The Immune-Enhancing Effect of the Herbal Combination Bouum-Myunyuk-Dan

Hyun-Ja JEONG,a,b Hwan-Suck CHUNG,a,b Hyo-Jin AN,a,b Sang-Wan SEO,a,b Tae-gyun KIM,c Jin-Hee WON,c Jo-Young SHIN,c Kyoo-Seok AHN,d and Hyung-Min KIM* a

a Department of Pharmacology, College of Oriental Medicine, Kyung Hee University; 1 Hoegi-Dong, Dongdaemun-Gu, Seoul, 130–701, Republic of Korea; b Department of Oriental Pharmacy, College of Pharmacy, VCRC of Wonkwang University; Iksan, Jeonbuk, 570–749, Republic of Korea; c College of Oriental Medicine, Wonkwang University; Iksan, Jeonbuk, 570–749, Republic of Korea; and d Department of Pathology, College of Oriental Medicine, Kyung Hee University; 1 Hoegi-Dong, Dongdaemun-Gu, Seoul, 130–701, Republic of Korea.

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The herbal formulation Bouum-Myunyuk-Dan (BMD) has long been used for various diseases. It has been shown to have antimicrobial and anti viral activity clinically. However, it is still unclear how BMD exerts these effects in experimental models. In this study, we investigated the effect of BMD on the production of cytokines in a human T cell line, MOLT-4 cells, and in mouse peritoneal macrophages. As a result, BMD significantly increased the viability and proliferation of splenocytes (p<0.05) and also significantly increased interleukin (IL)-2 and IL-4 production compared with media control (about 2.7-fold for IL-2 and 6.7-fold for IL-4, p<0.05) after 24 h. BMD increased the interferon (IFN)-γ production by 3.7-fold but there were no significant differences compared with controls. Maximal effective concentrations of BMD were 1 mg/ml for IL-2 and IL-4 and 0.1 mg/ml for IFN-γ. In addition, BMD (0.01 mg/ml) increased the production of tumor necrosis factor (TNF)-α and IL-12 in mouse peritoneal macrophages (by 2.7-fold for TNF-α and 42.5-fold for IL-12, p<0.05). In conclusion, these data indicate that BMD may have an immune-enhancing effect through the production of various cytokines.

Key words Bouum-Myunyuk-Dan; interleukin-2; interleukin-4; interleukin-12; interferon-γ; tumor necrosis factor-α

Although herbal medicines have long been used effectively in treating many diseases in Asian communities, the pharmacologic mechanisms of most herbs used have not been defined. Bouum-Myunyuk-Dan (BMD), a traditional Oriental medicine, has been used for the treatment of various diseases in Korea. However, its marked effect on various diseases has not been investigated experimentally.

T cells play a crucial role in immune functions as they act both as effectors (cytotoxic T cells, Tc cells) and regulators (helper and suppressor T cells, Th and Ts cells). Tc cells can kill virus-infected cells and cells that undergo malignant transformation. Their activation depends on antigen challenge and signals sent from activated Th cells. Th cells mediate the link between antigen-presenting cells and the triggering of other cellular (natural and lymphokine-activated killer cells, macrophages, granulocytes) and humoral (B cell-producing antibodies) components of the immune response. In particular, Th cells are known to have two different subsets, Th1 and Th2. They are distinguished by the cytokines they secrete. Th1 lymphocytes produce interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, which promote cell-mediated immunity. Th2 lymphocytes produce IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and granulocyte macrophage colony-stimulating factor (GM-CSF), which promote the humoral antibody-mediated immune response.

As described above, various cytokines such as IFN-γ, IL-2, IL-4, IL-12, and TNF-α are related to the immune reaction directly or indirectly. Macrophages are involved in many different processes such as tissue remodeling during embryogenesis, wound repair, removal of damaged or senescent cells subsequent to injury or infection, hemopoiesis, and homeostasis. Another function of macrophages is to provide a line of defense against microbial invasion and to recognize and kill tumor cells. Macrophages can accomplish this in a direct manner, involving the release of products such as oxygen radicals and TNF-α that are harmful to microorganisms or cancer cells. On the other hand, they play an indirect role in these antimicrobial or antitumor activities by secretion of cytokines (e.g., IL-12) or by antigen processing and presentation, thereby regulating the immune system.

To investigate the effect of BMD on the production of cytokines, in the present study we analyzed the production of IL-2, IL-4, IFN-γ, TNF-α, and IL-12 on BMD-treated MOLT-4 cells and mouse peritoneal macrophages.

MATERIALS AND METHODS

Reagents Avidin-peroxidase, Concanavalin-A (Con-A), and 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) tablets (ABTS) substrates were purchased from Sigma (St. Louis, MO, U.S.A.). RPMI 1640, ampicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Anti-human IL-2, IL-4, IFN-γ, and TNF-α, biotinylated anti-human IL-2, IL-4, IFN-γ, and TNF-α, and recombinant (r) human IL-2, IL-4, IFN-γ, and TNF-α were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Anti-mouse IL-12p70, biotinylated anti-mouse IL-12p70, and rmouse IL-12p70 were purchased from Pharmingen (San Diego, CA, U.S.A.).

MOLT-4 Cell Culture The human leukemic T cell line, MOLT-4 was used in this study. The cells were maintained in RPMI-1640 medium (Gibco BRL) with 10% FBS at 37°C under 5% CO2 in air.

Peritoneal Macrophage Culture Thioglycolate (TG)-elicited macrophages were harvested 3 d after intraperitoneal injection of TG 2.5 ml to mice and isolated as reported previously. Using 8 ml of Hank's balanced salt solution (HBSS),...
which contained heparin 10 U/ml, we performed peritoneal lavage. Then, the cells were distributed in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% heat-inactivated FBS, in 96 well tissue culture plates (3×10^5 cells/well), incubated for 3 h at 37 °C in an atmosphere of 5% CO\(_2\), washed 3 times with HBSS to remove nonadherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

**Isolation of Splenocytes** Splenic lymphocytes were obtained by gentle disruption of the spleen (male BALB/c mice) and repeated pipetting. Splenocytes were maintained in RPMI-1640 medium (Gibco BRL) with 10% FBS at 37 °C under 5% CO\(_2\) in air.

**Preparation of BMD** BMD (Table 1) was prepared by decocting the dried prescription of herbs with boiling distilled water. These plant materials were obtained from Oriental Medicine Hospital (Chonju, Chonbuk), Wonkwang University, and identified by E. J. Park, College of Oriental Medicine, Wonkwang University. Their voucher specimens have been deposited in the Herbarium in the College of Pharmacy, Wonkwang University. BMD was tested for their lipopolysaccharide (LPS) content with using an E-TOXATE kit (Sigma; detection limit, 0.25 EU/ml). BMD did not contain endotoxin.

**Cell Proliferation and Viability** Cell proliferation was analyzed based on \[^{3}H\]-thymidine incorporation. Cells (5×10^5) were grown in serum-free (0.5% FBS) media to arrest the cell cycle for 24 h, cell cycling was stimulated by adding 10% FBS, and they were incubated for 24 h after treatment with BMD. Cells were pulsed with \[^{3}H\]-thymidine 2 µCi/well for 3 h prior to measuring the proliferation value. After incubation, cells were harvested and washed twice with phosphate-buffered saline (PBS) and then washed twice with 10% trichloroacetic acid (TCA). Cells were solubilized in 0.2 m NaOH, 0.1% SDS, and 2 m Na\(_2\)CO\(_3\) for 30 min at 37 °C. Radioactivity of the \[^{3}H\]-thymidine incorporation was determined with a liquid scintillation counter (Beckman LS6500, U.S.A.). To investigate the viability of cells, an MTT assay was performed. Briefly, cell suspensions (3×10^5 cells) were cultured in 4-well plates for 24 h after treatment with BMD. Forty microliters of an MTT 5 mg/ml solution was added and the plates were incubated for 30 min at 37 °C. Wells were then washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate with recombinant IL-2, IL-4, IFN-γ, TNF-α, and IL-12 in serial dilutions.

**Statistical Analysis** The experiments shown are a summary of the data from four experiments and values are expressed as the mean±S.E.M. Statistical evaluation of the results was performed using ANOVA with the Tukey post hoc test. The results were considered significant at a value of \(p<0.05\).

**RESULTS**

**Effects of BMD on the Viability and Proliferation of MOLT-4 Cells and Splenocytes** To assess the effect of BMD on the viability and proliferation of cells, we performed the MTT assay and \[^{3}H\]-thymidine uptake assay. As a result, BMD increased the viability of MOLT-4 cells by 15±0.7% at a dose of 1 mg/ml (Fig. 1A). Proliferation of splenocytes was also increased by treatment with BMD or Con-A (Fig. 1B). BMD 1 mg/ml increased the viability of splenocytes by 34±0.7% (Fig. 1C).

**Effects of BMD on the Production of IL-2, IL-4, and IFN-γ by MOLT-4 Cells** To assess the effects of BMD on the production of various cytokines, MOLT-4 cells were treated with various concentrations of BMD for 24 h. The levels of IL-2, IL-4, and IFN-γ were analyzed using ELISA. As shown in Fig. 2, BMD 1 mg/ml significantly increased IL-2 and IL-4 production (0.67±0.17 ng/ml for IL-2 and 0.27±0.16 ng/ml for IL-4) compared with the media control (0.23±0.02 ng/ml for IL-2 and 0.04±0.02 ng/ml for IL-4). BMD 0.1 mg/ml also increased IFN-γ production (0.15±0.24 ng/ml) compared with the media control (0.04±0.02 ng/ml).

**Effect of BMD on the Production of TNF-α and IL-12 by Peritoneal Macrophages** We next examined whether BMD could stimulate potential mediators such as TNF-α and IL-12 in isolated mouse peritoneal macrophages. Mouse peritoneal macrophages were cultured with rIFN-γ for 6 h

<table>
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<th>Botanical name</th>
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<tr>
<td>Hedyotis diffusa</td>
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<tr>
<td>Astragalus membranaceus</td>
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<tr>
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<td>Angelica sinensis</td>
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<td>Adenophora triphylla var. japonica</td>
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<tr>
<td>Spaltholobus suberectus</td>
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<td>Total amount</td>
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and then stimulated with various concentrations of BMD for 24 h. Cells were then collected and assessed for viability and proliferation using MTT and [3H]-thymidine. A, MTT assay in MOLT-4 cells; B, [3H]-thymidine uptake in splenocytes; C, MTT assay of splenocytes. Values are the mean ± S.E.M. of duplicate determinations from four separate experiments (p<0.05).

**DISCUSSION**

In the present study, we showed that BMD increased the proliferation of splenocytes. BMD also induced the production of IL-2, IFN-γ, TNF-α, and IL-12 by MOLT-4 cells and mouse peritoneal macrophages. These results indicate that BMD has immune-enhancing effects.

The activation of tumor antigen-specific Th and Tc cells or nonspecific macrophages and natural killer (NK) cells using immunotherapeutic approaches may lead to the subsequent destruction of tumor tissue.⁹ Previous reports have demonstrated that the induction of Th1-promoting cytokines, using specific adjuvants, can enhance antitumor immunity and can reduce or even prevent tumor growth.¹⁰ The immune response can be broadly categorized into a cellular or humoral response. The production of IL-2, IFN-γ, TNF-α, and IL-12 by BMD also induced the production of IL-2, IL-4, TNF-α, and IL-12 by MOLT-4 cells and mouse peritoneal macrophages. These results indicate that BMD has immune-enhancing effects.
cancer vaccines, particularly in combination with immune adjuvants, elicit strong cellular immune responses leading to the production of Th1-type cytokines such as IL-2, IFN-γ, TNF-α, and IL-12.\(^{11}\) IL-2 (also known as T cell growth factor) has multiple immunoregulatory functions and biological properties. IL-2, together with other factors and in conjunction with antigens, mitogens, or anti-immunoglobulin antibodies, controls B cell proliferation and differentiation into antibody-producing plasma cells.\(^{12}\) NK and lymphokine-activated killer cells, monocytes, and macrophages all have the ability to respond to IL-2 with increased activity or proliferation.\(^{16,17}\) IFN-γ is also an important cytokine in the host defense against infection by viral and microbial pathogens.\(^{18}\) IFN-γ induces a variety of physiologically significant responses that contribute to immunity. IL-12, which is primarily produced by activated macrophages, and stimulates T cells and NK cells. It induces IFN-γ and plays a role in promoting Th1 cell responses. Immunoregulation by IL-12 is of central importance in cell-mediated immunity against those pathogens and tumors that are controlled by cell-mediated mechanisms.\(^{19}\) TNF-α participates in the host defense against pathogens. TNF-α also modulates the immune response by triggering the production of a number of other regulatory cytokines such as IL-1, IL-6, IFNs, transforming growth factors, and granulocyte-macrophage colony-stimulating factor.\(^{20}\) Previously, we reported that Th2 cytokine levels were higher than Th1 cytokine levels in various diseases including cerebral infarction, allergy, and asthma.\(^{21—23}\)

Proinflammatory cytokines involved in hemostatic and immunologic imbalance lead to the enhancement of various types of tissue damage. The proinflammatory cytokine IL-4 has also been called the prototypic immunoregulatory cytokine. Like many cytokines, it can affect a variety of target cells in multiple ways. IL-4 has an important role in regulating antibody production, hemopoiesis, and inflammation, and the development of effector T cell responses.\(^{24}\) In addition, IL-4 affects a number of other cells including granulocytes, fibroblasts, endothelial cells, and certain thymocytes as well as B cells. The biological effects of IL-4 are numerous and many of them are a result of indirect action whereby IL-4 stimulates various cell types to produce other cytokines.\(^{25—29}\)

In this study, we observed that BMD increased the production of IL-4, the Th2 cytokine. We can speculate that BMD improves the ability to respond to IL-2 with increased activity or proliferation, whereas the production of IL-4 by Th2 cells improves the ability to respond to IL-2 with increased activity or proliferation, whereas the production of IL-4 by Th2 cells.

On the other hand, others reported that IFN-γ produced by Th1 cells inhibits the development of Th2 cells as well as humoral responses, whereas the production of IL-4 by Th2 cells inhibits the development and activation of Th1 cells.\(^{30,31}\) In our study, BMD (0.01—1 mg/ml) increased IL-2 and IL-4, but IFN-γ production tended to increase with BMD at concentration of 0.01 and 0.1 mg/ml, but decreased at 1 mg/ml. These data indicate that the stimulated secretion patterns of cytokines differ with the concentration of BMD.

As shown in Table 1, BMD consists of 16 different herbs. Other studies reported that each medicinal herb has a different effect. \(\text{Hedyotis diffusa WILLD}\) and \(\text{Glycyrrhiza uralensis FISCH}\) have an antitumor effect.\(^{32,33}\) Cui et al. reported that \(\text{Astragalus membranaceus BUNGE}\) has a suppressive effect in chemical-induced hepatocarcinogenesis.\(^{34}\) The aqueous extract of \(\text{Ophiopogon jacinthus KER-Gawl}\) increased the spleen weight (immunity organ) of mice.\(^{35}\) Nyasol, \((Z)-1,3-	ext{bis}(4\text{hydroxyphenyl})-1,4-	ext{phenylenedi})\), purified from \(\text{Anemarrhena asphodeloides Bge}\), showed antimicrobial activity against fungi and bacteria.\(^{36}\) In this study, BMD increased the Th1 and Th2 cytokine production by immune cells. Therefore we speculate that BMD has an immune-enhancing effect leading to antitumor and antibacterial effects via Th1/Th2 cytokine regulation \textit{in vivo}. The information presented in the current study seems compatible with the purpose of the Oriental medicine prescription. However, since IL-2 and IL-4 were produced with high concentrations of BMD, the active components of BMD should be isolated in further studies to clarify whether the components may also be effective in a murine \textit{in vivo} model and in patients.

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**REFERENCES**