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EARLY-LIFE EXPOSURE TO LIPOPOLYSACCHARIDE REDUCES THE SEVERITY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN ADULTHOOD AND CORRELATED WITH INCREASED URINE CORTICOSTERONE AND APOPTOTIC CD4$^+$ T CELLS

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Abstract—Early-life exposure to bacterial endotoxins, such as lipopolysaccharides (LPS), can provide neuroprotection against experimental autoimmune encephalomyelitis (EAE) during adulthood, possibly through altering the responsiveness of the immune system. Here, we show that exposure of LPS to neonatal rats resulted in a sustained elevation of corticosterone level in urine when compared with saline-treated rats, and that the high level of urine corticosterone was maintained during the progression of EAE ($P<0.05$). This high level of production of corticosterone plays an important role in altering the predisposition to EAE-induced neuroinflammation, as a positive correlation occurred between the concentration of urine corticosterone and the increased apoptotic CD4$^+$ T cells from the peripheral blood. LPS-treated rats also showed a reduced number of CD3$^+$ T cells in the spinal cord. The splenic antigen-presenting cells showed a reduced expression of MHC II during EAE development in LPS-exposed rats when compared with rats exposed to the saline-treated control. Together, these findings suggest that treating neonatal rats with LPS evokes a sustained elevation of glucocorticoid, which may suppress immune response during EAE by increasing apoptosis of CD4$^+$ T cells and reducing the expression of MHC II on antigen-presenting cells. Therefore, exposing neonates to bacterial endotoxin may further be developed as an immunization strategy to prevent human multiple sclerosis and other inflammatory brain diseases. **Crown Copyright © 2011 Published by Elsevier Ltd on behalf of IBRO. All rights reserved.**

Key words: experimental autoimmune encephalomyelitis, multiple sclerosis, neuroprotection, lipopolysaccharide, corticosterone, immune regulation.

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the CNS and the most common cause of neurological disability in young adults. Mechanistically, the interplay between susceptibility genes and environmental factors contributes to the pathogenesis of MS (Ramagopalan et al., 2008; Sotgiu et al., 2004). However, currently there is a lack of disease-modifying therapeutic treatment for MS, and management of MS symptoms appears to be the only common clinical practice for treatment (Pittock et al., 2006; Sospedra and Martin, 2005; Thompson et al., 2010; Wingerchuk et al., 2001).

Exciting evidence emerged in recent years where modulation of the developmental plasticity of the immune system early in life can alter the predisposition to inflammation in adulthood. Indeed, helminth infections and more subtle exposure to environmental microbes have been shown to reduce the risk of relapses in MS and also protect the brain during experimental autoimmune encephalomyelitis (EAE), the principal model for MS (La Flamme et al., 2003; Sewell et al., 2003). Several recent studies, including those of our own (Li et al., 2010), also showed that treating neonatal rats during their first week of life with lipopolysaccharide (LPS) significantly suppresses EAE-induced spinal cord damage. The possible mechanisms are through altering the activities of hypothalamic-pituitary-adrenal (HPA) axis function, which in turn promotes tolerogenic dendritic cells and regulatory T cells (Ellestad et al., 2009; Wang and McCusker, 2006), and regulates the immune response in the CNS (Li et al., 2010).

The neuroendocrine system, such as HPA, plays a critical regulatory role on the immune system (Shanks et al., 1995, 2000). Modulation of the immune system, in turn, triggers the onset and development of EAE. Activation of CD4$^+$ autoreactive T cells and their differentiation into a Th1 phenotype are crucial events in the initial steps. These cells are also important players in the long-term evolution of the disease (Sospedra and Martin, 2005). Stress-induced glucocorticoids directly target the HPA and produce a pronounced anti-inflammatory effect. Glucocorticoids may exert their immunosuppressive activity by inducing

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Abbreviations: CFA, complete Freund's adjuvant; EAE, encephalomyelitis; HPA, hypothalamic-pituitary-adrenal; H&E, hematoxylin-eosin; LPS, lipopolysaccharide; MS, multiple sclerosis; SCH, spinal cord homogenate.

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apoptosis in activated lymphocytes (Herold et al., 2006; Reichardt et al., 2006; Zen et al., 2011). Most importantly, the HPA axis is subject to programming by early-life events, and exposure to pathogens is a common occurrence (Shanks et al., 1995). This has led to the suggestion that early-life exposure to endotoxins may alter the predisposition to neuroinflammation later in life. However, acute intercurrent infections or diseases often exacerbate autoimmune disorders such as MS. So, the timing of this plasticity of the HPA system is crucial in altering the predisposition to inflammation later in life.

In this study, we gave injections of LPS to neonatal rats within the first week they were born and monitored the level of corticosterone in their urine. We found a significant correlation of LPS treatment with the level of urine corticosterone and suppression of EAE-induced neurological deficits. Mechanistically, high levels of corticosterone were associated with the rising numbers of apoptotic $CD4^+$ T cells in the peripheral blood. Our study provides additional evidence to support the idea that an "immunization" strategy may be a viable approach for development as therapeutics to MS.

EXPERIMENTAL PROCEDURES

Animals and EAE induction

All animal procedures were in accordance with the Care and Use of Laboratory Animals guidelines stipulated by the US National Institute of Health Guide (1996). We also made every effort to minimize animal suffering and to reduce the number of animals used. Sprague–Dawley rats were obtained from the Laboratory Animal Center of Wenzhou Medical College and housed under controlled conditions (14-h light/10-h dark cycle, with lights on at 7 AM, and the room temperature was maintained at 22±2 °C). Litters at postpartum day 3 (P3) were reduced to 10–12 pups and randomly separated into two groups: an LPS-treated group and a saline-treated group. After 8 weeks, each group was further separated into two groups: one group receiving spinal cord homogenate (SCH) to induce EAE (LPS-EAE/saline-EAE) and another group injected with complete Freund's adjuvant (CFA) (LPS-CFA/saline-CFA) as control group.

Specifically, at P3 and P5, the pups were injected i.p. with either 0.05 mg/kg LPS (LPS, serotype *E. coli* 055:B5, Sigma-Aldrich, USA) in 0.05 ml sterile saline or an equivalent volume of sterile saline. Rats were provided with food and water *ad libitum* after weaning. When the rats were 8 weeks old, EAE was induced as previously described (Wang et al., 2009). Briefly, SCH suspended in 50% saline (w/v) containing CFA with 10 mg/ml bacillus calmette-guerin at a total volume of 400 μl, was injected into the hind limb footpads subcutaneously. The rats were then injected i.p. with 0.1-ml pertussis vaccine suspension (Shanghai Institute of Biological Products, PR China) at 0 and 48 h postimmunization.

Evaluation of neurobehavioral deficits

The neurobehavioral deficits caused by EAE were assessed according to a previously published scale (Kono et al., 1988) with a modification, as we previously published (Wang et al., 2009): grade 0, no obvious movement difficulties; grade 1, tail paralysis; grade 2, paresis of hind legs; grade 3, complete paralysis of hind legs; grade 4, tetraplegia; and grade 5, moribund state or death.

Measurement of urine corticosterone

Rats were individually housed in metabolic cages to collect urine samples. They were allowed to acclimate to the metabolic cage for 7 days prior to urine collection (Brennan et al., 2000). The metabolic cages allowed free access to rat chow and water as well as allowing for daily urine collection through the floor of the cage into 50-ml centrifuge tubes. After centrifugation, the supernatant was frozen at −80 °C until analysis.

Urine samples were diluted 1:20 with assay buffer, and analyzed for corticosterone levels using an enzyme-linked immunosorbent assay (Assay Designs, ADI-901-097, USA) following exactly the manufacturer's recommended procedures. To determine urine creatine levels, an enzymatic kit produced by Dongou Reagent (PR China) was used in an automatic biochemistry analyzer (ARCHITECT C8000, USA), with the ratio of urine corticosterone levels calculated against urine creatine levels.

Histology and immunohistochemistry

The rats were anesthetized with 10% chloral hydrate (Sigma Chemical Co., St. Louis, MO, USA) followed by perfusion through the left ventricle with saline. After fixation with 4% paraformaldehyde, the spinal cord was removed, further postfixed for 12 h, and embedded in paraffin as we described previously (Hou et al., 2008; Wang et al., 2009). Paraffin sections of 5-μm thickness were cut and stained with hematoxylin-eosin (H&E) to determine the histopathology of the spinal cord. A rabbit anti-CD3 polyclonal antibody (Abcam, USA) was used at a 1:150 dilution to detect inflammatory T cell infiltration. The method for immunostaining was exactly as previously described (Hou et al., 2006; Wang et al., 2009). Briefly, after treatment with 0.03% H_2O_2 to inactivate endogenous peroxidase, antigen unmasking was achieved using a microwave. Nonspecific binding was then blocked with 10% fetal bovine serum (GIBCO, Australia), and the sections were incubated overnight with the rabbit anti-CD3 polyclonal antibody at 4 °C. After being washed with PBS, the sections were incubated with a biotinylated goat antirabbit antibody (1:100), which was visualized with 3-3'diaminobenzedine tetrahydrochloride using a VECTASTAIN avidin-biotin kit following manufacturer's recommendation (Vector Laboratories, USA).

The degree of EAE-induced histopathological changes in the spinal cord was evaluated in a double-blind manner using H&E-stained sections according to the criteria as previously published (Okuda et al., 1999): grade 0, no inflammatory cells; grade 1, cellular infiltration occurred only around blood vessels and meninges; grade 2, mild cellular infiltration in the parenchyma (1–10 cells/section); grade 3, moderate cellular infiltration in the parenchyma (11–100 cells/section); grade 4, severe cellular infiltration in the parenchyma ($>$100 cells/section). The numbers of CD3 immunopositive cells on every section from three randomly selected high-power fields (×400) were counted in a double-blinded manner. Results were calculated as the number of infiltrating cells per mm^2.

Flow cytometry

Cells were surface stained with fluorochrome-conjugated antibodies recognizing CD11c/b, CD4, and MHC II. Antibodies to CD4, CD25, and MHC II were purchased from eBioscience (San Diego, CA, USA). Antibody to CD11c/b was obtained from Invitrogen (USA) to determine antigen-presenting cell (APC). Annexin V was obtained from MultiSciences Biotech Co. (PR China). For $CD4^+$ T cell apoptosis analysis, peripheral blood was collected by cutting the tail of anesthetized rats. Cells from the spleen were isolated using homogenization according to previously published protocol (van den Brandt et al., 2004). Cells were harvested, washed with PBS, and stained for surface markers. Stained cells were analyzed on a BD FACSCanto flow cytometer using FACSDiva soft-

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