A Newly Devised Formulation for Self-Medication Enhances Interferon-γ Production and Proliferation of Splenic Lymphocytes

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A newly devised formulation for self-medication in Toyama, PanaWang, is a new herbal medicine (so called Toyama original brand formulation) developed based on traditional philosophy and scientific evidence. We here tried to examine the effect of oral administration of PanaWang on the balance of type I helper T cells (Th1) and Th2 cells. Splenic lymphocytes from normal mice were stimulated with Concanavalin A (Con A) in vitro and the secretion of Th1- and Th2-type cytokines, interferon-γ (IFN-γ) and interleukin-4 (IL-4) respectively, was investigated. Con A-induced production of IFN-γ from spleen cells, but not IL-4, was enhanced by the administration of PanaWang. Increased production of IFN-γ was also detected in splenic lymphocytes from Th2-predominant BALB/c mice after DNP-immunization, without a change in antigen-specific IgE levels in vivo. Antigen-specific proliferative responses were also increased in lymphocytes from PanaWang-treated mice. These findings raise the possibility that PanaWang has Th1-stimulating activity and induces Th1-predominant immunity.

Key words Toyama original brand formulation; interferon-γ; interleukin-4; helper T cell

More than 70% of Japanese medical doctors are reported to use traditional Chinese/Japanese (Kampo) medicines; however, the mechanism of action of these Kampo medicines has not been completely elucidated. Some Kampo medicines are useful for enhancing immune responses. For example, juzentaihoto (Shi-Quan-Da-Bu-Tang in Chinese) has been used traditionally for the treatment of a depressed or weakened status including fatigue, loss of appetite, anemia and anorexia. At present, juzentaihoto is often used clinically in the treatment of cancers for prevention of adverse effects of chemotherapy and radiation therapy, blood disease, atopic dermatitis and so on. Several researchers have revealed pharmacological effects of juzentaihoto on the immune system, including the intestinal immune system, population of natural killer T cells (NKT) in the liver and type I helper T cell/type II helper T cell (Th1/Th2) responses. Abe also reported a protective effect of juzentaihoto on lethal Candida albicans infection in immunosuppressed mice.

CD4+ helper T cells can be subdivided into Th1 and Th2 cells. Th1 cells predominantly synthesize interferon-γ (IFN-γ) and interleukin-2 (IL-2), and induce cellular immunity (e.g. against cancer and infection). On the other hand, Th2 cells produce IL-4, IL-5 and IL-13, and induce humoral immunity. It has been demonstrated that the Th1 and Th2 types of immune response are reciprocally regulated in vivo. Th1 cytokine IFN-γ inhibits the proliferative response of Th2 cells, whereas Th2 cytokines IL-4 and IL-10 can inhibit the production of IL-2 and IFN-γ. Therefore, the balance of Th1 and Th2 cells is considered to be important for the regulation of immune functions. It has also been suggested that many diseases are partially caused by a skewed Th1 and Th2 cytokine balance. For example, an increase in Th2 type cytokine production is observed in patients with systemic lupus erythematosus (SLE) or asthma. Conversely, Th1 cells mediate inflammatory diseases such as graft versus host disease (GVHD).

A new formulation containing eleven herbs and crude drugs (Table 1), named “PanaWang”, was devised for self-medication to prevent lifestyle-related diseases including atherosclerosis, allergy and infection. Recent study demonstrated that PanaWang has strong scavenging activity for superoxide anion and hydroxyl radical, and protects against nitric oxide (NO)-mediated neuronal cell death in vitro, and prevented intimal thickening after endothelial injury in rats. Oral administration of PanaWang resulted in the inhibition of vasoconstriction induced by phospholipase A2, and a decrease in plasma levels of triglyceride and lipid peroxide in spontaneously hypertensive rats. It also had a positive effect on fatty metabolism in spontaneously diabetic rats. Ginseng Radix, a major constituent of PanaWang, is known to have anti-tumor anti-inflammatory and immunomodulating activities. In the present study, we examined the effect of oral administration of PanaWang on the balance of Th1/Th2 in immune responses, and found that it up-regulated IFN-γ production in splenic lymphocytes which is considered to be associated with the inhibition of tumor

Table 1. The Botanical Origins and Ratio of Crude Drugs in PanaWang

<table>
<thead>
<tr>
<th>Crude drug</th>
<th>Botanical origin</th>
<th>Dose (mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginseng Radix</td>
<td>Panax ginseng C. A. MEYER</td>
<td>450</td>
</tr>
<tr>
<td>Bezoar Bovis</td>
<td>Boe taurus L. var. domesus Gmelin</td>
<td>5</td>
</tr>
<tr>
<td>Corydalis Tuber</td>
<td>Corydalis yanhusuo W. T. WANG</td>
<td>60</td>
</tr>
<tr>
<td>Cnidii Monnieri Fructus</td>
<td>Torilis japonica A. P. DC</td>
<td>30</td>
</tr>
<tr>
<td>Oxoaminidin</td>
<td>Extraction of Allium sativum Liliaeae</td>
<td>100</td>
</tr>
<tr>
<td>Magniae Cortex</td>
<td>Magnolia obovata TRUM</td>
<td>90</td>
</tr>
<tr>
<td>Angelieae Radix</td>
<td>Angelica acutiloba KITAGAWA</td>
<td>200</td>
</tr>
<tr>
<td>Paenoeae Radix</td>
<td>Paenia lactifora DALLAS VAR</td>
<td>200</td>
</tr>
<tr>
<td>Cnidii Rhizoma</td>
<td>Cnidium officinale MAXIO</td>
<td>200</td>
</tr>
<tr>
<td>Cinnamomi Cortex</td>
<td>Cinnamonum cassia BLUME</td>
<td>200</td>
</tr>
<tr>
<td>Astragali Radix</td>
<td>Astragalus membranaceus BUNGE</td>
<td>200</td>
</tr>
</tbody>
</table>

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growth, metastasis and infectious diseases.

MATERIALS AND METHODS

Preparation of PanaWang  PanaWang, kindly provided by the Federation of Pharmaceutical Industries Association in Toyama, is composed of eleven crude drugs (Table 1) and was dissolved in water before oral administration. The quality of these crude drugs and HPLC profile of the preparation were described previously, to assess the homogeneity of the formulation and to prepare batches of constant formulation.

Animals  Specific pathogen-free female BALB/c mice, 6 to 10 weeks old, were purchased from Japan SLC (Hamamatsu, Japan), and maintained in the Laboratory for Animal Experiments, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, under laminar air-flow conditions. This study was conducted in accordance with the standards established in the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

Immunization  Dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) was prepared according to the method of Einsen et al. BALB/c mice were immunized intraperitoneally with 4 μg of DNP-KLH adsorbed on 4 mg of aluminum hydroxide gel (Alum) and boosted after 2 weeks with the same immunogen.

Preparation of Mouse Splenocytes  Normal or immunized mice were orally administered PanaWang (12.2 mg) or water for 4 weeks once a day. The administration started on the day of immunization. Mice were sacrificed and sera were obtained on day 28 and the amount of DNP-specific IgE in sera, suggesting that PanaWang did not affect the DNP-induced Th2 immunity.

Cell Proliferation Assay in Vitro  Splenocytes (2.5×10⁵ cells) suspended in 100 μl of complete medium were cultured in 96-well U-bottom culture plates with or without DNP-KLH for 72 h at 37°C. Proliferation was determined by WST-1 assay (DOJINDO, Place).

Quantification of Serum IgE  A 96-well plate was coated with LO-ME-3 PURIFIED (Technopharm Biotechnology, France). Standard monoclonal anti-DNP antibody (Sigma) and samples were added. The bound antibody was detected by biotin-conjugated DNP-BSA and peroxidase-conjugated avidin (DAKO, Place). The peroxidase reaction was carried out using TMB as a substrate.

Induction of Cytokine Production  Splenocytes (2.5×10⁶ cells/well) were prepared as described above, and then cultured in 24-well culture plates with Concanavalin A (Con A; 4 μg/ml) or DNP-KLH (1 μg/ml) for 24 h or 72 h. Cell-free supernatant was collected from each well and stored at −80°C until the ELISA. IFN-γ and IL-4 levels in the culture supernatants were determined using ELISA kits (BD Pharmingen, Place) according to the manufacturer's instructions.

Statistical Analysis  The statistical significance of differences between groups was determined with Student’s two-tailed t-test. Statistical significance was defined as a p value <0.05.

RESULTS

Effect of PanaWang on Th1/Th2 Cytokine Production in Normal Mice  To examine the effect of PanaWang on the balance of Th1/Th2 cytokines, ex vivo experiments were carried out using splenocytes from normal mice orally given PanaWang for 7 d. Splenocytes were stimulated with the T cell mitogen Con A for 24 h, and secretion of a Th1 cytokine (IFN-γ) and a Th2 cytokine (IL-4) was determined by ELISA. Figure 1 shows that IFN-γ production was significantly enhanced by treatment with PanaWang. On the other hand, IL-4 production was not affected. These results suggested that Th1 responses were up-regulated by PanaWang.

Effect of PanaWang on the Production of Antigen-Specific IgE  It has been shown that immunization of DNP together with Alum induces the production of antigen-specific IgE, which is a marker for Th2-mediated immune responses. To examine the effect of PanaWang on IgE production, mice were primed with DNP-KLH on day 0 and boosted on day 14. Daily oral administration of PanaWang started from the day of priming. DNP-specific IgE in serum was determined on day 28 after priming by ELISA (Fig. 2). However, there was no discernible difference between the PanaWang-treated group and control in the production of IgE in sera, suggesting that PanaWang did not affect the DNP-induced Th2 immunity.

Fig. 1. Effect of PanaWang on the Production of IFN-γ and IL-4 in Normal Splenocytes

Fig. 2. Effect of PanaWang on the Amount of DNP-Specific IgE in Serum

BALB/c mice were intraperitoneally immunized with DNP-KLH+Alum. Boosting was carried out on day 14. The daily oral administration of PanaWang or water started on the day of priming. Sera were obtained on day 28 and the amount of DNP-specific IgE was measured by ELISA. The results are expressed as the mean±S.D. for five BALB/c mice. N.D. means not detectable. Similar results were obtained in two independent experiments and a representative data is shown.
Mitogenic responses was detected in Panawang-treated splenocytes. As shown in Fig. 4, a significant increase in proliferation was determined by WST-1 assay. In addition, Panawang tends to suppress IL-4 production, although Panawang enhanced antigen-induced IFN-γ production. Allogenic responses in vivo.

Effect of Panawang on Th1/Th2 Responses of Immunized Splenocytes. As described above, Panawang enhanced Con A-induced production of IFN-γ in splenocytes of normal mice but not IL-4. We next investigated the effect of Panawang on antigen-induced IFN-γ and IL-4 production in splenocytes of DNP-immunized mice. Similarly, Panawang enhanced antigen-induced IFN-γ production. Although Panawang tended to suppress IL-4 production, the effect was not statistically significant (Fig. 3). Thus, oral administration of Panawang leads predominantly to enhanced production of Th1 cytokines in Th2-activating mice as well as normal mice.

Antigen-mediated stimulation is known to induce proliferative responses of lymphocytes. Therefore, we examined the effect of Panawang on the antigen-induced proliferation of splenocytes. Splenocytes from immunized mice were treated with DNP for 72h and cell proliferation was determined by WST-1 assay. As shown in Fig. 4, a significant increase in mitogenic responses was detected in Panawang-treated splenocytes.

DISCUSSION

Herbal prescriptions have been recognized as potentially valid by the scientific medical establishment, and their use has been increasing. Since traditional herbal prescriptions are generally prepared from combinations of many crude drugs on the basis of oriental prescriptions and herbalogy, they may have combined effects which differ from the sum of the effects of the individual constituent crude drugs. Panawang has been constructed based on traditional philosophy and scientific evidence. It consists of eleven herbs, six of which are also constituents of juzentaihoto, a typical Kampo medicine affecting the immune system. In the present study, we tried to obtain scientific evidence of the immunomodulating potential of Panawang focusing on lymphocyte function.

The balance of helper T cell subset Th1/Th2 in the host is considered to be important for the regulation and induction of immune functions. IL-12 is an initiation cytokine for cell-mediated responses and, in particular, induces the differentiation of bipotential Th precursors, or rather a selective priming and/or expansion of already committed Th1 and Th2 precursor cells. IL-12 in combination with IL-2 has several effects on Th1 cells, and whether a response will be a Th1 or Th2 type is regulated by the balance of IL-12 and IL-4 production at the early phase of the immune response. The balance of Th1 and Th2 type cytokines such as IL-2, IFN-γ, TNF-α, IL-4, IL-5 and IL-10 critically affects the expression of several immune responses. It has also been shown that NO down-regulates IFN-γ production and differentiation of Th1 cells. Oral administration of Panawang to normal and DNP-immunized mice caused the increased production of IFN-γ by splenocytes stimulated with Con A, as compared with untreated controls (Fig. 3). In addition, Con A-stimulated proliferation of splenocytes from immunized mice was enhanced by Panawang. These results indicate that oral administration of Panawang can lead to the dominant induction of Th1-type immune responses, possibly through the induction of IFN-γ production. Since Panawang has been shown to have a protective effect on NO-induced neuronal cell death, it is possible that down-regulation of NO function is related to the enhancement of Th1 responses by Panawang.

Th1 cytokines induce cellular immunity by activating CD8+ T cells, natural killer cells and macrophages. Th1-dominant immune responses are responsible for the induction of protection against bacterial and viral infections and anti-tumor effect. On the other hand, it is well accepted that allergic reactions are controlled by Th2-dominant immune responses. Therefore, enhancement of IFN-γ production by Panawang may potentiate Th1-mediated immunity and consequently reduce Th2-mediated allergic pathogenesis. Matsumoto et al. reported that juzentaihoto enhances Con A-induced IFN-γ production in normal splenic lymphocytes. This suggests that six common herbs in Panawang and juzentaihoto contribute to the Th1-inducing activity. However, Panawang without Magnoliae Cortex and Corydalis Tuber, herbs not contained in juzentaihoto, did not enhance IFN-γ production in immunized lymphocytes (data not shown), indicating that common herbs and additional herbs cooperate to induce the Th1 activity.

In summary, we demonstrated that Panawang stimulates Th1 cytokine production from splenic lymphocytes.
this study; therefore, it is necessary to further clarify the enhanced Th1 responses \textit{in vivo}, especially in some pathogenic animal models.

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