Acupuncture Stimulation Enhances Splenic Natural Killer Cell Cytotoxicity in Rats

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Abstract: Acupuncture stimulation of the anterior tibial muscle, which is equivalent to Tsu-sanli point (S36) stimulation and which induces acupuncture analgesia, enhanced YAC-1 cell lytic activity of the splenic natural killer cells (NK activity) from pentobarbital anaesthetized Wistar rats. On the other hand, abdominal muscle stimulation, which does not induce acupuncture analgesia, did not affect NK activity. The serum from rats treated with acupuncture stimulation (acupuncture-stimulated rats) also enhanced the NK activity of other intact rats. The reinforcement effect of serum remained after heat inactivation (56°C, 30 min). The serum from acupuncture-stimulated rats also had more potent complement activity than that from control rats. These results indicate the following considerations: 1) the Tsu-sanli point may be one of the specific points modulating immune activity, 2) this immune modulation system may share a common nervous pathway with the acupuncture analgesia-producing system, 3) acupuncture stimulation may modify NK activity through unknown heat stable humoral factors as well as the nervous system, and 4) acupuncture stimulation may activate the complement system. [Japanese Journal of Physiology, 46, 131–136, 1996]

Key words: acupuncture stimulation, splenic natural killer cell, Tsu-sanli, complement.

Interaction between the nervous system and the endocrine system is well known, however it has been thought that the nervous system and immune system are independent of one another. Recently, reciprocal communication between these systems has gained attention [1–3]. It is reported that the psychological state affects the survival time of cancer patients [4], natural killer (NK) or T lymphocyte activity during or after bereavement [5–7] and T lymphocyte activity of medical students who received examination [8]. Acupuncture stimulation as well as moxibustion are known to modify immune activity [9–11]. But the mechanism of the interaction between the nervous and immune systems has not been completely clarified. We had been studying the central mechanism of acupuncture analgesia [12] and we are investigating the relationship between the acupuncture analgesia-producing system and immune function which includes the activity of B lymphocytes, T lymphocytes, macrophages and NK cells. The possibility that the central nervous system may affect the immune system through the autonomic nervous system [13, 14] as well as the humoral system has been reported. In the present study, we investigate the effects of acupuncture stimulation applied to the anterior tibial muscle (Tsu-sanli point stimulation), which is known to induce acupuncture analgesia [15], and the effects of abdominal muscle stimulation, which does not cause acupuncture analgesia [15], on cellular immune activity. Cellular immune activity was estimated based on the antitumor activity of the splenic natural killer cell which plays numerous important roles in the immune system.

MATERIALS AND METHODS

Forty-six male Wistar rats weighing 300–600 g from 8 to 12 weeks old were used. Generally, the control and...
test animals were from one litter to minimize individual variation.

**Methods of acupuncture stimulation.** Electrical stimulation with a 1 Hz biphasic wave, using an acupuncture stimulator (BT701, Shanghai, China), was applied to the tibial muscle for stimulation of the Tsu-sanli point and to an abdominal muscle for stimulation of other points. The acupuncture needles (0.22 mm in diameter) were inserted perpendicular to the muscle surface to the opposite side of the muscle. The intensity of acupuncture stimulation was adjusted to cause minimum muscle contraction through two needles about 5 mm apart in the muscle under restriction in an acrylic rectangular box. The intensity was varied from 5 to 20 V with a duration of 1 ms. The details of the stimulation method and the acrylic box were reported in our previous paper [16]. Two hours of acupuncture stimulation (from 3:00 p.m. to 5:00 p.m.) was applied to the rats for 3 d. The control animals were also restricted in acrylic rectangular box but without acupuncture stimulation under the same condition.

**Measurement of the antitumor activity of the splenic natural killer cells.** Preparation of the effector cells: The rats were anesthetized by pentobarbital sodium (i.p. 50 mg/kg). After collection of 10 ml of blood from the portal vein, the spleen was isolated between 10:00 a.m. and 12:00 a.m. on the day after completion of 3 d of acupuncture stimulation. The dissected spleens were disrupted into a single cell suspension using stainless steel tweezers. Suspension without debris was centrifuged at 400×g for 5 min. After centrifugation, the cell pellets were treated with 5 ml of 0.83% Tris-ammonium chloride solution (pH = 7.65) for 2-5 min to lyse red blood cells. The cells were washed 3 times in phosphate-buffered saline (PBS) and then suspended in tissue culture medium (RPMI-1640 with NaHCO₃ and L-glutamine, Nipro, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, Grand Island, NY). In order to deplete the macrophages (adherent cells), 15 ml of the cell suspension (5-10×10⁶/ml) in a plastic Petri dish (10 cm in diameter) was incubated for 1 h at 37°C. The resulting suspensions were centrifuged at 400×g for 5 min and adjusted to 10⁷ cells/ml in RPMI-1640 for assay.

Preparation of target cells: YAC-1, an in vitro cell line derived from a Moloney virus-induced lymphoma in A/Sn mice, was used as the target cell. Before lytic assays, YAC-1 cells were adjusted to 3-5×10⁶/0.9 ml in RPMI-1640 containing 10% FBS and added to 0.1 ml Na₂⁵²⁵CrO₄ (37 MBq/ml, Amersham Japan, Tokyo, Japan). The cells were incubated for 1-1.5 h at 37°C in a humidified incubator with 5% CO₂ and were washed three times in PBS. In order to minimize the spontaneous release of ⁵¹Cr from labeled YAC-1 cells, the cells were suspended in RPMI-1640 containing 10% FBS and incubated for 1 h at 37°C in a humidified incubator with 5% CO₂ and washed again three times in PBS. The labeled YAC-1 cells were adjusted to 2×10⁶/ml in RPMI-1640 for assay.

**Cytotoxic assay.** NK cell activity was measured in a standard 4-h chromium-5¹ release assay [17]. One hundred microliters of spleen cell suspension and 50 μl of 2×10⁵ ⁵¹Cr-labeled cells were cocultured in 96-well V-bottom microculture plates (A/S Nunc, Kamstrup, Denmark) at 100:1, 50:1, or 25:1 effector to target (E:T) ratios. Seventeen microliters of FBS and/or rat serum was added according to the experimental design. Plates were incubated for 4 h at 37°C with 5% CO₂, centrifuged at 400×g for 5 min, and 100 μl of supernatant was collected for measurement with a gamma scintillation counter. Percent specific lysis was calculated by the following formula.

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\text{Percent specific lysis} = \left[ \frac{(\text{experimental } ⁵¹\text{Cr release}) - \text{spontaneous } ⁵¹\text{Cr release}}{(\text{maximum } ⁵¹\text{Cr release}) - \text{spontaneous } ⁵¹\text{Cr release}} \right] \times 100
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To determine the spontaneous release, aliquots of 100 μl of supernatant from the wells which contained non-labeled YAC-1 cells were incubated with ⁵¹Cr-labeled target cell at ratio of 100:1, 50:1, or 25:1. The maximum release was determined by incubating target cells with 1 N HCl. Spontaneous release ranged from 8 to 26% of the maximum ⁵¹Cr release.

**Preparation of serum.** Before isolation of the spleen, 10 ml of the blood was collected from the portal vein under pentobarbital anaesthesia and was placed in a test tube containing blood coagulant (VENOJECT II, TERUMO Co., Tokyo). The test tubes were centrifuged at 1,870×g for 20 min and the supernatant was collected. The serum was filtered to eliminate bacterial contamination. In some experiments, the serum was heated at 56°C for 30 min to inactivate the complement system.

**Measurement of the complement activity.** CH50 (50% hemolytic unit of complement) value was measured as the complement activity by a commercial laboratory (SRL Co., Tokyo).

**Statistics.** Data are expressed as mean±SE. The data were statistically analyzed using ANOVA followed by Fisher PLSD as post-hoc test (Super
ANOVA, Abacus Concepts, Inc., Berkeley, CA). \( p < 0.05 \) was considered significant.

**RESULTS**

The effect of acupuncture stimulation applied to the tibial and abdominal muscle on the splenic NK cell cytotoxicity

The cytotoxic activity of the splenic NK cells cultured with FBS was increased after 3 d of electroacupuncture stimulation to the tibial muscle (Tsusunai point stimulation) compared to the NK activity of non-stimulated control. Percent specific lysis of acupuncture stimulated rats were 19.2±2.1, 30.6±1.8, 50.3±1.9 and the values of the non-stimulated rats were 17.2±2.3, 26.3±1.9, 42.3±2.6 (mean±standard error) at an E: T ratio of 25:1, 50:1, and 100:1, respectively. Statistical significance \( (p<0.05) \) was observed at an E: T ratio of 100:1 (Fig. 1). However, acupuncture stimulation applied to the abdominal muscle did not show a significant effect on the splenic NK cell activity at any E: T ratio. The percent specific lysis of abdominal-stimulated rats was 20.5±5.8, 28.0±7.6, 40.4±6.3 at each E: T ratio, respectively (Fig. 1).

The effect of serum collected from tibial muscle-stimulated rats on the lytic activity of NK cells prepared from other non-stimulated rat spleen

The serum (at a concentration of 10%) prepared from tibial muscle (S36)-stimulated rats revealed a higher NK cell activating effect than the serum (at a concentration of 10%) from abdominal muscle-stimulated rats at each E: T ratio \( (p<0.05) \) and compared to the serum (at a concentration of 10%) from non-stimulated rats at an E: T ratio of 100:1 \( (p<0.05) \) (Fig. 2). The percent specific lysis of tibial muscle-stimulated rats was 50.0±6.9, 51.4±5.0, 60.4±3.3, the values of abdominal muscle-stimulated rats were 37.3±3.3, 41.3±2.9, 51.4±2.0 and the values of non-stimulated control rats were 41.5±2.7, 44.5±1.9, 52.6±1 at E: T ratios of 25:1, 50:1, and 100:1, respectively (Fig. 2).

However, the serum itself showed YAC-1 cell lytic activity under NK cell free condition \( (E: T \text{ ratio } 0:1) \) (Fig. 2) and tibial muscle stimulation enhanced the lytic activity against YAC-1 cells compared with abdominal muscle stimulation \( (p<0.05) \). The percent lytic activities of the 10% serum from tibial muscle-stimulated, non-stimulated, and abdominal muscle-stimulated rats were 47.2±8.0, 38.5±2.4, and 33.3±4.1, respectively.

![Graph showing effects of acupuncture stimulation on NK cell activity](image)

**Fig. 1.** Effects of acupuncture stimulation applied to the tibial and abdominal muscle on NK activity. NK activity of tibial stimulated rats was enhanced compared with the NK activity of control rats at an E: T ratio of 100:1. * Indicates \( p<0.05 \). Abdominal stimulation did not show a significant effect on NK activity at any E: T ratio.

**Fig. 2.** Effect of the serum (at a concentration of 10%) taken from tibial (S36) or abdominal acupuncture and non-acupuncture control rats on the splenic NK activity of non-stimulated rats. The serum from the tibial acupuncture group significantly enhanced the splenic NK activity of other non-stimulated rats compared with the serum from the abdominal acupuncture group at each E: T ratio (* indicates \( p<0.05 \) between the tibial acupuncture and abdominal acupuncture groups) and with the serum of the control group at E: T = 100:1 (* indicates \( p<0.05 \) between the tibial and non-stimulated groups). Even non-stimulated rat serum itself had YAC-1 cell lytic activity under NK cell-free conditions at an E: T ratio of 0:1. The serum from tibial acupuncture rats showed significantly higher lytic activity on YAC-1 cells than the serum from abdominal acupuncture rats.

The elevation of YAC-1 cell lytic activity of the serum collected from tibial muscle-stimulated rats and abolishment of the lytic activity by heat inactivation

Even the serum from non-stimulated Wistar rats showed lytic activity on YAC-1 cells, which originated from mice, under an NK cell-free condition (open square in Fig. 3) and the intensity of lytic activity was dependent on the serum concentration. The YAC-1 cell lytic activity of the serum collected after tibial muscle stimulation (open circle in Fig. 3) was elevated compared with the lytic activities of control serum. The percent lytic activity of serum from tibial muscle-stimulated rats was 0, 3.5±1.6, 12.7±2.5, 25.9±5.6, 44.2±7.0, and 46.2±7.0, the values of the serum from non-stimulated rats were 0, 4.3±1.0, 7.8±2.8, 15.3±1.1, 28.8±5.5, and 33.7±4.5, and at rat serum concentrations of 0, 2, 4, 8, and 10%, respectively. The YAC-1 cell lytic activity of the serum itself from both acupuncture-stimulated and non-stimulated rats was abolished by heating at 56°C for 30 min (solid circle and square in Fig. 3). The percent lytic activities of the heat-inactivated serum from tibial-stimulated rats were 0, 3.3±1.2, 3.9±1.6, 4.5±0.7, 5.6±0.5, and 5.5±0.2, and the values of heat-inactivated serum from non-stimulated rats were 0, 4.5±0.8, 2.1±1.0, 2.8±1.2, 2.9±1.3, 3.5±1.9 at each serum concentration, respectively.

Complement activity of the serum from acupuncture-stimulated and non-stimulated rats

CH50 was measured as an indicator of complement activity. The CH50 value of the serum from acupuncture-stimulated rats (25.1±0.6 U/ml) was higher than that of the serum from non-stimulated rats (20.2±1.6 U/ml) (Fig. 4).

**Fig. 4.** Effect of tibial (S36) acupuncture stimulation on serum complement activity estimated by CH50 value. The serum CH50 value increased significantly by acupuncture stimulation compared with control serum (* indicates p<0.05).

![Graph showing % specific lysis vs. % rat-serum concentration](image1)

**Fig. 5.** Effect of heat-inactivated rat serum (56°C for 30 min) taken from tibial (S36) acupuncture and control rats on splenic NK cell activity of other non-stimulated rats at an E:T ratio of 100:1. The final concentration of serum in medium is adjusted to 10% by supplementation of heat-inactivated FBS. The serum from acupuncture-stimulated rats showed less of an inhibitory effect than the serum from non-stimulated rats (* indicates p<0.05 by two-way ANOVA).
The effect of heated serum from acupuncture-stimulated rats on the NK activity of other rats

The heated serum from both acupuncture-stimulated and non-stimulated rats diminished the NK cell activity in a concentration-dependent manner at an E:T ratio of 100:1 (Fig. 5). However, the NK cell activity was higher in the medium containing the serum from acupuncture-stimulated rats than from non-stimulated rats (p<0.05 by two-way ANOVA) (Fig. 5). The cytotoxic activity of NK cells cultured with serum from acupuncture-stimulated rats was 52.3, 46.1±1.4, 38.1±2.9, 34.8±0.9, 34.7±2.3, and 32.5±1.3, and from non-stimulated rats was 52.3, 42.4±2.4, 32.9±2.1, 31.0±0.35, 29.2±4.7, 26.9±4.5 at a rat serum concentration of 0, 2, 4, 6, 8, and 10%, respectively.

DISCUSSION

In traditional Chinese medicine, numerous acupuncture points are known in humans and in animals. Stimulation of those points is also known to cause many biological effects according to the point stimulated. The present study revealed that tibial muscle stimulation, which is equivalent to Tsu-sanli stimulation and which is known to induce acupuncture analgesia [15], enhanced NK cell cytotoxicity. However, abdominal muscle stimulation, which does not cause acupuncture analgesia [15], did not affect NK cell cytotoxicity. These results indicate that Tsu-sanli may be one of the specific points which modulate immune activity as well as an analgesic effect. Furthermore, these observations indicate that the immune modulating system involves a common pathway with the acupuncture analgesia-producing system. We already reported that the afferent pathway from the tibial muscle is connected with the acupuncture analgesia-producing pathway via the dorsolateral funiculus in the spinal cord and the dorsal part of the periaqueductal centralgray but abdominal muscle did not connect with that pathway [15]. Two mechanisms may be considered to regulate NK cell activity. One mechanism is mediated by the sympathetic nerve which depresses NK activity [13, 14]. The present observation supported this possibility, since acupuncture stimulation to the Tsu-sanli point affected the tumor cell (YAC-1 cell) lytic activity of splenic NK cells cultured with FBS medium without rat serum, whereas acupuncture stimulation enhanced NK activity. Acupuncture stimulation may inhibit the splenic sympathetic nerve activity, or part of the splenic sympathetic nerve may activate NK activity in the spleen. Another mechanism is mediated by a humoral factor, since the serum from acupuncture-stimulated rats also elevated the apparent activity of NK cells from a separate non-stimulated rat spleen.

Regarding a sympathetic mechanism, we observed that splenic sympathetic denervation abolishes the enhancing effect of acupuncture stimulation on the splenic NK cell activity (unpublished data).

In relation to a humoral mechanism, the serum from acupuncture-stimulated rats increased the $^{51}$Cr release by NK cell cytotoxicity in an E:T ratio-dependent manner; however, the $^{51}$Cr release above the spontaneous basal level at an E:T ratio of 0:1 remained (Fig. 2). This observation showed the possibility that the serum itself might lyse YAC-1 cells under the NK cell-free condition and that augmentation of lysis of YAC-1 cells by serum from an acupuncture-stimulated rat might be due to only the lytic activity of the serum itself, not to NK activity. Therefore, we investigated the lytic activity of rat serum on YAC-1 cells in an NK cell-free condition and the effect of heat-inactivated serum on NK activity. According to the results, rat serum itself lysed YAC-1 cells in a concentration-dependent manner. To determine the factor in rat serum which had lytic activity on YAC-1 cells, the activity of complement, which was considered to be a candidate, was measured. The complement activity (CH50 value) of the serum from acupuncture-stimulated rats was found to be higher than that of the serum from non-stimulated animals. The possibility that YAC-1 cell lytic factor in rat serum might be complement was also supported by the result that heat-inactivation inhibits this activity of the serum, since complement is heat-unstable. Other effects of heat-inactivated serum were studied and the following considerations were revealed.

The enhancing effect of acupuncture stimulation on complement and NK activity were considered to be independent of one another, since after heat inactivation, which completely abolishes the serum complement activity, the serum from both acupuncture-stimulated and non-stimulated rats had a rather inhibitory effect on NK cell cytotoxicity in a concentration-dependent manner (Fig. 5). These data indicate that the serum may contain an intrinsic heat-stable inhibitory factor against NK cells from other individuals, however the inhibitory factor is unknown at the present time. Further, acupuncture stimulation attenuated the inhibitory effect of serum on NK activity. This loss of inhibition suggests the following two possibilities to explain the enhancement of NK activity by acupuncture stimulation: 1) a decrease in the unknown heat-stable inhibitory factor, and 2) an increase in another
heat-stable stimulating factor including cytokines. However, the possibility remains that heat-inactivated serum from acupuncture-stimulated rats might affect the sensitivity of target cells to NK cells.

In conclusion, Tsu-sanli stimulation, which induces acupuncture analgesia, enhanced NK activity possibly through both a nervous and a humoral mechanism. On the other hand, abdominal muscle (another acupuncture point) stimulation, which does not cause acupuncture analgesia, did not affect NK activity. Independent of the effect on NK activity, Tsu-sanli stimulation enhanced complement activity but abdominal stimulation did not. Both complement and NK cells are known to be activated in the early stage of immune responses. The fact that acupuncture stimulation to the Tsu-sanli point enhanced these early immune responses indicates the usefulness of Tsu-sanli stimulation to activate the immune system.

There are many reports indicating that noxious stimulation or stress diminishes NK cell activity; however, reports of enhanced NK cell activity are few. To elucidate the central mechanisms which enhance NK cell activity, further studies on the relationship of the acupuncture analgesia-producing central mechanism and the unknown humoral factors are in progress.

REFERENCES
