Enhancement of Splenic Interferon-γ, Interleukin-2, and NK Cytotoxicity by S36 Acupoint Acupuncture in F344 Rats

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Abstract: The effect of Tsusanli acupuncture point (S36 acupoint) stimulation on splenic natural killer (NK) cytotoxicity was examined in Fischer 344 (F344) rats. Electro-acupuncture stimulation (voltage intensity, 1 to 5 V; duration, 1 ms; frequency, 1 Hz) was applied to bilateral S36 acupoints once a day (1 h) for 3 d. NK cytotoxicity was measured by the standard 4-h 51Cr release assay. Successive acupuncture treatment for 3 d significantly enhanced splenic NK cytotoxicity (p<0.001) on the first day after final treatment as compared to that of the control. However, similar stimulation to abdominal muscle did not influence splenic NK cytotoxicity. We also examined endogenous cytokine activities in aqueous spleen extracts prepared from acupunctured and control rats. The extracts from rats acupunctured at the S36 acupoint contained high levels of interleukin (IL)-2 and interferon (IFN)-γ as compared to those of abdominal muscle acupunctured and non-acupunctured control rats (p<0.01). Furthermore, a significant positive correlation (p<0.01) was observed between the levels of each cytokine tested and splenic NK cytotoxicity. The same positive correlation was also observed between the levels of IL-2 and IFN-γ (p<0.01). These observations indicate that electro-acupuncture stimulation of the S36 acupoint enhances splenic NK cytotoxicity and that IL-2 and IFN-γ may function, at least in part, in the regulation of NK cell activity in this system. [Japanese Journal of Physiology, 47, 173–178, 1997]

Key words: acupuncture, Tsusanli (S36) point, natural killer cytotoxicity, interferon-γ, interleukin-2.

Acupuncture is well known as one of the traditional Chinese medical treatments and is used for health maintenance throughout the world [1-4]. In recent years, it has been reported that acupuncture stimulation applied to a specific location on the human body, such as the Tsusanli acupuncture point (S36 acupoint), favorably modulates the immune function of HBsAg carrier and decreases the HBsAg-positive rates [5]. Combined acupuncture and moxibustion at supplementary acupoints inhibits the development of cancer cells and results in the prolonged survival of cancer patients [6, 7].

There is an established concept that NK cells are important as the first line of host defense and as one of the final effector cells against certain tumors, viruses, and infections [8, 9]. Several cytokines have been shown to affect NK cell proliferation or cytolytic activity. Of these, IL-2 and IFN-γ have been the most extensively studied. They have been proven to augment the cytolytic activity of NK cells to attach to tumor cells and viruses and kill these organisms [10, 11]. These reports suggest that acupuncture enhances NK cell activity and results in favorable modification of the clinical conditions of the patients described above.

Our previous report [12] and unpublished data revealed that successive acupuncture treatment applied to the S36 acupoint (but not to the abdominal muscle) for 3 d enhanced splenic NK cytotoxicity in Wistar rats, peaking (p<0.01) on the first day after final treatment and gradually declined thereafter. However, the mechanism of acupuncture on NK cell activity is un-
known [12]. This study was designed to examine the possible mechanism of acupuncture on NK cell activity in an inbred strain of F344 rats.

MATERIALS AND METHODS

Rats. Inbred F344 rats were purchased from Charles River Japan Inc. (Atsugi, Japan). They were all male and 8 to 10 weeks of age at the start of the experiment. They were housed individually in stainless cages at our animal facilities (12 h dark/12 h light cycle, 25±2°C, 55±10% humidity) and given food and water ad libitum.

Acupuncture at S36 acupoint and abdominal muscle. Electro-acupuncture stimulation was applied to the S36 acupoint and abdominal muscle as described previously [12]. Briefly, two sterilized acupuncture needles (length, 3.8 cm; diameter, 0.22 mm; TCM Supply Co. Ltd., Yokohama, Japan) were inserted perpendicularly about 5 mm into the anterior tibial muscle at the S36 point and the external oblique abdominal muscle. Electrical stimulation pulse with voltage ranging from 1 to 5 V, duration of 1 ms, and frequency of 1 Hz delivered from an acupuncture stimulator (Model G6805, Shanghai Medical Equip. Co. Ltd., Shanghai, China) was applied by two outlets through the two needles. The intensity of electrical stimulation was determined to be the minimum voltage to cause moderate muscle contraction. Electro-acupuncture stimulation at the S36 acupoint was applied to the left S36 acupoint for 1 h, followed by one 1 h stimulation to the right acupoint in the same rat. Electro-acupuncture stimulation at the abdominal muscle was applied bilaterally for 2 h. The stimulation protocol was repeated each day for three consecutive days on rats under restriction in acrylic rectangular boxes.

Reagents. RPMI-1640 (No.87-630; Nipro, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium) was used throughout this study. The following reagents were obtained from the companies as indicated: Pentobarbital sodium for injection (Abbott Lab., North Chicago, IL, USA); 3H-thymidine (specific activity: 2.92 TBq/mmol, Amersham, Buckinghamshire, UK); 51Cr (sodium chromate, 1 mCi/ml, Amersham); recombinant rat interferon-γ (Genzyme Corp., Cambridge, MA, USA); and rat natural IL-2 (Immuno-Biol. Institute, Fujioka, Gunma, Japan).

Experimental design. Electro-acupuncture stimulation was carried out between 3:00 P.M. and 5:00 P.M. for 3 d. Non-acupunctured control rats were restrained in acrylic rectangular boxes with no special treatment. On the first day after the final treatment, rats were anesthetized by intraperitoneal injection with 50 mg/kg pentobarbital sodium. The spleens were removed aseptically, divided into two portions and weighed. One portion was used to measure splenic NK cytotoxicity and the remainder was stored at −80°C until used for cytokine assay.

NK cytotoxicity assay. Splenic NK cytotoxicity was measured in a standard 4-h 51Cr release assay as described previously [12]. Briefly, spleens were dissociated into single-cell suspensions in 10 ml of complete medium. After allowing red blood cells and adherent cells to settle out, the effector cell suspensions were washed twice in phosphate-buffered saline (PBS) and resuspended in complete medium at appropriate concentrations. Radiolabeled YAC-1 murine lymphoma cells were used as target cells. Mixtures of 100 μl of appropriate concentrations of effector cells and 50 μl of 51Cr-labeled target cells (2×105 cells/ml) were co-cultured in 96-well V-bottomed microtiter plates (Nunc, Kamstrup, Denmark) at various ratios of effector and target cells (ranging from 12.5:1 to 100:1). Plates were incubated for 4 h at 37°C with 5% CO2. After centrifugation at 400×g for 5 min, 100 μl of supernatant was harvested from each well and 51Cr release was determined using a gamma scintillation counter (Packard Instrument Company Inc., Downers Grove, IL, USA). Aliquots of 100 μl of supernatant from wells in which only radiolabeled YAC-1 cells were incubated in either complete medium or in 1 M HCl served to determine spontaneous and maximum release. Spontaneous lysis ranged from 8 to 18% of maximum release. The percent specific lysis was calculated by the following formula for a mean of triplicate wells:

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\text{Percent specific lysis} = \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}]}{\times 100}
\]

The results of the NK cytotoxicity are expressed in lytic units (LU). One LU is defined as the number of effector cells required for 50% specific target lysis (determined from dose-response curve of individual animal with r>0.95), and results are expressed as number of LU per 107 effector cells [13].

Preparation of aqueous extract of spleen (extract). To prepare the extract, frozen spleen tissues were dissolved, weighed, immersed in 500 μl cold RPMI 1640 medium, and homogenized by a glass tissue homogenizer in an ice-cold water bath. The homogenates were then centrifuged at 8,000×g
for 1 h at 4°C and the supernatants were collected, sterilized by passing through a 0.22 μm filter (Millipore, Bedford, MA, USA), and stored at −80°C until use.

**Cytokine assays.**

*IL-2 assay.* IL-2 in extracts was analyzed according to the ability to support the growth of IL-2-dependent T-cell line (CTLL-2 cell line) [14]. Briefly, CTLL-2 cells were washed 3 times with complete medium and adjusted to 5×10^5 cells/ml. The cells (100 μl) were distributed into microtiter plates with 100 μl of a serial 2-fold dilution of the extracts. Cultures were incubated for 24 h and 0.5 μCi of 3H-thymidine was added to each well 6 h before harvest. 3H-thymidine incorporation was measured with a liquid scintillation counter (Canberra Packard International S.A., Zurich, Switzerland). IL-2 activities were calculated by probit analysis using natural rat IL-2 and expressed as mean units per g of spleen tissue (U/g) of duplicate assays.

*IFN-γ assay.* IFN-γ in extracts were measured by the Mouse IFN-γ ELISA Test Kit (Genzyme). This kit employs antibodies raised against mouse IFN-γ cross-reacting with rat IFN-γ [15]. The assay was performed according to the manufacturer’s recommended procedure. The ELISA was run in duplicate, and the level of IFN-γ was calculated by recombiant rat IFN-γ. The results were expressed as mean pg per g of spleen tissue (pg/g).

**Statistical analysis.** Data are presented as mean±standard error of mean (±SEM). Statistical analysis was performed with Statview ver.4.11 software (Abacus Concepts Inc., Berkeley, CA, USA) on a Macintosh Quadra 840 AV computer. The curve for determining LU in each experiment was calculated from the dose-response determinations with 4 different effector: target ratios, by simple linear regression. One-way analysis of variance (ANOVA) for factorial models (difference between experimental groups and control) was used to test the differences of splenic NK cytotoxicity and levels of cytokine. Significance was assessed by Fisher’s protected least significant difference (PLSD) test. NK cytotoxicity and cytokine were also examined by correlation and simple linear regression analysis.

**RESULTS**

The effect of acupuncture applied to the S36 acupoint on splenic NK cytotoxicity

The present experiments were designed to examine splenic NK cytotoxicity affected by acupuncture applied to the S36 acupoint in F344 rats. NK cells were prepared from rats on the first day after final treatment and cultured with target cells at E:T ratios of 100:1, 50:1, 25:1, and 12.5:1. The experiments were repeated five times with similar results. The data expressed in Fig.1 represents the mean±SEM of combined LU data from all rats. The splenic NK cytotoxicity of the S36 acupoint acupunctured group (17.7±0.5 LU/10^7 effector cells) was significantly higher (p<0.001) than that of the abdominal muscle acupunctured group (13.4±0.9 LU/10^7 effector cells) and the non-acupunctured control group (13.7±0.6 LU/10^7 effector cells). In contrast, there was no difference between the abdominal muscle acupunctured group and non-acupunctured control group.

The effect of acupuncture applied to the S36 acupoint on IL-2 and IFN-γ levels of splenic aqueous extracts

Since T-cell cytokines IL-2 and IFN-γ are generally believed to be responsible for the enhancement of NK cytotoxicity, this experiment was carried out to examine whether or not acupuncture at the S36 acupoint enhances T-cell cytokine production and results in an increase in NK cytotoxicity. To do this, extracts were prepared from acupunctured and control F344 rats on the first day after final treatment and endogenous cytokine activities were examined. As shown in Table 1,

<table>
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<th>Table 1. Effect of acupuncture on the IL-2 and IFN-γ levels of splenic aqueous extracts in F344 rats</th>
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<td>Group</td>
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<tr>
<td>S36 acupoint acupunctured</td>
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<td>Abdominal muscle acupunctured</td>
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<td>Non-acupunctured control</td>
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Extracts of spleen were taken from acupunctured and control rats. The levels of IL-2 and IFN-γ in these extracts were detected. Data presented here are mean±SEM of three of four different experiments which gave reproducible results. One-way ANOVA followed by Fisher’s PLSD test revealed a significant enhancement of acupuncture at the S36 acupoint on IL-2 level (F(2, 30)=10.6, p<0.001) as well as IFN-γ level (F(2, 30)=12.2, p<0.001). ** p<0.01 for S36 acupoint acupunctured group vs. abdominal muscle acupunctured group. *** p<0.001 for S36 acupoint acupunctured group vs. abdominal muscle acupunctured group and non-acupunctured control group.
the IL-2 level in the extracts of S36 acupoint acupunctured rats (31.5±2.3 U/g) was significantly higher than that of abdominal muscle acupunctured rats (18.5±3.3 U/g, p<0.001) and non-acupuncture control rats (21.3±1.1 U/g, p<0.001). The level of IFN-γ in S36 acupoint acupunctured rats (617±39 pg/g) also showed a significantly higher level than that of abdominal muscle acupunctured rats (460±37 pg/g, p<0.01) and non-acupuncture control rats (419±19 pg/g, p<0.001). However, there was no difference between the abdominal muscle acupunctured and non-acupuncture control rats.

To determine whether the increase in splenic NK cytotoxicity after acupuncture treatment at the S36 acupoint was related to the increase in the levels of IL-2 and IFN-γ, the splenic NK cytotoxicity was plotted against the IL-2 and IFN-γ production for individual rats. As shown in Fig. 2, the shift from splenic NK cytotoxicity correlated not only with the shift in the IL-2 level (simple linear regression, r=0.62, p<0.01) but also with the increase in IFN-γ production (simple linear regression, r=0.49 p<0.01). In addition, as shown in Fig. 3, the increase in IFN-γ production was accompanied by an increase in the IL-2 level (simple linear regression, r=0.53, p<0.01).

**DISCUSSION**

Traditional Chinese acupuncture, especially at the S36 acupoint, is used as one type of supplementary therapy for the treatment of acute and chronic diseases such as viral infectious disease and cancer in China [1]. Several mechanisms have been proposed to explain the therapeutic effect of acupuncture. These include: 1) regulation of the endocrine control system in the body (hormone regulation); 2) regulation of nervous control system (neurotransmitter regulation); and 3) increase in function of the immune system. However, the mechanism(s) by which acupuncture favorably modifies the clinical conditions of these diseases is not well known [16]. The present results clearly show that electro-acupuncture stimulation of the bilateral S36 acupoint once a day (1 h) for 3 d significantly increased splenic NK cytotoxicity as compared to that of abdominal muscle stimulated and non-stimulated control rats (Fig. 1). It is also shown that acupuncture at the S36 acupoint enhanced the levels of IL-2 and IFN-γ in the spleen (Table 1). There are significantly positive correlations between the levels of cytokines and their splenic NK cytotoxicity (Figs. 2, 3).

IL-2 is primarily released by activated helper T cells and acts as the central role in the regulation of NK cell activity [11]. IFN-γ is released not only by helper T cells but also by IL-2-activated NK cells and serves as one of the important factors in the up-regu-

![Fig. 1. Effect of acupuncture on splenic NK cytotoxicity in F344 rats.](image1)

![Fig. 2. Correlation of the splenic NK cytotoxicity with two cytokine levels in individual S36 acupoint acupunctured (●; n=13), abdominal muscle acupunctured (○; n=6), and non-acupuncture control (△; n=14) rats. A: The splenic NK cytotoxicity plotted against the IL-2 level (r=0.62, p=0.001 by simple linear regression analysis). B: The splenic NK cytotoxicity plotted against the IFN-γ production (r=0.49, p=0.004 by linear regression analysis).](image2)
Correlation of the production of IFN-γ and IL-2 in the extracts of individual acupunctured and control rats. Results presented are the production of IFN-γ plotted against IL-2 level (r=0.53, p=0.002 by simple linear regression analysis).

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