Abstract. The purpose of this study was to evaluate the modifying effects of electroacupuncture (EA) and moxibustion (Mox) through the communication networks of the neuro-immune system using the two-step bacterial stimulation method. When EA or Mox treatment was implemented immediately following injection of the Streptococcus pyogenes preparation OK-432, a significant suppression of the tumor necrosis factor (TNF)α production by the peritoneal macrophages induced by lipopolysaccharide (LPS 1 µg/ml) was observed. When the stimulation with EA or Mox was reduced in volume, the degree of suppression decreased correspondingly. Naloxone antagonized to some extent the suppression of TNFα induced by EA, but did not compete with the suppression of TNFα release induced by Mox. These results suggest that activated macrophages are an important target of the immuno-suppressive effects of EA and Mox and that μ-opioid receptor-mediated mechanisms are responsible, to some extent, for the suppressive effect of EA, although Mox may not be dependent on these mechanisms.

Acupuncture (electroacupuncture; EA) and moxibustion (Mox) have come into use as alternative treatments in the management of diseases. EA is a mechanical somato-sensory stimulatory technique, while Mox is a thermal somato-sensory stimulatory technique. Health care professionals employing acupuncture and moxibustion use appropriate acupuncture points in treating various diseases, which include excessive inflammation and abnormalities of the immune system. Many people have experienced acupuncture which is becoming, however slowly, accepted by Western practising physicians. Furthermore, acupuncture experiments have also been performed in animals. It is thought that when EA and Mox are applied to specific body areas, for instance the fourth governor vessel (GV4) acupuncture point, which is on the post meridian line, between the second and third lumbar processes, they may modulate immunological functions and maintain mechanisms by which the body defends itself against disease. The authors previously reported that, in tests conducted on mice, when EA was administered at the treatment point corresponding to the GV4 point in humans, the delayed type of hypersensitivity to 2,4,6-trinitro-chlorobenzene (TNCB) was either enhanced or suppressed, depending on the timing of treatment administration (1). We also reported that EA, administered a single time at an acupuncture point corresponding to the GV4 point in humans, immediately prior to the challenge of TNCB, suppressed ear swelling that had been induced by the delayed type of hypersensitivity, through a central opioidergic mechanism (2, 3). In another report, we indicated that a single administration of Mox at an acupuncture point corresponding to the GV4 point in mice affected the production/release of lipopolysaccharide (LPS)-induced cytokines such as the cytotoxic factor (TNF) and interferon in mouse sera (4).

In this study, we demonstrated that EA and Mox modulated the reactivity against LPS of macrophages that were infiltrated following treatment with the Streptococcus pyogenes preparation OK-432 (5), modifying the ability of the macrophages to produce cytokines. We also showed that the effects of EA and Mox were similar, but that they had different mechanisms of action.

Materials and Methods

Mice. Specific pathogen-free, male C57BL/6 mice were obtained from Nihon SLC Co., Ltd. (Hamamatsu, Japan). The mice were eight weeks old and were kept in plastic cages, given food and water ad libitum and maintained in a temperature-controlled room (23±1°C) with an LD of 12:12.

Electroacupuncture (EA) treatment. Each mouse was held in a mouse holder (KN-330, Natsume Seisakusho Co., Ltd., Tokyo, Japan) and EA with low-frequency mechanical stimulation (1 Hz,
500 µs or 50 µs in duration) was applied at a point on the posterior meridian line, between the second and third lumbar processes, anatomically equivalent to the GV4 acupuncture point in humans. A wave 500 µs in duration was generated from a pulse generator (NC-707, Kimura Med. Ins. Co., Ltd., Tokyo, Japan) and applied to each EA-15 group for 15 minutes.

For the weak pulse electroacupuncture (PA)-15 group, a wave 50 µs in duration was generated from a pulse generator (PG 701, Suzuka Iyoki, Tokyo, Japan) and applied for 15 minutes. In order to standardize conditions, the mice were normally treated with EA or PA between 1:00 and 3:00 p.m.

Moxibustion (Mox) treatment. Thermal stimulation using moxa (moxa grass products) was also applied to a point anatomically equivalent to the GV4 acupuncture point in humans. Small pieces of moxa, turned in the shape of a cone by hand (moxa cones), were placed on the skin at the acupuncture point and the moxa was burned. One cone comprising two mg of moxa was applied each time for a total of five times on each mouse in the Mox-10 group, while a cone of two mg of moxa was applied once on each mouse in the Mox-2 group. In order to standardize conditions, the mice were normally treated with moxa between 1:00 and 3:00 p.m.

Animal priming and collection of peritoneal cells. To induce in vivo priming (primary immunization) and in vitro induction, we used the two-step stimulation method (6). As the priming agent, we used a preparation consisting of an inactivated and lyophilized low-virulence strain of Streptococcus pyogenes (OK-432, provided by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan). Peritoneal cells were primed through an i.p. injection at a dose of 0.1 mg of dry streptococci for each mouse, suspended in an isotonic sodium chloride solution (Otsuka Pharmaceuticals, Tokyo, Japan). Immediately following immunization, EA or Mox was administered a single time. After 48 hours, the peritoneal cells that had infiltrated into the peritoneal cavity were harvested from the cavity using ice-cold Hanks’ Balanced Salt Solution (HBSS). After being washed twice with cold HBSS, the cells were suspended in RPMI 1640 (NIPRO, Osaka, Japan) supplemented with heat-inactivated 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 10 mM Hepes. This medium was used for the experiment. The washed abdominal cavity cells from each mouse were counted using a blood cell counter, and the survival rate was determined using the trypan blue method of exclusion.

Isolation of macrophages and induction of cytokine production. Macrophages were suspended in the medium at a density of 1x10^6/ml. The cells were plated in the flat-bottomed wells (100 µl/well) of 96-well culture plates (Iwaki Glass, Chiba, Japan) and cultured in a humidified, 5% CO₂ atmosphere at 37°C. Cells were left to adhere for 2 hours, and non-adherent cells were removed by washing. Using latex minute-particle phagocytosis and Diff Quik stain, it was found that more than 70% of the remaining monolayer consisted of macrophages. After washing three times with HBSS, the macrophage monolayers were cultivated for 1 to 48 hours in medium with 1 µg/ml of lipopolysaccharide (LPS, Escherichia coli 026 : B6, Sigma Chemical Co., St. Louis, MO, USA).

Following incubation, the culture plates were centrifuged and the cell-free culture supernatants were used to determine the cytokines that had been released. These samples were sterilized using a filter and were stored at -30°C until assayed.

Measurement of tumor necrosis factor (TNFa). The TNFα level of cell-free supernatants was measured using an enzyme-linked immunosorbent assay kit (Mouse ELISA TNFα, Endogen, Inc., MA, USA).

Systemic injection of naloxone. Five minutes prior to treatment with EA and Mox, each mouse was injected i.p. with naloxone hydrochloride (Sigma Chemical Co.), in the form of an isotonic sodium chloride solution (Otsuka Medicine, Tokyo, Japan) at a dose of 10mg / kg.

Statistical analysis. Data were expressed as the mean±standard deviation (SD). The statistical significance of the difference between two groups was determined using the Student’s t-test. The statistical significance of the differences among three groups was determined using an analysis of variance followed by Fisher’s LSD test. The results were considered significantly different if p<0.05.

Results

Cytological analysis of peritoneal cell suspensions. Cytological analysis was performed on peritoneal cell suspensions. The number of peritoneal cells in the control group was 9.8±2.0 x 10^6 cells/mouse (n=27), and in the EA (EA-15, PA-15) group was 10.3±1.6 x 10^6 (n=21). For the Mox (Mox-10, Mox-2) group, the number of peritoneal cells was 9.2±0.5 x 10^6 (n=21). There were no significant differences among the three groups.

The mean percentages of macrophages in the peritoneal cells were 73±2.0 for the control group, 74±3.0 for the EA group and 71±3.0% for the Mox group. The infiltration of...
cells into the peritoneal cavity during the immunization process with the i.p. injection of the streptococcal preparation OK-432 was not affected by EA or Mox.

Effects of EA and Mox on TNFα applied at an acupuncture point corresponding to GV4. It was not possible to detect the TNFα level over time in the macrophage culture supernatant when LPS was not added. When 1 μg/ml of LPS was added, however, it became possible to detect TNFα approximately one hour later, with a maximum level of 3,861 ± 213 pg/10⁶ macrophages being detected 4 hours after LPS exposure (Figure 1).

The time-course of the level of TNFα induced by LPS in the EA and the Mox groups approximated that of the level induced by the LPS in the control group. The EA and Mox treatments did not affect the time-course of the TNFα level (Figure 2).

The TNFα level at 4 hours in the EA-15 group, after treatment with a continuous wave duration level of EA500 μs, was about 52% that of the control group (Figures 2, 3). It was also found that the TNFα level of the group treated with a continuous wave duration of EA 50 μs (PA-15) was suppressed, but it was significantly less than that of the EA-15 group (Figure 3). The wave duration of the EA treatment closely correlated with the suppression of the macrophage function.

Following Mox (Mox-10), the TNFα level at 4 hours was about 55% of the control group (Figures 2, 3). While in Mox-2 treatment, the TNFα level at 4 hours was higher (about 80% of the control) than that of the Mox-10 group (Figure 3). It is shown that the dose of moxa used in the Mox treatment closely correlated to the level of suppression of the macrophage function.

Effect of naloxone on suppression of TNFα levels induced by EA or Mox. The suppression of the TNFα production at 4 hours following EA-15 treatment was incompletely antagonized by prior administration of a single i.p. injection of naloxone (Figure 4). We also observed suppression of the TNFα level change 4 hours after Mox treatment, but there was no antagonism in response to prior injection of a single dose of naloxone (Figure 4).

Discussion

The effects of EA and Mox with respect to the production of TNFα in response to immunized peritoneal macrophages were investigated. The method used for this study was the two-step bacterial (OK-432 and LPS) stimulation method. The *Streptococcus pyogenes* detoxicated pharmaceutical preparation OK-432 is an active Biological Response Modifier (BRM) (5).
*I.p.* injections of OK-432 brought about an inflammatory microenvironment in the abdominal cavity. Cross-talk of the neuro-endocrine-immune system is supposed to occur involving the peritoneal macrophages (7). Circulating monocytes infiltrated the abdominal cavity in response to the *i.p.* injection of OK-432. The peritoneal macrophages were activated by OK-432 over a period of 24~72 hours. The abdominal cavity was infiltrated first by neutrophils, then by macrophages, and then by lymphocytes. We collected peritoneal cells 48 hours subsequent to the injection, which was the point at which macrophage infiltration reached its peak.

Next, macrophages were treated *in vitro* with LPS to induce a more strongly activated state in which pro-inflammatory immune mediators such as the cytokine, TNFα, were released in extremely large amounts. This experiment is appropriate for testing macrophage function, since macrophages begin to synthesize cytokines such as TNFα only when immunized macrophages are induced with LPS *in vitro* (5).

In this experiment, we applied EA or Mox immediately after *i.p.* injection of OK-432. Subsequently, LPS-induced TNFα was measured to see whether or not the immunization process caused any inhibitory effects. If the EA or Mox modified the cross-talk of the neuro-endocrine-immune system, one would expect a change in the cytokine production in the peritoneal macrophages.

No effects were observed after EA or Mox on the number of cells infiltrating the abdominal cavity and on the percentage of macrophages. The percentage of macrophages was also confirmed using flow cytometric analysis (data not shown). Therefore, we assumed that the change in the TNFα production by the macrophages in the mice treated with EA and Mox, using a point equivalent to GV4 in humans as a stimulus point, was not caused by a change in the number of macrophages, but rather was in response to a change in the TNFα production.

In our experiment, it was shown that the LPS-induced TNFα produced by the macrophages of mice treated with EA-15 and Mox-10 decreased to about 50% of that of the corresponding control group. We then considered the question of how the immunization process of the macrophages was affected by EA and Mox treatment. We did not examine the differences between various acupuncture points in this study; rather, we compared the degree of stimulus at the acupuncture point, which was the equivalent to the GV4 point in humans. When EA was used, the wave duration was 500 μs (EA-15) and 50 μs (PA-15). With Mox, we used 10 mg (Mox-10) and 2 mg (Mox-2) of moxa.

In recent years, a greater understanding has been gained of the theory of the communication networks of the neuro-endocrine-immune system and how peripheral stimulatory procedures, such as EA and Mox, affect those communication networks. The hypothesis of the action...
mechanisms of EA and Mox have been grouped and demonstrated under the collective name of "acupuncture". Acupuncture (EA and Mox) is being used to treat various conditions apart from pain control (8-10).

Various types of information taken in by living organisms are controlled by the hypothalamus, and pass through the hypothalamic-pituitary-adrenal (HPA) axis and hypothalamic-autonomic nervous system (HANS) axis to the endocrine and neurologic tissues to maintain homeostasis (11, 12). Endorphinergic, monoaminergic, cholinergic transmitters and endocrinological factors are involved in the course of that transmission route (10).

It still has not been clarified how the production of various inflammatory factors by the macrophages is controlled. With respect to TNF-α production, when LPS binds to CD14 and the Toll-like receptor 4 (TLR-4) of a macrophage, a signal is transmitted to the nucleus and then a transcription factor NF-κB is activated. It is thought that the activated NFκB induces gene expression so that inflammatory cytokines such as TNF-α are produced (13, 14).

There are various reports concerning endogenous factors that control TNF-α production. These theories are classified into four categories, in accordance with the hypothesis of the action mechanisms of EA and Mox, as follows;

1. **HPA axis and glucocorticoids**: Glucocorticoids down-regulate the immune system and repress TNF-α genetic transcription activation (15). In experiments that we have conducted on mice, a 200% increase in adrenocorticotropic hormone (ACTH) was observed in the plasma 15 minutes after the EA-15 treatment. Additionally, there was a 250% increase in ACTH 30 to 60 minutes after Mox-10 treatment (unpublished data).

We earlier reported the results of a study comparing EA and morphine in delayed type hypersensitivity in mice, showing that the HPA axis became involved based on the timing of the biological response of the suppression of delayed type hypersensitivity resulting from EA-15 treatment (3, 16). Consequently, there may be a possibility that the production of glucocorticoids through the HPA axis may be strongly enhanced by EA and Mox.

2. **HPAS axis and catecholamines**: Numerous reports have been published concerning the endogenous catecholamines that control TNF-α production. It is thought that the sympathetic nervous system modulates immune response. It has been reported that adrenaline regulates the TNF-α production in sepsis (17). LPS-induced TNF-α production in vivo is said to be mediated by the release of endogenous catecholamines. In that process, β-adrenoceptors may act as the suppressor, while α-adrenoceptors may act as the enhancer (18). The ligands of β-adrenoceptors increase intracellular cAMP levels, while the ligands of α-2 adrenoceptors decrease them. Therefore, it is conceivable that the mechanism by which catecholamines are produced through the HANS axis and the β-adrenergic mechanisms may be strongly enhanced by EA and Mox.

3. **Parasympathetic system and acetylcholine**: The autonomic nervous system, through the vagus nerve, can also inhibit the release of macrophage TNF. Acetylcholine released from vagus nerve endings is bound to the nicotinic acetylcholine receptor α7 subunit on the surface of macrophage and inhibits the release of TNF (19). Consequently, it is possible that the mechanism by which acetylcholine is released through the vagus nerve, as well as the mechanism activating the nicotinic acetylcholine receptor, may be strongly influenced by EA and Mox.

4. **Endogenous opioids**: Numerous reports have been published stating that endogenous opioids (endorphins) and exogenous opioidergic medicines work as biological response modifiers (20). In this study, we demonstrated that suppression of the TNF-α level following EA-15 treatment was antagonized to some extent by naloxone, although incompletely. It has also previously been reported that the EA-evoked delayed-type hypersensitivity suppression was blocked in a dose-dependent manner by systemic pretreatment with naloxone, indicating that opioid receptor-mediated mechanisms are involved in this immune response (3). Therefore, the mechanism by which endogenous opioids are produced, as well as the opioid receptor-mediated mechanisms, may be strongly enhanced by EA.

In summary, we investigated the process by which macrophage activation, using the streptococcal preparation OK-432 as the immunizing agent, was modified after EA or Mox treatment by examining the LPS-induced TNF-α production in vitro. The neuro-endocrine-immune system has many feedback responses in the body as a whole. EA and Mox are thought to modify these feedback responses.

For both EA and Mox, when treatment was administered at an acupuncture point corresponding to the GV4 point in humans, macrophages were influenced in a way that the LPS reactivity was suppressed by 50%. The production of TNF-α is thought to be suppressed when the degree of macrophage activation is suppressed by 50%. However, this does not necessarily mean that the body’s defenses against infection are suppressed completely, as they are, for instance, when an anti-TNF-α antibody reagent is used for treatment. Therefore, side-effects such as increased susceptibility to infection are not likely to occur.

With respect to morbid conditions in which chronic TNF-α production is seen, meaning diseases such as rheumatoid arthritis and auto-allergic diseases, treatment using EA or Mox is a means of achieving normalization of the homeostasis maintenance mechanism (21, 22). Further research is necessary to elucidate the mechanisms of action of EA and Mox.
References


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