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Exposure to 50-Hz electric fields on stress response initiated by infection with the protozoan parasite, *Toxoplasma gondii*, in mice

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ABSTRACT

The present study was aimed to examine whether a 50-Hz electric field (EF) affects biological stress responses. Male C57BL/6J mice were exposed to either 50-Hz EF (45 kV/m) or given a sham exposure for 30 minutes per day for 1, 2 or 4 weeks and then either mock inoculated or inoculated with the protozoan parasite, *Toxoplasma gondii* (*T. gondii*) PLK strain. Survival rate was calculated in mice infected with *T. gondii* after exposure to EF and the serum antibody levels to heat shock protein 60 (Hsp-60), and mRNA levels for Hsp-60, IFN-γ and IL-12p35 in mouse peripheral leucocytes were determined. As a result, no statistically significant difference was found in survival rate between mice exposed to EF and those in the sham exposure group. When mice were inoculated with *T. gondii*, plasma level of anti-Hsp-60 antibody increased gradually until day 16 after inoculation and remained at an elevated value until the end of the experimental period. On day 2 and 9 after inoculation, the increment in anti-Hsp-60 antibody level was significantly suppressed in mice exposed to EF as compared to those in the sham exposure group ($p<0.05$). Differences in changes in mRNA levels in Hsp-60, IFN-γ and IL-12p35 were not statistically significant. Taken together, our findings indicate that exposure to 50-Hz EF does not affect viability of mice infected with *T. gondii* but seems to slightly play inhibitory on the early phase of stress responses.

Key words: 50-Hz, electric field, Hsp-60, stress response, survival rate

INTRODUCTION

A number of studies have been conducted to determine whether exposure to electric fields (EF), magnetic fields (MF) or electromagnetic field (EMF) affects plasma levels of the hormone, corticosterone, a stress response indicator. Although some investigators have reported suppressive effects of EF on mitogenic and proliferative responses in the immune system, others did not support their conclusions (Murthy *et al.* 1995; Tremblay *et al.* 1996). Three types of reports exists up to date: 1) indicating that the stress marker levels are reduced (Hackman *et al.* 1981; Marino *et al.* 1977); 2) stress markers are not influenced (Free *et al.* 1981; Quinlan *et al.* 1985); and 3) stress markers show increment due to exposure to EF or MF (de Bruyn *et al.* 1994). Mice exposed to MF exhibited increased natural killer cell cytotoxic activity that was dependent in a complicated manner on the age of the mice at the commencement of exposure, field strength and the location of the laboratory from which the measurements were made (de Seze *et al.* 1993), while, no effects of EMF were found on natural killer and lymphokine activated killer activities and production of INF-γ, TNF-α, IL-2 and IL-10. Cytokine production in human peripheral blood mononuclear cells (Ikeda *et al.*

2003) and mice exposed to 60-Hz MFs intermittently for 21 weeks exhibited no changes in number or function of natural killer cells (McLean *et al.* 1991). In accordance with the results of the latter negative study, reliable- or dose-related- statistically significant alterations in immune function and host defense could not be demonstrated in mice exposed to 60-Hz MF (House *et al.* 1996). Intermittent exposure to a 50-Hz MF for up to 24 hours had no effect on the distribution of lymphocyte subpopulations in humans (Selmaoui *et al.* 1997). Thus, there remains controversy regarding the effects of EF on both the stress response and the immune system.

Recently, we reported that exposure to 50-Hz EF influences in biological stress responses including reduction in the plasma ACTH level increment induced by space restriction in rats or suppression of plasma lipid peroxide levels in rats treated with oxidising agent, 2,2'-azobis(2-aminopropane) dihydrochloride (Harakawa *et al.* 2005; Harakawa *et al.* 2004). Heat Shock Proteins (Hsps) are evolutionary highly conserved polypeptides that appear to be produced by many cells to preserve cellular functions under a variety of conditions of stress, including infection. Hsp has variety functions in immune system, such as the activation of T cell, stimulating autoimmune diseases, carrier of antigenic peptide, and trigger in the innate immune system. *Toxoplasma gondii* (*T. gondii*) is an obligate intracellular protozoan parasite that infects up to a third of the world's population (Dubey 1996). Hsp-60 was expressed in mice peripheral blood cell in the early stages of infection with the protozoan parasite, *T. gondii* (Nagasawa *et al.* 1992). IFN- γ has been shown to be effective against *T. gondii* infection (Suzuki *et al.* 1988). Hsp-60 affects the production of cytokine pattern, e.g., IFN- γ (Ausiello *et al.* 2006; Flohe *et al.* 2003). Exposure to MF is thought to elicit stress responses including expression of Hsps, especially Hsp-70, in human acute myeloid leukaemia cells (HL-60) (Tokalov *et al.* 2003). But, the effects of pure EF (MF-free) on Hsp-60 in immune responses have not been studied. In the present study, Hsp-60, IFN- γ and IL-12 production, which is enhanced by infection with *T. gondii*, were examined as the effects of EF on stress responses.

MATERIALS AND METHODS

Experimental animals

Male C57BL/6J mice were purchased at 8 weeks of age from CLEA Japan (Tokyo, Japan). All animals were housed in polycarbonate cages, and maintained in a specific pathogen-free environment in light-controlled (lights on from 07:00 to 19:00) and air-conditioned rooms (temperature: 24±1ºC, humidity: 50±10%). Animals had free access to standard laboratory chow (CE-2; CLEA Japan) and water except for the period of EF exposure. The animals were cared for and used in accordance with the Guiding Principles for the Care and Use of Research Animals circulated by Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan.

EF exposure system

The EF exposure system was composed of three major parts: a high-voltage transformer (Hakuju Institute for Health Science, Tokyo, Japan), a constant voltage unit (Tokyo Seiden, Tokyo, Japan) and EF exposure cages, which were described previously (Harakawa *et al.* 2004; Hori *et al.* 2005). Briefly, the exposure cages, which were designed for mice or smaller animals, were composed of a cylindrical plastic cage (diameter: 200 mm; height: 200 mm) with two electrodes made of stainless steel (600 x 600 mm) placed over and under the cylindrical cage. To form a 50-Hz sine waveform EF of 45 kV/m RMS intensity in

the cage, a stable alternating current (9 kV) was applied to the upper electrode. Experiments were carried out at normal room temperature (25±0.4ºC). To estimate the intensity of EF in each set for EF exposure, the voltage between the electrodes was measured using a Fluke 83 (Fluke Co. Ltd., WA, USA) and the voltage applied was set to 9 kV. Each exposure cage housed one mouse during each experimental session to avoid any imbalance in EF induced by housing two or more mice at the same time.

Parasite preparation

The PLK strain of *T. gondii* was grown in Vero cells. Confluent cell cultures (75 cm^2) were infected with 5 x 10^7 tachyzoites in minimal essential medium (Sigma, UK) supplemented with fetal calf serum (8% (v/v), Biological Industries, Israel). Just before infection, cells were broken by passage through a 27G needle, and parasites were purified through a Syringe-Driven filter unit (5 mm, Millipore, MA, USA).

Schedule for field exposure and infection of parasites

Forty mice were divided into two groups: group 1 (n=20) which were exposed to 50-Hz, 45 kV/m EF in the electric field cage for 30 minutes per day for 4 weeks; and group 2 (n=20) in which mice were not exposed to EF in the EF cage for 30 minutes per day for 4 weeks. After EF exposure, mice were inoculated with the parasites at one of two doses: 10^2 or 0 parasites. The sham control group was also divided into two groups and inoculated with parasites at the same doses. After inoculation of parasites, blood was taken from each mouse at 2, 9 and 16 days. Serum levels of antibody to Hsp-60 and the expression of Hsp-60, IFN-γ and IL-12p35 mRNA in the blood were measured. 180 µl of blood were taken from the tail vein with hematocrit capillary tubes. Aliquots of 60 µl of blood in the capillaries were centrifuged at 1,500 xg for 5 minutes at room temperature and serum was obtained and stored at –30 ºC. The remaining samples of 120 µl of blood were used for RNA extraction.

Detection of anti-Hsp-60 antibody

Ninety-six-well plates were coated with 0.8 µg/well of recombinant human Hsp-60 antigen, which was obtained from Calbiochem (MA, USA). After washing and blocking with 1% BSA-TBS, wells were incubated in 100 µl of serum samples diluted 1:80 with TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl). Anti-HSP60 mouse IgG (BD Biosciences, CA, USA) was used as a positive control. Binding of antibodies to Hsp-60 was measured using anti-mouse IgG-alkaline phosphatase conjugate (Caltag, CA, USA), anti-mouse IgM-alkaline phosphatase conjugate (Caltag) and 4-nitrophenyl phosphate (Boehringer Mannheim, Mannheim, Germany). The antibody level was measured as absorbance at 415 nm.

Quantitative real-time PCR

RNA was extracted from 60 µl of blood with a QIAamp RNA blood Mini Kit (Qiagen, Hilden, Germany) in accordance to the protocol provided from manufacture. To quantify expression of Hsp-60 and IL-12p35 mRNA, we used an Assays-on-DemandTM kit (Applied Biosystems, CA, USA) as primer sets and probes. A PDERS kit (Applied Biosystems) was used to quantify expression of IFN-γ mRNA. The expression of β-actin mRNA was used as an internal control, detected using an MGB-probe (ATC AAG ATC ATT GCT CCT C) (Applied Biosystems) and the sense primer beta-actin955F (GCT CTG GCT CCT AGC ACC AT) and antisense primer beta-actin1029R (GCC ACC GAT CCA CAC AGA GT) were used. To make standard curves of these mRNAs, male C57BL/6J mice were inoculated with *T. gondii* PLK strain. At 5 days after infection, the spleen was removed and the cells were incubated in 8% FCS MEM with Concanavalin A (2.5 µg/ml). Total RNA was extracted from the spleen cells using an Rneasy mini kit

(Qiagen) 2 days after incubation. Total RNA was used as a positive control. Real-time PCR was carried out using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, CA).

Survival rate in mice, which were exposed to EF and then were infected with parasite

In order to study effects in survival rate due to exposure to EF, forty mice were separated into four groups: 1) 10 mice were exposed to EF for 4 weeks; 2) 10 mice were not exposed to EF for the first 2 weeks and then exposed to EF for the last 2 weeks; 3) 10 mice were not exposed to EF for the first 3 weeks and then exposed to EF for the last 1 week; 4) 10 mice were not exposed to EF for 4 weeks. After EF exposure, mice were inoculated i.p. with $1x10^2$ tachyzoites of the PLK strain. As positive control, mice were inoculated i.p. with 1×10^5 tachyzoites of the PLK strain.

Statistical analyses

Analyses of levels of anti-Hsp-60 antibody were performed by repeated measures ANOVA. The analysis of mRNA expression was performed by Student's *t*-test. Statistical analyses of survival curves were performed by the log-rank test. A p value of 0.05 or less was considered significant. All computations for the statistical analyses were carried out using Prism Version 4.0c (GraphPad Software Inc., San Diego, CA).

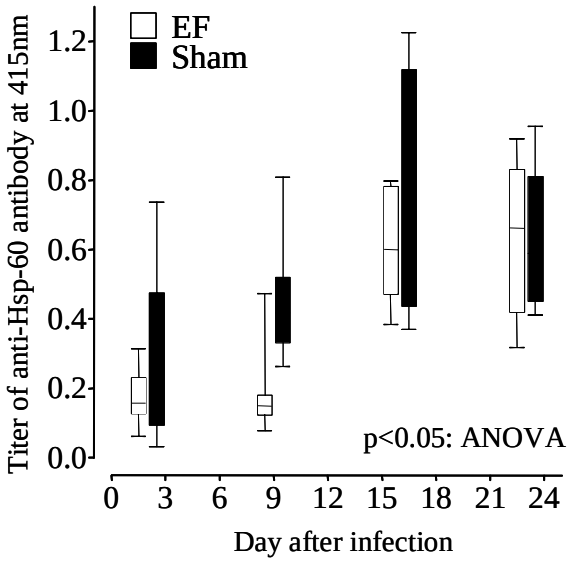

Figure 1: The effects of EF on anti-Hsp-60 antibody levels using mice infected with a dose of 10^2 *T. gondii*. Binding of IgG + IgM was measured using ELISA with mixed secondary antibody. Forty mice were divided into two groups: group 1 (n=20) which were exposed to 50-Hz, 45 kV/m EF in the electric field cage for 30 minutes per day for 4 weeks; and group 2 (n=20) in which mice were not exposed to EF in the EF cage for 30 minutes per day for 4 weeks. After EF exposure, mice were inoculated with the parasites at one of two doses: 10^2 or 0 parasites. The sham control group was also divided into two groups and inoculated with parasites at the same doses. For the parasite-infected mice, the antibody level was significantly lower in the EF-exposed group than in the sham control group ($p<0.05$: repeated measures ANOVA).

RESULTS

Hsp-60-antibody, IFN- γ and IL-12p35

The effects of EF on anti-Hsp-60-antibody level in mice infected with a dose of 10^2 *T. gondii* were outlined in figure 1. For the parasite-infected mice, the antibody level was significantly lower in the EF-exposed group than in the sham control group ($p<0.05$: repeated measures ANOVA). On days 9 after inoculation, the antibody level was significantly lower in the EF-exposed group than in the sham control group ($p<0.05$: Bonferroni post tests). In contrast, for non-infected mice, the anti-Hsp-60-antibody level did not show any changes in the EF and sham control groups. In the study utilized the real-time PCR method regarding to effects of EF on expression of Hsp-60, IFN- γ and IL-12p35 in mouse peripheral leucocytes, there were no significant differences in expression of these genes between the EF-exposed and the sham control mice.

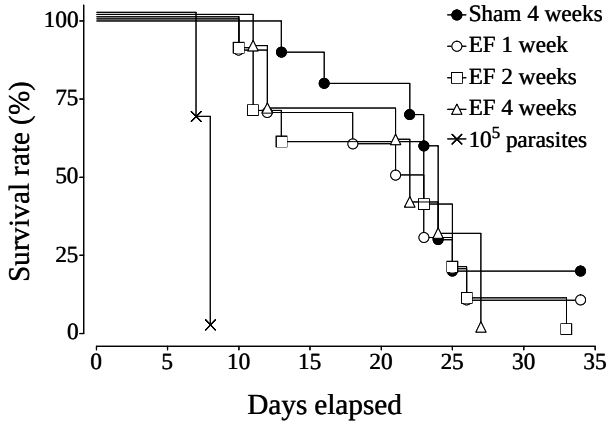

Figure 2: Effects of EF on survival rate of male C57BL/6J mice inoculated with 1 x 10^2 *Toxoplasma gondii* tachyzoites. Controls were inoculated with 1 x 10^5 tachyzoites. Forty mice were separated into four groups: exposed to 45 kV/m EF for 4 weeks, 2 weeks, or 1 week for 30 minutes per day and a sham control group. EF did not affect survival rate in any of these experimental groups.

Survival rate

Mice in the EF-exposed group were inoculated with a dose of 10^2 parasites of *T. gondii* and those in the sham control group were inoculated with 10^5 parasites. Follow-up observation was continued for 4 weeks. Exposure to EF was made for 1, 2 or 4 weeks. As a result, there were no significant differences in the effects of EF on survival of the three groups exposed for different periods ($p>0.05$: Log-rank test) (Figure 2).

Table 1. effects of EF on expression of Hsp-60, IFN-γ and IL-12p35 of male C57BL/6J mice inoculated with 1×10^2 *Toxoplasma gondii* tachyzoites using mouse peripheral leucocytes by the real-time PCR

	2 days after infection		
	EF(+) infected	EF(-) infected	EF(-) uninfected
HSP60	0.420± 0.600	0.056±0.049	0.052±0.034
IFN-gamma	0.00075±0.00017	0.00046±0.00036	0.00102±0.00002
IL-12p35	1.30±1.80	0.71±0.77	0.36±0.03

	9 days after infection		
	EF(+) infected	EF(-) infected	EF(-) uninfected
HSP60	0.15± 0.04	0.85±0.79	0.32±0.12
IFN-gamma	0.0065±0.0003	0.0098±0.0120	0.00003±0.00006
IL-12p35	0.032±0.030	0.310±0.360	0.319±0.056

	16 days after infection		
	EF(+) infected	EF(-) infected	EF(-) uninfected
HSP60	0.082±0.075	0.120±0.091	0.442±0.54
IFN-gamma	0.0025±0.0020	0.0039±0.0030	0±0
IL-12p35	0.058±0.048	0.190±0.180	0.291±0.068

Each value was expressed as the mean value and ± standard deviation.

DISCUSSION

The anti-Hsp-60 antibody levels in mice infected with *T. gondii* showed markedly increments and such the increments were inhibited due to exposure to EF ($p<0.05$ by repeated measures ANOVA; $p<0.05$ on day 9 by Bonferroni post tests in figure 1). In mock-infected mice, anti-Hsp-60 antibody level did not show any changes during test period (data not shown), suggesting that exposure to EF would depressively act in the production of anti-Hsp-60 antibody. Cross-reactive epitopes are shown to serve as autoimmune targets (Perschinka *et al.* 2003). Hsp-60 is expressed on plasma membrane surface in stressed human endothelial cells (Pfister *et al.* 2005). Poor expression of self-Hsp peptides in the thymus could allow T cells specific for self-Hsp to an evade selection, and an excess expression of conserved epitopes from pathogen-derived Hsp could break tolerance and activate immune reactions against self-Hsp determinants, in the periphery (Zugel *et al.* 1999). Thus, suppression in anti-Hsp-60 antibody level in this study suggest participant with some of autoimmune diseases. In order to elucidate whether EF depresses the anti-Hsp-60 antibody titer through the inhibition of Hsp-60 expression on membrane surface, further studies will be required.

The present study showed that the mean expression levels of Hsp-60, IL-12p35 and IFN-γ in the EF-exposed group tended to be higher at 2 days after infection with *T. gondii* in comparison with the sham control group, whereas these levels were all lower after 9 and 16 days (Table 1), suggesting a possibility of relation of exposure to EF with Th1 response. Th1 response is defined as the increment of Th1 cell population, which secrete IL-2, IL-12 and IFN-γ (Sudo *et al.* 2004). Another earlier study demonstrated in

mice that an infection with toxoplasma induced an increment of Th1 response within 1 week after infection and then subsequently Th2 response (Lee *et al.* 1999). Exposure to EF is seemed to participate in the immune responses containing Th1 and Th2 responses. It is likely that EF affects the cytokine pattern through its effects on Hsp-60.

It was demonstrated that EF has no effects on survival rate in mice infected with *T. gondii* (Figure 2). In addition, both hematocrit value and body weight were also not influenced by an exposure to EF (data not shown) in mice. These suggest that the biological effects of EF are likely to be mild if there is. The present study indicates that exposure to EF of 50-Hz slightly inhibit the production of anti-Hsp-60 antibody, which is related at the earlier phase in stress response, and that EF may participates in the pattern of cytokine production.

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