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Bladder Dysfunction in Mice with Experimental Autoimmune Encephalomyelitis

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Abstract

The vast majority of patients with multiple sclerosis (MS) develop bladder control problems including urgency to urinate, urinary incontinence, frequency of urination, and retention of urine. Over 60% of MS patients show detrusor-sphincter dyssynergia, an abnormality characterized by obstruction of urinary outflow as a result of discoordinated contraction of the urethral sphincter muscle and the bladder detrusor muscle. In the current study we examined bladder function in female SWXJ mice with different defined levels of neurological impairment following induction of experimental autoimmune encephalomyelitis (EAE), an animal model of central nervous system inflammation widely used in MS research. We found that EAE mice develop profound bladder dysfunction characterized by significantly increased micturition frequencies and significantly decreased urine output per micturition. Moreover, we found that the severity of bladder abnormalities in EAE mice was directly related to the severity of clinical EAE and neurologic disability. Our study is the first to show and characterize micturition abnormalities in EAE mice thereby providing a most useful model system for understanding and treating neurogenic bladder.

Keywords

EAE/MS; autoimmune; micturition; neurogenic bladder

1. Introduction

Multiple sclerosis (MS)⁶ is an inflammatory disease of the central nervous system (CNS) widely believed to involve T cell mediated demyelination and neurodegeneration (Steinman, 2001; Hafler et al., 2005). Although MS patients develop a variety of neurologic abnormalities,

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⁶Abbreviations: CNS, central nervous system; DSD, detrusor-sphincter dyssynergia; EAE, experimental autoimmune encephalomyelitis; FMOC, N-alpha-(9-fluorenylmethyloxycarbonyl); LNC, lymph node cells; MBP, myelin basic protein; MS, multiple sclerosis; PLP, myelin proteolipid protein; PMC, pontine micturition center.

the majority of MS patients have bladder control problems of both storage and voiding types including urgency to urinate, urinary incontinence, high frequency of urination, and retention of urine (Litwiller et al., 1999; Kalsi and Fowler, 2005; Yamamoto et al., 2006). Although genitourinary symptoms are not often life threatening, they create substantial morbidity and patient frustration and dramatically impact the overall quality of life experienced by affected MS patients (Blaivas and Barbalias, 1984; Nortvedt et al., 2001; Fernandez, 2002).

Experimental autoimmune encephalomyelitis (EAE) is an animal model commonly used in MS research (Steinman and Zamvil, 2005). EAE is typically induced by immunization of animals with myelin proteins or their derived encephalitogenic peptides, and clinical disease develops when primed T cells enter the CNS and recognize encephalitogenic peptide determinants presented in the context of major histocompatibility complex molecules by resident antigen presenting cells. Within a few weeks after immunization with the encephalitogenic p139-151 peptide of myelin proteolipid protein (PLP 139-151), SJL and SWXJ mice develop acute EAE often leading to severe multi-limb weakness and/or paralysis (Tuohy et al., 1989; Sobel et al., 1990; Yu et al., 1996). Affected mice typically undergo an incomplete recovery from the initial attack with residual neuroparalytic signs. Recovery is soon followed by a series of multiple relapse/remission cycles with each neuroparalytic episode leaving the mice progressively more impaired. By 8-12 weeks after immunization, the incremental accumulation of neurologic deficit makes it increasingly more difficult to observe relapses. This chronic-progressive stage of EAE is characterized by sustained limb paresis or paralysis and marked CNS demyelination and neurodegeneration particularly pronounced in the spinal cord.

In the current study we examined bladder function in female SWXJ mice with different defined levels of neurological impairment following induction of EAE. We found that EAE mice develop profound bladder dysfunction characterized by significantly increased micturition frequencies and significantly decreased urine output per micturition. We also found that the severity of bladder abnormalities in EAE mice are directly related to the severity of clinical EAE and neurological disability. Our results are the first to show and characterize bladder dysfunction in EAE mice and provide a novel animal model for examining the underlying features of neurogenic bladder in MS and in developing new strategies for treating these bladder abnormalities more effectively.

2. Materials and Methods

2.1 Peptide synthesis

The encephalitogenic p139-151 peptide of myelin proteolipid protein (PLP 139-151 HSLGKWLGHDPKF; serine for cysteine at residue 140) was synthesized by the Molecular Biotechnology Core Facility of the Lerner Research Institute using standard solid phase methodology and FMOC side chain protected amino acids. The peptide was purified >97% by reverse phase HPLC, and amino acid composition was confirmed by mass spectrometry. Female SWXJ (H-2^{q,s}) mice were purchased at 6 weeks of age from the Jackson Laboratory (Bar Harbor, ME) and were immunized for induction of EAE at 8-10 weeks of age.

2.2 Induction of EAE

EAE was induced as previously described (Yu et al., 1996). Briefly, SWXJ mice were immunized *s.c.* in the abdominal flank on day 0 with 200 µg of PLP 139-151 and 400 µg *Mycobacteria tuberculosis* H37RA (Difco, Detroit, MI) in 200 µl of an emulsion of equal volumes of water and Freund's adjuvant (Difco). On days 0, 3, and 7 each mouse was injected *i.p.* with 0.2 µg of purified *Bordetella pertussis* toxin (List, Campbell, CA). Mice were weighed and scored daily for neurologic signs according to the following scale: 0, no disease; 1,

decreased tail tone or slightly clumsy gait; 2, tail atony and/or moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; 5, moribund state. At the termination of each experiment, mice were euthanized by asphyxiation with CO₂ followed by cervical dislocation. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Cleveland Clinic and were performed in compliance with the Public Health Service policy on humane care and use of laboratory animals.

2.3 Frequency-Volume Chart (FVC) Analysis

Twenty-four hours prior to micturition testing, solid food was removed from cages and replaced with lactose-free milk (Lactaid 100 Whole Milk; McNeil Nutritionals, Fort Washington, PA). This strategy substantially reduces the frequency and weight of the feces generated during testing and thereby prevents skewing of the urine collection and aberrations of data analysis (Liu et al., 2006). Twenty-four hour micturition and drinking habits of mice was measured by placing the mice individually in metabolic cages (MED-CYT-M; Med-Associates, St. Albans, VT). Urine was collected in a plastic tray located on an analytical balance (VI-3 mg; Acculab, Huntingdon Valley, PA) placed directly underneath each cage. Balances were connected to a data acquisition software program designed by the vendor for measuring the weight in mg of urine collected over the defined time period. During testing, mice were provided with free access to lactose-free milk and water, and the testing room was maintained on a light/dark cycle identical to normal housing conditions. At completion of testing, milk and water bottles were examined to measure consumption over the 24 hour time period. To ensure unobstructed access to water and milk, all liquids were provided to mice with impaired mobility in easy access low lying containers.

2.4 Histological analysis

Bladders and spinal cords were removed and were fixed in 10% neutral formalin overnight. Paraffin-embedded tissue was cut into sections 5 mm in thickness, then stained with hematoxylin and eosin. The severity of tissue injury and inflammation was analyzed by researchers masked to sample identity. Images were collected using a Leica DM5000 microscope with large field-of-view (mosaic tile) capability (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with a Retiga SRV Cooled CCD camera and liquid crystal tunable RGB filter (QImaging, Surrey, BC Canada). Data was stored using ImagePro Plus software (Media Cybernetics, Inc, Bethesda, MD) and Objective imaging Mosaic Tile Software with encoded scanning stage (Objective Imaging Ltd, Kansasville, WI).

2.5 Real-time RT-PCR

Total RNA was extracted from spinal cords and bladders with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was synthesized with random hexamers (Applied Biosystems, Foster City, CA) using M-MLV reverse transcriptase (Promega, Madison, WI). Expression of the genes encoding IFN γ , TNF α , IL-1 β , and IL-17a were quantified with the SYBER Green PCR Master Mix kit (Applied Biosystems) using primer pairs selected for amplification of each individual cytokine (Table 1). Relative gene-expression was determined as the ratio of cytokine to β -Actin gene expression levels for each tissue and data are expressed in percentage points.

2.6 Biostatistical analysis

Micturition frequencies and weights as well as relative gene expression levels were compared using the Student's *t*-test.

3. Results

3.1 Bladder dysfunction in EAE mice

EAE was induced by immunization of female SWXJ mice with PLP 139-151. After immunization, mice were weighed and examined daily for neurologic deficit on a score of 1 through 5 (Figure 1a). At different times after EAE onset, mice with mild to moderate grade 1-2 EAE as well as mice with severe grade 3 disease were tested over a 24 hour period for micturition frequencies and urine output per micturition measured in grams. Age- and sex-matched SWXJ mice immunized with CFA were used as neurologically unimpaired controls and tested at the same time after immunization. Compared to 5 control mice immunized with CFA, 24 hour micturition frequencies were significantly higher in 10 mice with mild to moderate grade 1-2 EAE ($P=0.02$) and in 6 mice with severe grade 3 EAE ($P=0.02$; Figure 1b and 2). Although micturition frequencies in mice with severe grade 3 EAE were higher than those observed in mice with mild to moderate grade 1-2 EAE, these differences approached but did not achieve significance ($P=0.09$). Urine output/micturition was inversely related to frequency of urination. Compared to control mice immunized with CFA (vehicle), mean urine output per micturition were significantly lower in mice with mild to moderate grade 1-2 EAE ($P=0.003$) and in mice with severe grade 3 EAE ($P=0.0004$; Figure 1c and 2). Also, urine output per micturition in mice with severe grade 3 EAE was significantly lower than those measured in mice with mild to moderate grade 1-2 EAE ($P=0.004$). It is important to note that mice with severe grade 4 or grade 5 EAE failed to urinate over their 24 hour testing period perhaps because of their inability and/or unwillingness to drink, and not because of urinary retention.

3.2 Histological analysis of spinal cords and bladders

To determine whether the bladder abnormalities observed in EAE may be the result of bladder inflammation perhaps resulting from bystander or crossreactive immunity, H&E stained sections from each organ were examined under light microscopy. Spinal cords from mice immunized with PLP 139-151 consistently showed conventional signs of CNS inflammation often marked by large perivascular infiltration of dorsal and ventral columns (Figure 3a, b, and c). CFA immunized control mice never developed spinal cord inflammation. In contrast to spinal cords, bladders from both EAE and CFA immunized control mice never showed any signs of inflammation (Figure 4d and e). Our failure to observe inflammation in EAE bladders was confirmed by real-time RT-PCR analysis of inflammatory cytokines in spinal cord and bladder tissues. Expression levels of IFN γ , TNF α , IL-1 β , and IL-17a were significantly elevated ($P<0.0005$ in every instance) only in spinal cords from EAE mice and were not elevated in bladders from EAE animals (Figure 4). Our data indicate that the bladder dysfunction occurring in EAE is the result of a neuromuscular CNS disorder and not due to local inflammation of the bladder.

4. Discussion

Our results are the first to show and characterize bladder dysfunction in EAE mice and thereby provide a most relevant relapsing-remitting MS-like animal model for enhancing our understanding and improving the treatment of the neurogenic bladder. The majority of MS patients experience bladder dysfunction of both storage and voiding types including urgency, frequency, urinary incontinence, and/or urinary retention. Over 60% of MS patients show detrusor-sphincter dyssynergia (DSD), an abnormality characterized by obstruction of urinary outflow caused by disordinated contraction of both the bladder detrusor and urethral sphincter muscles (Litwiller et al., 1999; Kalsi and Fowler, 2005). Management of MS patients with bladder dysfunction and specifically DSD remains a clinical dilemma, as no effective treatment modality exists. Current treatments involve palliative measures such as intermittent

catherization, use of antimuscarinic medication, and in some cases surgical interventions such as external sphincterotomy, implantation of urethral stents, or injections with botulinum toxin (Ahmed et al., 2006). Prolonged DSD is suspected to contribute to urinary upper track damage in patients with neurogenic bladder. The built up pressure eventually affects the ureters leading to dilation of the upper track collecting system or hydronephrosis, and complicating urinary tract infections. Lack of a valid DSD animal model for evaluating pathogenic details and examining novel therapies has contributed to continuation of this clinical dilemma.

It appears that bladder dysfunction occurs equally in both males and females with MS. However, sex differences are prominent since males often have symptoms related to obstruction including hesitation, interrupted or weak streams, and incomplete emptying, and women more often have symptoms related to incontinence and leaking (Blaivas and Barbalias, 1984; Koldewijn et al., 1995).

Various CNS networks are involved in the two major functions of the bladder, namely, storage (Athwal et al., 2001; Matura et al., 2002), and voiding (Blok et al., 1997; Blok et al., 1998). However, activation or inhibition of the pontine micturition center (PMC) ultimately controls bladder function. The PMC directly innervates the S2-S4 sacral spinal cord directly activating parasympathetic excitation of detrusor motoneurons resulting in bladder contraction/voiding and indirectly inhibiting parasympathetic urethral sphincter motoneurons by activation of GABA-ergic interneurons (Blok, 2002). Various degrees of PMC dysregulation of the sacral spinal region may occur in MS leading to varying levels of uncontrolled and uncoordinated detrusor/sphincter contraction patterns (Birder et al., 1998; Anderson, 2001; Abrams et al., 2003).

Prior studies have shown micturition abnormalities in Lewis rats with EAE. One early study showed that urinary retention was due to the presence of tightly impacted mucous plugs in the bladder neck (Lifson et al., 1983). Using cystometric analysis, Mizusawa and colleagues (2000) showed micturition abnormalities in 64% (12/33) of female Lewis rats with EAE with detrusor areflexia as the predominant disorder occurring in 52% (17/33) of rats with EAE. Only 12% (4/33) of their EAE rats showed detrusor hyperactivity. More recently, Vignes and colleagues (2007) also used cystometric analysis to show that the majority of female Lewis rats with EAE had detrusor areflexia prior to EAE onset but that detrusor hyperactivity was prominent at onset of disease. Moreover, after EAE onset these investigators observed an equivalent incidence of detrusor areflexia and detrusor hyperactivity.

Our current study complements the cystometric findings observed in Lewis rats with EAE by showing micturition differences that are directly related to the neurologic impairment of SWXJ mice with EAE. However, we did observe that mice with severe paralytic grade 4-5 EAE did not urinate. This failure to urinate was directly related to their failure to drink despite having free and unobstructed access to both water and milk prior to and during the testing period. Thus, it is clear that the failure to drink was not due to a lack of availability and access and may have been the result of dysphagia, a debilitating condition often afflicting MS patients (Prossiegel et al., 2004).

Although the mouse provides anatomic challenges for cystometric studies, the PLP 139-151 mouse model provides distinct features in common with MS that are not characteristic of the Lewis rat EAE model. MBP induced EAE in Lewis rats is primarily a monophasic inflammatory disease with limited relapses and with relatively modest levels of demyelination and neurodegeneration (Swanborg, 2001). In contrast, following immunization with PLP 139-151, SWXJ hybrid mice and their paternal SJL strain develop a relapsing-remitting course of clinical EAE with an ensuing chronic progression of neurologic impairment accompanied by severe CNS demyelination and axonal degeneration (Tuohy et al., 1989; Sobel et al.,

1990; Yu et al., 1996; Wujek et al., 2002), all features common to MS (Peterson and Fujinami, 2007). Thus, our studies provide a most useful animal model for studying the relationship between micturition abnormalities and fluctuations in neurologic impairment within individual mice over the course of relapsing-remitting autoimmune demyelinating disease that ultimately shows progression to severe disability.

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Figure 1.

Bladder dysfunction in EAE mice. (a) EAE was induced by immunization of female SWXJ mice with PLP 139-151. After immunization, mice were weighed and examined daily for neurologic deficit on a score of 1 through 5. Data show mean clinical score over time in 10 mice actively induced with EAE. Error bars show $\pm$ SE. (b) Mice with defined differential levels of neurologic disability were evaluated over a 24 hour period for urination frequencies and output/micturition measured in grams. Compared to control mice immunized with CFA (n=5), 24 hour micturition frequencies were significantly higher in mice with mild to moderate grade 1-2 EAE (n=10; $P=0.02$) and in mice with severe grade 3 EAE (n=6; $P=0.02$). Although micturition frequencies in mice with severe grade 3 EAE were higher than those observed in

mice with mild to moderate grade 1-2 EAE, these differences did not achieve significance ($P=0.09$). (c) Urine output/micturition was inversely related to frequency. Compared to control mice immunized with CFA, mean urine outputs/micturition were significantly lower in mice with mild to moderate grade 1-2 EAE ($P=0.003$) and in mice with severe grade 3 EAE ($P=0.0004$). Outputs/micturition in mice with severe grade 3 EAE were also significantly lower than those measured in mice with mild to moderate grade 1-2 EAE ($P=0.004$).

Figure 2.

Representative micturition frequencies and outputs. Each vertical line represents a micturition event and the height of each vertical line indicates the micturition output measured in grams for a CFA immunized control mouse with no neurologic impairment (upper panel), for an EAE mouse with moderate grade 2 EAE (middle panel), and for an EAE mouse with severe grade 3 EAE (lower panel).

Figure 3.

Histologic analysis of spinal cords and bladders. *(a)* Representative section of upper spinal cord from a control mouse showing no inflammatory infiltration three weeks after immunization with CFA; *(b)* representative section of upper spinal cord from a mouse with grade 3 EAE showing perivascular infiltration of the dorsal column (upper arrow) and ventral cord (lower arrows) three weeks after immunization with PLP 139-151; *(c)* enlarged image of dorsal column infiltration during acute grade 3 EAE; representative sections of normal appearing non-inflamed bladder from *(d)* control mouse immunized with CFA and from *(e)* mouse with grade 3 EAE three weeks after immunization with PLP 139-151.

Figure 4.

Real time RT-PCR analysis of spinal cords and bladder. RNA was isolated from bladders and spinal cords of control mice immunized with CFA and EAE mice immunized with PLP 139-151. cDNA was generated and analyzed for expression of inflammatory cytokines by real-time RT-PCR using primer pairs specific for (a) IFN γ , (b) TNF α , (c) IL-1 β , and (d) IL-17a. Compared to bladders from untreated or CFA immunized control mice, bladders from EAE mice showed no increased gene expression for inflammatory cytokines. However, compared to spinal cords from untreated or CFA immunized control mice, spinal cords from EAE mice showed significantly increased gene expression for each inflammatory cytokine tested ($P < 0.0005$ in every instance). Error bars show $\pm$ SE.

Table 1

Primer pairs used for real-time RT-PCR analysis of gene expression.

Protein		Sequence (5'-3')
IFN γ	Sense	TGATGGCCTGATTGTCTTTCAA
	Antisense	GGATATCTGGAGGAAGTGGCAA
TNF α	Sense	CAAAGGGAGAGTGGTCAGGT
	Antisense	ATTGCACCTCAGGGAAGAGT
IL-1 β	Sense	GAGTGTGGATCCAAGCAAT
	Antisense	AGACAGGCTTGTGCTCTGCT
IL-17a	Sense	CTCCACCGCAATGAAGAC
	Antisense	CTTCCCTCCGCATTGAC
β -Actin	Sense	GGTCATCACTATTGGCAACG
	Antisense	ACGGATGTCAACGTCACACT