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Effect of Ninjin-Youei-To on Th1/Th2 Type Cytokine Production in Different Mouse Strains

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Abstract: Ninjin-Youei-To (NYT; Ren-Shen-Yang-Rong-Tang in Chinese) is a traditional herbal formula, which is widely used in Japan, Korea and China to modulate physiological immunity. The effects of oral administration of NYT on cytokine production from splenocytes were investigated in both C57BL/6 and BALB/c mice in which Th1 and Th2 were dominant, respectively. Splenocytes from C57BL/6 and BALB/c mice, which took NYT orally for four weeks, were cultured with anti-mouse CD3 mAb, and the supernatant was examined for cytokine production using enzyme-linked immunosorbent assay (ELISA). Administration of NYT to C57BL/6 mice, increased the production of interleukin-4 (IL-4) significantly, and slightly decreased interferon-γ (IFN-γ) production from splenocytes. In contrast, the same treatment significantly increased IFN-γ secretion from splenocytes of BALB/c mice. No remarkable changes of IL-12 production from splenocytes were observed in either strain of mice. These results suggest that oral administration of NYT ameliorates the excessive inclination of Th1 and Th2 type cytokine production, and NYT may provide a beneficial effects for the treatment of diseases caused by a skewed Th1-Th2 balance in the immune system.

Keywords: Ninjin-Youei-To, IFN-γ; IL-4; Th1-Th2 Balance; Biological Response Modifier.

Introduction

Traditional herbal medicines (Japanese name: Kampo) are widely used for the treatment of many kinds of acute and chronic diseases in East Asia. Ninjin-Youei-To (NYT; Ren-Shen-Yang-Rong-Tang in Chinese) is a traditional herbal medicine which is a hot water extract of 12 different kinds of herbs. Clinically, NYT has been used to treat patients with reduced physical strength, cold constitution, anemia and anorexia. Recently, various biological

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activities of NYT have been reported, namely cytokine induction (Okamura *et al.*, 1991), augmentation of the host resistance to infection (Yonekura *et al.*, 1992; Miura *et al.*, 1992), anti-inflammatory effect (Aoki *et al.*, 1994) and therapeutic effect on autoimmune animal models (Zhou *et al.*, 1994; Nakai *et al.*, 1993; Harigai *et al.*, 1995). These studies, taken together, suggested that one of the pharmacological effects of NYT might be applied by immunomodulation.

Murine and human $CD4^+$ helper T cells can be subdivided into Th1 and Th2 subsets based on their profile of cytokine production (Mosman and Sad, 1996). Th1 cells produce interferon-γ (IFN-γ), tumor necrosis factor-β (TNF-β), interleukin-2 (IL-2) and stimulate the cellular immune response. In contrast, Th2 cells produce IL-4, -5, -6, -10 and -13 and stimulate the humoral immunity. Furthermore, evidence suggests that the Th1 and Th2 types of immune response are reciprocally regulated *in vivo* (Heinzel *et al.*, 1989; Romagnani, 1991). It has also been suggested that many diseases are partially caused by a skewed Th1 and Th2 cytokine balance (O'Garra and Murphy, 1995; Liblau *et al.*, 1995). For example, increase in Th2 type cytokine production is observed in patients with systemic lupus erythematosus (SLE) or asthma (Richaud-Patin *et al.*, 1995; Humbert *et al.*, 1999). Conversely, Th1 cells mediate inflammatory diseases such as graft versus host disease (GVHD) (Blazar *et al.*, 1997). It has also been demonstrated that this Th1/Th2 balance is genetically regulated according to the strains such as BALB/c mice or C57BL/6 mice (Schulte *et al.*, 1997).

The purpose of this study was to observe the change of the Th1 and Th2 type cytokine production *in vivo* using NYT, and these are different according to genetically controlled immune constitution. We compared the effects of NYT on IFN-γ and IL-4 productions which are the major Th1 and Th2 type cytokines, respectively, between the different mouse strains. The production of cytokines was different according to the genetic immune background.

Materials and Methods

Drugs

Medicinal plants (crude drugs) used for the preparation of Ninjin-Youei-To are listed in Table 1. Angelicae Radix, Hoelen, Rehmanniae Radix, Ginseng Radix, Astragari Radix, Paeoniae Radix and Schisandrae Fructus were purchased from Uchida Wakan-Yaku Co. Ltd. (Tokyo, Japan). Atractyloides Lanceae Rhizoma, Cinnamomi Cortex, Polygarae Radix and Glycyrrhizae Radix were obtained from Tochimoto Tenkaido Co. Ltd. (Osaka). Auratii Nobilis Pericarpium was obtained from Tsumura & Co. Ltd. (Tokyo, Japan). The Astragali Radix used is classified into extra high-grade in the Japanese market, and the quality of other crude drugs is controlled by the Japanese Pharmacopoeia 13th Edition. NYT used in this study was prepared according to the prescription book of the Oriental Medicine Research Center of the Kitasato Institute. The NYT extract was prepared as follows: A mixture of 12 crude drugs was decocted with 600 ml of boiling water for 40 minutes to half volume. The extracted solution was centrifuged at 6000 rpm for 20 minutes, and then the supernatant was filtered and frozen for stock.

Table 1. Composition of Ninjin-Youei-To (NYT)

Medicinal Plants	Amount (g)
Angelicae Radix	4.0
Atractyloides Lanceae Rhizoma	4.0
Hoelen	4.0
Rehmanniae Radix	4.0
Ginseng Radix	3.0
Cinnamomi Cortex	2.5
Astragari Radix	2.0
Auratii Nobilis Pericarpium	2.0
Paeoniae Radix	2.0
Polygarae Radix	2.0
Glycyrrhizae Radix	1.0
Schisandrae Fructus	1.0

Animals

Six-to seven-week-old male BALB/c mice and C57BL/6 mice were purchased from Japan SLC Co. Ltd. (Shizuoka, Japan). The animals were maintained in specific pathogen free conditions and housed with a lighting schedule (12 hours of light and 12 hours of darkness) in a controlled temperature (22 ± 1°C). Mice were administered NYT orally with drinking water for four weeks. The control mice were provided tap water alone. The average dose of NYT was about 20-fold per kg body weight corresponding to humans, according to the water consumption.

Reagent and Chemicals

RPMI-1640 medium was supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). Purified hamster anti-mouse CD3e monoclonal antibody (CD3e mAb, 145-2C11: purchased from Pharmingen, CA, USA) and lipopolysaccharide (LPS, from *Escherichia coli* serotype 0127:B8: purchased from Sigma, St Louis, MO, USA) were used for splenocytes stimulation. Enzyme-linked immunosorbent assay (ELISA) kit were used for IL-4, IFN-γ (Amersham International PLC, Buckinghamshire, UK) and IL-12 (Biosource International CA, USA) measurement.

Preparation of Splenocytes

At autopsy, the spleens were immediately removed and pressed with slide glasses in phosphate buffered saline PBS (–). The cell suspension was passed through a #200 stainless steel sieve. Red blood cells were dissolved by 0.83% Tris-NH_4Cl hemolysis buffer and splenocytes were washed three times with PBS (–).

Measurement of Cytokine Production

Splenocytes (5×10^6/ml) were suspended in RPMI-1640 complete medium and stimulated

by purified hamster anti-mouse CD3e mAb (1 μg/ml) for IL-4 or IFN-γ measurement, or stimulated by LPS (10 μg/ml) for IL-12 measurement, in 24-well culture plate (FALCON 3097 24-well plate; Becton Dickinson). After 42 hours of incubation at 37°C, the supernatant was harvested and assessed cytokine production by ELISA kit.

Statistical Analysis

The data were analyzed using Student's t-test. A value of $p < 0.05$ was accepted as statistically significant.

Results

Cytokine Production of C57BL/6 and BALB/c Mice

We first examined the difference in cytokine production between C57BL/6 mice and BALB/c mice. Th1 (IFN-γ) and Th2 (IL-4) type cytokine production by splenocytes from both mouse strains was shown in Table 2. The splenocytes were cultured for 42 hours in the presence of anti-CD3e mAb (1 μg/ml) and the supernatant was subjected to cytokine assay. There was no great difference in IFN-γ production from splenocytes between C57BL/6 mice and BALB/c mice (Table 2). On the contrary, in BALB/c mice, increased IL-4 production in culture supernatant was observed compared to that of C57BL/6 mice (Table 2).

Table 2. Th1-Th2 Type Cytokine Production from Splenocytes of C57BL/6 and BALB/c Mice

Strain	IFN-γ (ng/ml)		IL-4 (pg/ml)	
Expt. 1				
C57BL/6	52.5 ± 5.3	*	151.4 ± 16.3	+
BALB/c	65.0 ± 8.7		1167.0 ± 70.6	
Expt. 2				
C57BL/6	127.6 ± 10.7	+	138 ± 25.8	+
BALB/c	150.2 ± 7.8		1049.3 ± 131.2	
Expt. 3				
C57BL/6	41.6 ± 9.7		107 ± 20.3	+
BALB/c	51.1 ± 12.8		842 ± 66.3	

Splenocytes (5×10^6/ml) were cultured with anti-CD3 MAb (l μg/ml) in 24-well culture plate. After 42 hours of incubation at 37°C, the supernatant was harvested and assessed for IFN-γ and IL-4 production as described in the "Materials and Methods" section. Each value represented as mean ± S.E. (n = 5). Significantly different from control group, $^*p < 0.05$ and $^+p < 0.01$.

Effect of NYT on Cytokine Production

We next examined the effect of NYT on cytokine production of both Th1 and Th2 type cytokine. Mice were administered NYT orally with drinking water for four weeks. There was no difference in body weight gain between control and NYT-treated groups (data not shown).

The *in vivo* effects of NYT on cytokine production by splenocytes from C57BL/6 and BALB/c mice are shown in Fig.1. The cultured splenocytes secreted both Th1 (IFN-γ) and Th2 (IL-4) type cytokines during the 42-hour incubation in the presence of anti-CD3e mAb (1 μg/ml). In the C57BL/6 mice orally treated with NYT, the production of IL-4 was significantly increased ($p < 0.01$) compared to that in the control mice (Fig. 1A), while IFN-γ was slightly decreased by NYT treatment (Fig. 1A). On the other hand, in the BALB/c mice, IFN-γ production was significantly increased, and IL-4 production was slightly decreased by the administration of NYT (Fig. 1B).

It is known that IL-12 is the major determinant of the differentiation of naive T cells into Th1 cells (Jacobson *et al.*, 1995). It is assumed that NYT modulates Th1/Th2 cytokine balance via modulation of IL-12 production. We next examined that effect of NYT on IL-12 production of splenocytes from both strains. The splenocytes were cultured for 42 hours in the presence of LPS (10 mg/ml). No significant difference of IL-12 production between NYT-treated and control groups was observed in both C57BL/6 and BALB/c mice (Fig. 2).

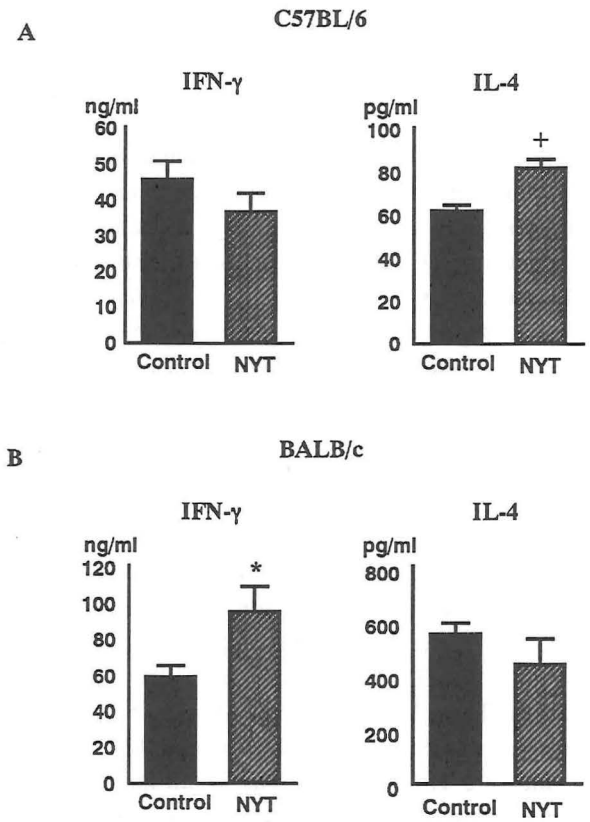

Figure. 1. The effects of NYT on cytokine production from splenocytes of C57BL/6 and BALB/c mice. NYT was administered orally with drinking water for 4 weeks. Splenocytes (5×10^6/ml) were cultured with anti-CD3 MAb (1 μg/ml) in a 24-well plate for 42 hours and supernatants were harvested and measured for the IFN-γ and IL-4 production as described in the "Materials and Methods" section. Each value represented as mean ± S.E. ($n = 5$). Significantly different from control group, $^*p < 0.05$ and $^+p < 0.01$.

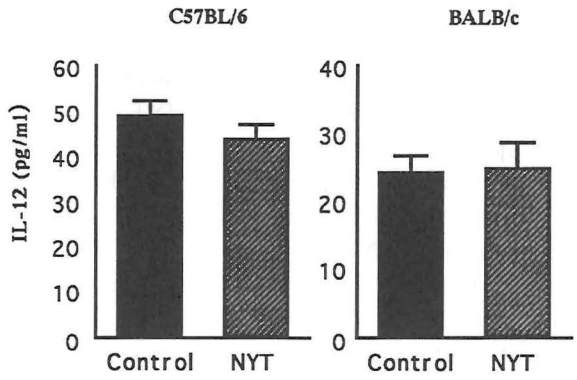

Figure. 2. The effect of NYT on IL-12 production from splenocytes of C57BL/6 and BALB/c mice. NYT was administered orally with drinking water for 4 weeks. Splenocytes (5×10^6/ml) were cultured with LPS (10 μg/ml) in a 24-well plate for 42 hours and supernatants were harvested and measured for the IL-12 production as described in the "Materials and Methods" section. Each value represented as mean ± S.E. (n = 5).

Discussion

BALB/c and C57BL/6 mice behave differently in their immune response. The former strain exhibits Th2-dominant immunity, as susceptive to *Leishmania major* infection, whereas the latter strain, manifesting Th1 dominant immunity, is resistant to the same infection (Heinzel *et al.*, 1993). Conversely, C57BL/6 mice are sensitive to *Propionibacterium acnes* and LPS-induced Th1-dependent liver injury, whereas BALB/c mice are resistant (Tanaka *et al.*, 1996). Furthermore, BALB/c mice develop allergic immune response more easily than C57BL/6 mice because of their genetic background (Tamura *et al.*, 1986).

It has been reported that NYT modulates serum IFN-γ and IL-6 concentrations of MRL/lpr mice which spontaneously develop autoimmune disease resembling SLE (Nakai *et al.*, 1996). IFN-γ and IL-6 are cytokines produced by Th1 and Th2 cells, respectively. Furthermore, It has also been reported that NYT enhanced natural killer activity in normal individuals (Kamei *et al.*, 1994). It is widely accepted that Th1-associated cytokines, especially IFN-γ and IL-2, enhance natural killer cell activity (Itoh *et al.*, 1985). Therefore, their action of NYT might be related to Th1/Th2 type cytokine production.

In Th1 dominant C57BL/6 mice, administration of NYT significantly increased IL-4 production. Enhancement of the IL-4 production from splenocytes suggested that NYT activated and/or induced the Th2 cells, leading to the stimulation of the humoral immunity. On the other hand, the same treatment significantly increased IFN-γ secretion of splenocytes from BALB/c mice which are Th2 dominant. This result indicated that NYT activated and/or induced Th1 and stimulated cellular immunity of BALB/c mice in contrast to C57BL/6 mice. These results suggested that oral administration of NYT improved the excessive inclination of both Th1 and Th2 cytokine production. This suggested that NYT played the role of biological response modifier (BRM).

Lentinan is another well-studied BRM. Lentinan is a fully purified b-1,3-D-glucan with b-1,6-branches obtained from Lentinus edodes. It has strong antitumor activity against rariouw tumors, and prevents chemical and viral carcinogenesis (Chihara *et al.*, 1970; Rose *et al.*, 1984). Since it does not show any direct cytotoxicity against tumor cells, its antitumor action is considered host-medicated. Lentinan has been described as a T cell oriented adjuvant (Chihara *et al.*, 1987), but according to others, it stimulates the natural killer cell activity, and several macrophage/monocyte functions, e.g. IL-1 and superoxide anion production, phagocytosis and cytotoxicity (Gergely *et al.*, 1988; Freunhauf *et al.*, 1982).

The effectiveness of lentinan differs among various strains of inbred mice, suggesting the influence of genetic factors of the host for the inducibility of the antitumor action of lentinan (Abel *et al.*, 1989; Suga *et al.*, 1984). The effect of the administration of lentinan on the TNF secretion from the peritoneal macrophages is different in different mice (Kerekgyarto *et al.*, 1996). The expression of biological activities of BRMs may be under multigenic control. Kampo medicine is used according to the patient's condition. This experiment showed that the genetic background affected the effect of Kampo medicines.

It is known that IL-4 is a major determinant of the differentiation of naive T cells into Th2 cells. IL-12 produced by an antigen-presenting cell (APC), such as dendritic cell or macrophage, is a major determinant of the differentiation of naive T cells into Th1 cells (Constant and Bottomly, 1997). Ninjin-Youei-To did not affect the IL-12 production from the splenocytes stimulated with LPS in both strains. This data indicated that the modulation ability of NYT on Th1/Th2 balance was not through the IL-12 production. It has been reported that Th differentiation is modified by a lot of factors, i.e. expression of co-stimulatory factor on APC (Palmer and van Seventer, 1997) and humoral factors such as IL-18 (Murphy, 1998; Barbulescu *et al.*, 1998). It is not known whether the oral administration of NYT modulates Th1/Th2 cytokines production via those factors or not. To elucidate the mechanism of NYT on Th cytokine production, further studies are necessary.

In summary, we demonstrated for the first time that NYT modulated Th1 and Th2 cytokines production in C57BL/6 and BALB/c mice, and its effect was different in the different strains. Furthermore, effects of NYT on Th1-Th2 cytokine production were not through the IL-12 production in both strains. These results suggested that NYT ameliorated the excessive inclination of both Th1-Th2 cytokine production, leading to the beneficial treatment of diseases caused by Th1-Th2 imbalance of the immune system.

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