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The effect of electroacupuncture on T cell responses in rats with experimental autoimmune encephalitis

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ABSTRACT

Successive electroacupuncture (EA) stimulation on Zusanli ST36 acupoints of rats with experimental autoimmune encephalitis (EAE), which is an inflammatory disease mediated by autoreactive T cells, relieved disease severity, inhibited specific T cell proliferation and rebuilt the CD4+ T cell subset balance. In addition, EA-treated rats had significantly higher ACTH concentrations in vivo compared to untreated EAE rats. These results indicated that EA stimulation could relieve the severity of EAE by restoring balance to the Th1/Th2/Th17/Treg Th cell subset responses by stimulating the hypothalamus to increase ACTH secretion.

1. Introduction

Multiple sclerosis (MS) is a chronic, inflammatory disease mediated by autoreactive T cells characterized by multifocal damage to the myelin sheath of the central nervous system. Experimental autoimmune encephalomyelitis (EAE), which is commonly induced by immunizing susceptible strains of laboratory rodents with myelin proteins or peptides, is a useful model that has provided considerable insights into the pathogenesis of MS (Paterson, 1986; Dittel, 2008). A delicate balance exists between the different CD4+ Th cell subtypes and an imbalance, for example, between pro-inflammatory Th1 cells that secrete IFN-γ and IL-2 and Th2 cells that secrete anti-inflammatory cytokines like IL-4 and IL-10, can result in disease. In the case of MS, a Th1/Th2 imbalance results in myelin sheath destruction accompanied by episodes of neurological dysfunction (Bebo et al., 2001). Recently, a new Th subset defined as Th-17 cells has been shown to secret the highly pro-inflammatory cytokine IL-17 and studies have now revealed that Th-17 cells are the major pathogenic T cell subset associated with the progression of EAE (previously considered to be a Th1-mediated disease) (Komiyama et al., 2006; Suryani and Sutton, 2007). IL-17 is not only a micro-adjusting pro-inflammatory cytokine, but also can induce human fibroblasts to express intercellular adhesion molecule (ICAM-1), thereby to stimulate T-cell proliferation. Additionally, a subset of Th regulatory cells (Tregs) that can inhibit certain immune cell responses has also been identified and are believed to play a critical role in down-regulating inflammatory responses. In vitro, Tregs have the ability to inhibit proliferation and production of cytokines by responder (CD4+ CD25− and CD8+) T cells (Jonuleit et al., 2001; Thornton and Shevach, 1998) to polyclonal stimuli, as well as to down-modulate the responses of CD8+ T cells, NK cells and CD4+ cells to specific antigens (Dieckmann et al., 2001; Wing et al., 2003). TGF-β affects Treg T cell differentiation, down-regulates the function of Th1 and Th17 cells, and ameliorates autoimmune disease (Afzali et al., 2007).

Acupuncture originated in ancient China and is utilized as a clinical treatment for various diseases. In addition to the analgesic effects of acupuncture, an increasing number of studies have demonstrated that acupuncture can control autonomic nerve system (ANS) functions including blood pressure, the sphincter of Oddi and immune modulation (Chiu et al., 1997; Joos et al., 2000; Lee et al., 2001). Electroacupuncture (EA) is a form of acupuncture that electrically stimulates acupoints via the use of acupuncture needles. Acupuncture is currently flourishing in the US and Europe both as primary and adjunctive therapy for a variety of chronic conditions (Kavoussi and Ross, 2007).

Chinese medicine is based upon a philosophy of balance and harmony between the body and the universe. It believes a formless energy, called “chi” within the body that allows all the vital organs to maintain the body balance. If the invisible flow is disturbed, then a person will be in a state of illness. The objective of acupuncture is to stimulate the flow of chi to relieve any blockage in the body and thereby restore a person's balance with the universe. The purpose of the present study was to examine whether EA could provide protection against EAE in a rat model for MS in addition to assessing the effects of acupuncture on EAE CD4+ Th cell profiles.

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2. Materials and methods

2.1. Animals

Eight-week-old female Lewis rats were purchased from the Peking Vital River Laboratory Animal Ltd. (Peking, China). All rats used in these studies were bred and maintained in accordance with the guidelines for the Care and Use of Laboratory Animals published by the China National Institute of Health.

2.2. Antigens

The MBP_{68-86} (myelin basic protein) peptide (YGSLPQKSQRSQ-DENPV) was synthesized using solid phase techniques and purified by high performance liquid chromatography (HPLC) at Sangon Ltd. (Shanghai, China). A synthetic peptide corresponding to the 97–116 region of the rat AChRα subunit (DGDFAIVKFTKVLLDYTGHI) was synthesized by AC Scientific, Inc. (Xian, China).

2.3. EA induction and evaluation

Animals were divided into four treatment groups: (1) The complete Freund's adjuvant (CFA) (St. Louis, MO, USA) only group received CFA emulsified with an equal volume of phosphate buffered saline (PBS) in a final volume of 200 µl subcutaneously (CFA contained *Mycobacterium tuberculosis* strain R37RA at a concentration of 20 mg/ml; (2) the EAE group was immunized subcutaneously in the tail with 0.2 ml of 0.025 mg MBP_{68-86} emulsified in CFA; (3) the Zusanli acupoint (EA) immunization group that was immunized as group 2 but treated with EA; and (4) rats immunized as group 2 but receiving Non-acupoint (NA) therapy. The Zusanli acupoint (ST36) is located 5 mm below and lateral to the anterior tubercle of the tibia. Electrical stimulation was applied to the ST36 acupoint using two outlets via two needles. The needles (length 30.0 mm, diameter 0.20 mm) were inserted perpendicular to the skin, 5 mm apart and 5 mm to one side of the anterior tibial muscle. Rats in the NA group received electroacupuncture 5 mm below the Zusanli acupoint at a frequency of 1 Hz with a square waveform at a pulse duration of 0.5 mm. 3–5 V were applied at an intensity just below the threshold of muscle contraction. EA stimulation was applied for 30 min, started on the day of immunization and repeated each day for a period of 7, 14 and 21 d. Rats were scored for EAE as follows: 0, no disease; 1, piloerection; 2, loss in tail tonicity; 3, hind leg paralysis; 4, paraplegia, and 5, moribund or dead. Mean clinical scores at separate days and mean maximal scores were calculated by adding scores of individual rats and dividing by number of rats in each group.

2.4. Histopathology

Spinal cords from euthanized rats were removed, fixed in 4% paraformaldehyde and embedded in paraffin. Vibratome sections were stained with H&E or Luxol Fast Blue. The infiltration of inflammatory cells was evaluated in a blinded manner.

2.5. Lymphocyte cultures

Lymphocytes were obtained from the lymph nodes of EAE rats 7, 14 or 21 d post-immunization with MBP_{68-86}. Cells were washed 3 times in RPMI 1640 and then cultured in lymphocyte culture medium RPMI 1640 supplemented with 1% normal rat serum (Jackson ImmunoR-esearch, USA), 1% (v/v) minimum essential medium (MEM, Hyclone, Thermo Fisher Scientific, Waltham, MA), 2 mM glutamine (Sigma), 1 mM sodium pyruvate, 50 mM 2-ME (Amresco, Solon, OH), 100 U/ml penicillin, 100 mg/ml streptomycin and 10 mg/ml MBP_{68-86}. Lymphocytes were then adjusted to 2×10^6/ml.

2.6. Cell proliferation assays

Lymphocytes were resuspended at a final concentration of 2×10^5 cells/well and cultured in 96-well flat-bottomed microplate wells. After exposure to MBP_{68-86} (20 µg/ml), Concanavalin A (ConA, 5 µg/ml, Sigma), $AchR_{97-116}$ (10 µg/ml) or PBS for 72 h cell viability was measured using a CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) as described previously (Chang et al., 2006). Briefly, 10 µL of CCK-8 solution was added to each well, incubated for 1–4 h at 37 °C and the absorbance measured at 450 nm.

2.7. Cytokine detection

Cell culture supernatants were recovered at 72 h and frozen at −20 °C until needed for the cytokine detection assay. Measurement of IFN-γ, IL-4, TGF-β and IL-17 was performed using ELISA kits (BD Biosciences, San Jose, CA). Standard curves for each assay were generated using recombinant mouse cytokines and the concentration of cytokines in the cell supernatants was determined by interpolation from the respective standard curves.

2.8. Immunofluorescent staining for flow cytometry

Intracellular and extracellular cytokine detection was carried out by flow cytometry as described previously (Lohr et al., 2006). Briefly, to distinguish between the Th subsets, purified single-cell suspensions from 3 different groups were incubated for 5 h with Brefeldin A (1:1000 dilution, eBioscience Inc., San Diego, CA), an inhibitor of intracellular protein transport. T cells were first incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rat-CD4 (eBioscience Inc.) for 30 min at 4 °C. After fixation and permeabilization, intracytoplasmic staining was carried out using one of the following fluorescently-labeled antibodies (Abs): Phycoerythrin (PE)-conjugated anti-rat-IFN-γ (BD Biosciences), anti-rat-IL-4 (BD Biosciences), anti-Foxp3 (BD Biosciences) or rabbit-anti-rat-IL-17 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with a Cy3-conjugated anti-rabbit-IgG (Caltag Laboratories, Burlingame, CA) as the secondary antibody. The fixation and permeabilization kit used for flow cytometry was purchased from eBioscience (San Diego, CA). Samples were analyzed within 24 h with a BD FACScan (BD Biosciences) using Cell Quest software (BD Biosciences). Isotype matched, PE-and FITC-conjugated mAbs of irrelevant specificity were used as negative controls.

2.9. Determination of plasma and hypothalamus ACTH concentrations

Rats from the respective treatment groups were sacrificed 7, 14 and 21 d post-MBP_{68-86} immunization under ether anesthesia and blood from 5 rats/group was obtained by cardiac puncture and collected in heparinized tubes containing EDTA-2Na. Plasma was separated by centrifugation at 4 °C and stored at −80 °C until used. ACTH samples from the hypothalamus were extracted as described by Javadi et al. (2003) and stored at −80 °C until analyses were performed. Plasma and hypothalamus ACTH concentrations were determined using the ACTH ^{125}I RIA kit (Tianjin Hope Year Medical Products Co., Ltd., China). The ACTH assay sensitivities were 3.5 pg/ml.

2.10. Statistical analysis

Data are expressed as the mean ± standard error (SE) of 5–7 observations. Statistical analyses were performed using the SPSS (Statistical Package for Social Sciences, Chicago, IL) software. One-way analysis of variance (ANOVA) was used to determine statistical differences between groups. Clinical scores were analyzed using the non-parametric Kruskal–Wallis test. The level of significance was set as $p<0.05$.