery. The EAE lesion is characterized by infiltration of T cells, macrophages, and some bystander cells. During the recovery from EAE, apoptotic elimination of inflammatory cells is one of the most important phenomena in the recovery of the damaged central nervous system (Pender *et al.*, 1992; Schmied *et al.*, 1993).

Nitric oxide (NO) is a gaseous free-radical that mediates a variety of biological functions, including vasodilation, neurotransmission, and cytotoxicity (Moncada *et al.*, 1991; Nathan *et al.*, 1994). NO is

produced from L-arginine by the enzyme NO-synthase (NOS), and there are different isoforms of NOS in the CNS. Constitutive forms of NOS (cNOS) are Ca2+ and calmodulin-dependent, while inducible forms (iNOS) are Ca2+ and calmodulin-independent and continuously produce NO (Galea *et al.*, 1994; Lowenstein *et al.*, 1992; Lyons *et al.*, 1992). In addition to iNOS, our previous competitive PCR analysis revealed that TNF-α mRNA was significantly increased in the spinal cord in rat EAE (Tanuma *et al.*, 1997).

Functionally, both iNOS (Fenyk-Melody *et al.*, 1998; Tanuma *et al.*, 1997) and TNF-α (Liu *et al.*, 1998; Tanuma *et al.*, 1997) have both detrimental and beneficial effects in EAE. During the initiation of brain inflammation, such as occurs in EAE, both iNOS (Garcion *et al.*, 1997) and TNF-α (Tanuma *et al.*, 1997) genes are up-regulated. In this connection, iNOS has been studied extensively at its transcriptional level (Koprowski *et al.*, 1993; Tran *et al.*, 1997) and has functional significance in EAE via the inhibition of iNOS activity (Zhao *et al.*, 1996). EAE induced in either iNOS knockout mice (NOS2) (Fenyk-Melody *et al.*, 1998) or in mice lacking TNF-α (Liu *et al.*, 1998) is worse than in wild-type control mice. The only consensus is that either iNOS (Zhao *et al.*, 1996) or TNF-α (Tanuma *et al.*, 1997) exacerbates host tissue injury in autoimmune disorders, including EAE, during the induction stage.

The addition of PT when EAE is triggered worsens the CNS effects [1]. The mechanism by which PT accelerates the development of the disease is poorly understood. Arimoto et al. reported that IL-10 suppression by PT administration might induce the activation of, or deviation to, encephalitogenic T cells, and result in the development of more severe EAE (Arimoto *et al.*, 2000). Little is known about the functional roles of iNOS and TNF-α in EAE that is triggered with PT administration.

The purpose of this study was to elucidate the involvement of two major pro-inflammatory mediators, iNOS and TNF-α, in the exacerbation of rat EAE with or without pertussis toxin (PT) administration

Material and Methods

1. Animals

Lewis rats were purchased from Harlan (Sprague Dawley, Inc., Indianapolis, IN) and bred in our animal facility. Female rats weighing 160-200 grams, aged 7-12 weeks, were used throughout the experiments.

2. EAE induction

Each rat was injected in the hind footpads bilaterally with an emulsion containing equal parts of fresh rat spinal cord homogenate (SCH) in phosphate buffer(g/mL) and complete Freunds adjuvant (CFA; Mycobacterium tuberculosis H37Ra, 1 mg/mL; Difco) (CFA). Immunized rats were observed daily for clinical signs of EAE. At the time of immunization, some rats received an intraperitoneal injection of 500 ng pertussis toxin (Sigma, St. Louis, MO). The progress of EAE was divided into seven clinical stages (Grade (G) 0, no signs; G1, floppy tail; G2, mild paraparesis; G3, severe paraparesis; G4, tetraparesis; G5, moribund condition or death; R0, recovery stage). For the histological and Western blot analyses, rats were killed under ether anesthesia at the peak stage (G3) of the EAE.

3. Tissue sampling

Tissue samples were taken on days 14 and 21 post-immunization (PI), during the peak and recovery stages of EAE, respectively. Experimental rats (n=3) in each group were sacrificed under ether anesthesia, and the spinal cords were removed and frozen in a deep freezer (-70 oC) for protein analysis. Pieces of the spinal cords were processed for paraffin embedding after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4.

4. Competitive PCR analysis

For the semi-quantitative analysis of the TNF-α gene in the spinal cord, total RNA was extracted from spinal cord tissue using RNAzol B (Biotecx Lab, Houston, TX). cDNA was then synthesized by reverse transcription using a SuperScript Preamplification System (Life Technologies, Gaithersburg, MD) and amplified in a thermal cycler (Perking Elmer, Norwalk, CT) using primer pairs for TNF- (sense: 5′ TAC TGA ACT TCG GGG TGA TTG GTC C 3′, antisense: 5′ CAG CCT TGT CCC TTG AAG AGA ACC 3′, 295 bp). Quantification of TNF- mRNA levels was done by competitive PCR as described in our previous report (Tanuma *et al.*, 1997).

5. Western blot analysis

Frozen spinal cords were thawed at room temperature, minced, lysed in a buffer consisting of 40 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 0.1% Nonidet P-40 (polyoxyethylene [9] p-t-octyl phenol) containing the protease inhibitors leupeptin (0.5 g/ml), PMSF (1 mM), and Aprotinin (5 g/ml), and then homogenized. Samples were electrophoresed under denaturing conditions in 7.5% SDS-PAGE, and the separated proteins were transferred to PROTRAN nitrocellulose transfer membrane (Schleicher and Schuell, Keene, NH). iNOS binding was detected with the primary antibody (rabbit anti-iNOS (Transduction Laboratories, Lexington, KY) diluted with TBS-T, 1:5000). The reaction was visualized by labeling with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Vector, Burlingame, CA). Visualization was achieved using Amersham ECL reagents (Arlington Heights, IL). Quantification was performed by a densitometer (M GS-700 Imaging Densitometer, Bio-Rad, CA).

6. Immunohistochemistry

Five-micron sections of paraffin-embedded spinal cord were deparaffinized and allowed to react with anti-iNOS polyclonal antibody (Transduction Laboratories, Lexington, KY). To identify macrophages, mouse ED1 (Serotec, London, U.K.) was applied. The immunoreaction was visualized with the avidin-biotin peroxidase complex Elite kit(Vector, Burlingame, CA). Peroxidase was developed with a diaminobenzidine substrate kit (Vector). Before being mounted, the sections were counterstained with hematoxylin

Results

1. Clinical observation of EAE

Fifteen Lewis rats were immunized with either SCH/CFA plus PT (PT(+)) or with SCH/CFA alone (PT(-)) and checked daily for clinical signs. As shown in Fig. 1, all the PT(+) rats (n = 5) died around 14 days post-immunization, while the PT(-) EAE rats recovered from the paralysis (n = 10) (Fig. 1).

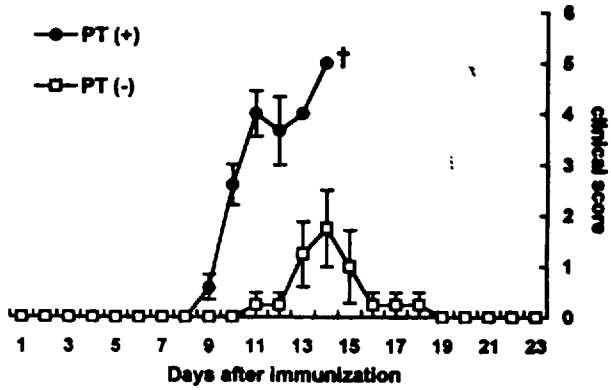

FIG. 1. Clinical course of EAE induced in Lewis rats with or without pertussis toxin (PT) administration. EAE rats with PT (n = 5) died around 14 days post-immunization, while EAE rats without PT administration recovered from the paralysis (n = 10).

Histopathological examination revealed infiltration of inflammatory cells in the spinal cord parenchyma in both the PT(+) and PT(-) groups. The inflammation was less severe in the PT(-) group (figure not shown)

2. Competitive PCR analysis

In order to confirm the differences of TNF-α in the exacerbation of EAE, TNF-α gene expression was quantified in the spinal cords of PT(+) and PT(-) rats. As shown in Fig. 2, TNF-α increased markedly in the spinal cord of PT(+) EAE (5 fold) ($p<0.01$).

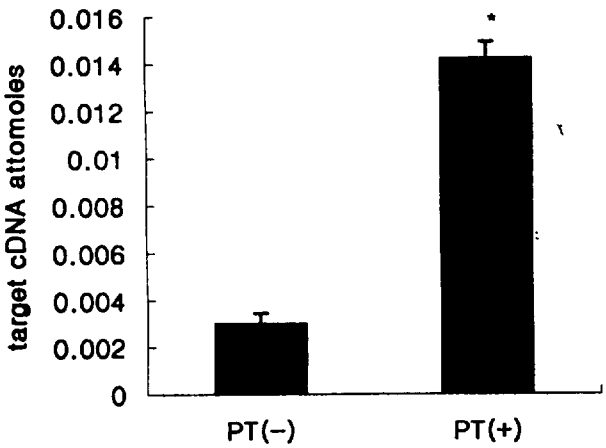

FIG. 2. Competitive PCR analysis of TNF- in the spinal cord. Spinal cord tissues taken from PT(-) and PT(+) rats at the peak stage of EAE were subject to competitive PCR analysis for TNF-. Five rats were examined. ** Significantly different by Students t-test ($p<0.01$)

3. Western blot analysis

As with TNF-α expression, iNOS expression was significantly increased in the spinal cords of PT(+) and PT(-) EAE rats (about a 3-fold increase) (Fig. 3).

4. Immunohistochemistry

Histological examination at the peak stage in the PT(-) (Fig. 4A) and PT(+) (Fig. 4B) groups showed inflammatory lesions in the spinal cord. Comparison showed that there were many more inflammatory cells infiltrating the perivascular lesions in the PT(+) group. As shown in Fig. 4, there were a large number of iNOS-positive cells in the spinal cords of PT(+) rats (Fig. 4B). In PT(-) rats, there were few iNOS-positive cells in the perivascular lesions (Fig 4A). The inflammatory cells consisted primarily of ED1+macrophages (Fig. 4C, 4D). Inflammatory cells were less abundant in the PT(-) group than in the PT(+) group. There were significantly more iNOS-positive cells in the PT(+) group (Table 1).

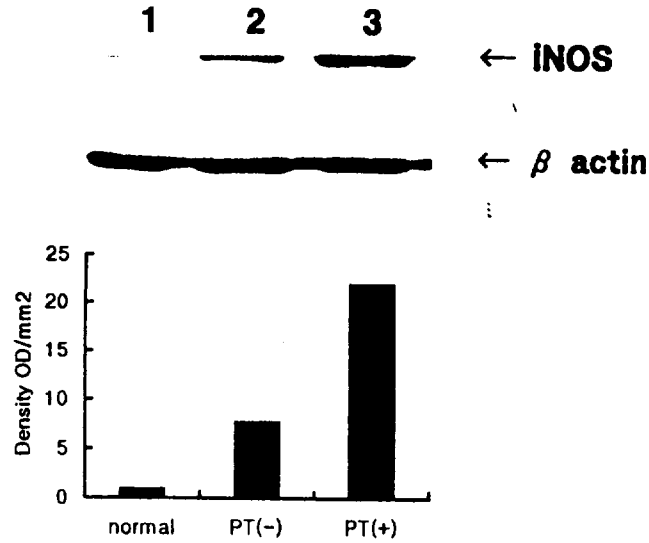

FIG. 3. Western blot analysis of iNOS in the spinal cord of normal (A, lane: 1), PT(-) (A, lane: 2) and PT(+) (A, lane: 3) rats. At the peak stage of PT(+) EAE, iNOS was significantly increased (lane: 3). There was much more iNOS in the PT(+) rats (lane: 3) than in the PT(-) rats (lane: 2). The molecular mass of iNOS is 130 kDa.

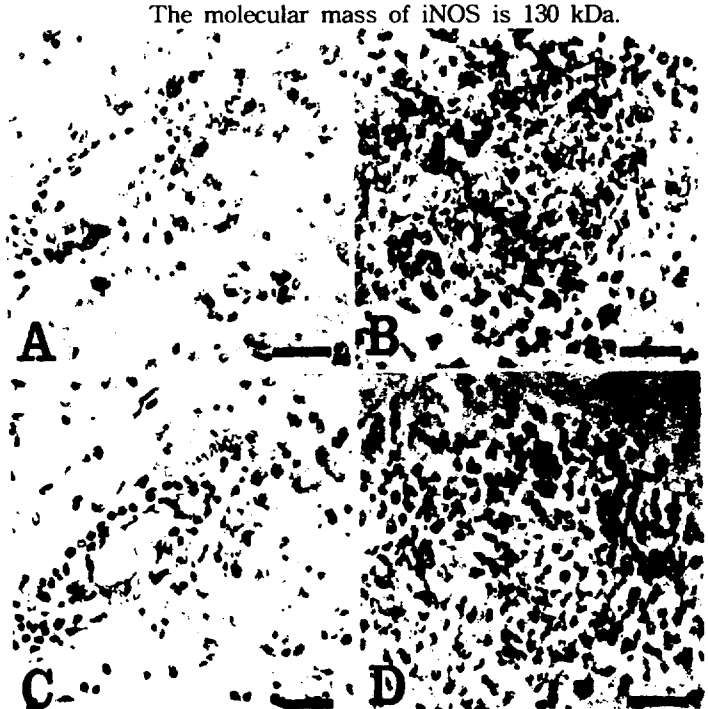

FIG. 4. Immunohistochemical examination of the spinal cord of rats immunized with SCH/

CFA alone (PT(-)) (A, C) or with SCH/CFA plus PT (PT(+)) (B, D). In PT(+), there are several iNOS-positive cells in the perivascular lesion (B), while in PT(-) there are few iNOS-positive cells (A). iNOS labeling is observed on macrophages (C, D). Counterstained with hematoxylin. Scale bar = 30m.

Table 1. Histological findings of inflammatory lesions in the spinal cords of rats with EAE with or without PT administration[a]

Pertussis Toxin	Mean clinical score at histological examination[b]	Mean iNOS+ cells in perivascular lesions[c]
-	0.75 ± 0.366	137 ± 0.88
+	3.8 ± 0.374*	1739 ± 1.372**

[a] Data are expressed as mean ± S.E.

[b] On day 13 post-immunization (PI), rats (n = 5) were killed under ether anesthesia and segments of the lumbar spinal cord were processed for hematoxylin-eosin staining.

[c] The mean iNOS-positive cell count in perivascular lesions at 13 days PI. The count was roughly 13 times higher in the PT(+) group than in the PT(-) group.

* Difference in the mean clinical scores of the PT(+) and PT(-) groups is statistically significant $p<0.001$.

** Difference in the mean number of iNOS-positive cells in perivascular lesions between the PT(+) and PT(-) groups is extremely statistically significant, $p<0.0001$, Students unpaired, two-tailed t-test.

Discussion

This study found that Lewis rats immunized with SCH/CFA plus PT developed severe EAE, whereas those immunized with SCH/CFA alone did not. We tried to elucidate the factor(s) accelerating the disease processes by comparing the expression of two important pro-inflammatory mediators, iNOS and TNF-α, in the two groups. One marked difference was that both iNOS and TNF-α expression were much greater in the spinal cords of PT(+) rats than in PT(-) rats. We postulate that either iNOS or TNF-α, or both, increase during the induction stage of EAE, which in turn exacerbates the progression of EAE paralysis. However, we do not exclude the possible involvement of TNF-α in the amelioration of myelin oligodendrocyte glycoprotein-induced EAE (Liu *et al.*, 1998).

The possibility that PT contributes to changing the blood brain barrier (BBB) cannot be excluded (Reiber *et al.*, 1984). PT-sensitive G protein is a vasoactive substance that affects the permeability of the BBB via interaction with an adhesion molecule in the absence of increased vascular permeability in the CNS, and it enhances the development of autoimmune diseases (Hickey *et al.*, 1991). Several reports have demonstrated that blockage of CD14 or G protein function is associated with ablation of endotoxin-mediated iNOS protein expression (Schroeder *et al.*, 1997). More recently, activation of p21ras, which binds G protein, was shown to be involved in the induction of iNOS in activated primary astrocytes (Pahan *et al.*, 2000). Moreover, Arimoto et al. (2000) examined the nature of T cells isolated from PT(+) and PT(-) rats. Administration of PT increased the proliferative responses of lymph node T cells, but not the switch to encephalitogenic T cells. A comparison of the immunological features of PT(+) and PT(-) rats revealed that downregulation of IL-10 by PT may be the major factor determining outcome in F344 rats (Arimoto *et al.* 2000). Based on this finding, and considering our findings, PT administration may induce the activation of either iNOS or TNF-α, or both, from macrophages, resulting in the development of more severe EAE.

These findings suggest that the enhanced expression of iNOS and TNF-α with PT administration in rat

EAE is an important factor in the exacerbation of central nervous system inflammation as far as rat EAE is concerned.

Acknowledgment

This work was supported by in part by the Korean Science and Engineering Foundation(KOSEF) through the subtropical Horticulture Research Center at Cheju National University.

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초 록

자기면역성뇌척수염에서 inducible nitric oxide synthase와 tumor necrosis factor-alpha의 역할

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자기면역성 뇌척수염(experimental autoimmune encephalomyelitis, EAE)은 뇌조직항원을 면역한 후 야기되는 염증성 질병으로 사람 다발성경화증의 한 모델로 연구되고 있다. 자기면역성 뇌염의 시작은 뇌조직항원에 반응하는 림프구가 중추신경계에 침윤되면서 마비를 나타내는데 이 과정 중에는 여러 종류의 pro-inflammatory mediator (tumor necrosis factor-alpha (TNF-α)와 inducible nitric oxide synthase (iNOS)등)가 관여하는 것으로 알려지고 있다. 이 연구에서는 염증의 진행 단계에 따라 염증 유도 또는 염증 억제의 상반된 기능을 갖는 것으로 알려진 TNF-α와 iNOS가 심급성 뇌척수염 진행에 어떠한 영향을 미치는지를 조사하였다.

뇌염을 유도하기 위한 항원으로는 랫트 척수 조직 유제를 complete Freund adjuvant와 혼합하여 뒷 발바닥에 주사하였으며 심한 뇌척수염을 유도하기 위하여 pertussis toxin(500ng/ea)을 면역하는 날 복강내로 주사하고 매일 체중과 마비 정도를 확인하였다.

독소를 주사한 실험군에서는 대조군(11일)에 비해 마비의 시작이 빨랐으며(9일), 대조군은 자연 회복하는 반면 독소를 주사한 실험군에서는 모두 폐사하였다.

척수 조직 내 TNF-α와 iNOS의 양적인 변화를 조사하기 위하여 Competitive PCR과 Western blot을 이용하였으며, 세포형을 구분하기 위하여 면역염색을 이용하였다.

Competitive PCR결과 TNF-α는 PT를 투여한 자기면역성뇌척수염의 심한 마비기(EAE, G3)에서 PT를 투여하지 않은 대조군보다 약 5배가 증가하였으며($p<0.01$), Western blot결과 iNOS는 PT를 투여한 군에서 정상조직에 비해 약 6배가 증가하였고, PT를 투여하지 않은 군에 비해서는 약 3배가 증가하였다($p<0.01$).

면역염색결과 PT를 투여하지 않은 랫트보다 투여한 랫트의 척수조직에서 iNOS 양성 세포가 약 15배가 증가하였으며($p<0.01$), 또한 연속절편에서 이들 세포가 큰포식세포임을 확인하였다.

이상의 결과를 종합해 볼 때, 자기면역성 뇌척수염의 초기 유도과정에는 TNF-α와 iNOS는 염증의 악화에 관여됨을 알 수 있었다.

주요어 : 자기면역성뇌척수염, inducible nitric oxide synthase, tumor necrosis factor alpha, pertussis toxin