

## Bing De Ling, a Chinese Herbal Formula, Stimulates Multifaceted Immunologic Responses in Mice

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### ABSTRACT

**Bing de ling** is a Chinese herbal formula most commonly used in complementary medical settings against viral disorders. We have found that bing de ling potentiates upregulation of immune activity when administered to mice in dosages proportional to those used clinically. These mice demonstrated significant elevation of interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ) production in splenocytes and enhancement of macrophage, natural killer cell, and lymphokine-activated killer cell cytotoxicity. These data are consistent with bing de ling's clinically observed efficacy against viruses and identify the formula as a promising candidate for clinical trials against diverse diseases that may respond to increased immunologic activity.

### INTRODUCTION

**B**ING DE LING IS A CHINESE HERBAL MIXTURE formulated to boost the body's immune responses and resistance to viral infection and to preserve its homeostatic balance. Most commonly prescribed in complementary medical settings against common colds, influenza, chronic fatigue syndrome, herpes simplex, herpes zoster, and other viral disorders, bing de ling is derived from both modern knowledge of its ingredients' biochemical activities and traditional Chinese herbal formulatory principles to boost body's immune system. While its mechanisms remain largely unresolved, favorable patient testimonials suggest that bing de ling may potentiate general cellular immunologic responses.

The immunologic stimuli effected by certain ingredients of bing de ling have been well documented. *Astragalus membranaceus* potentiates lymphokine-activated killer (LAK) cell cytotoxicity (Chu *et al.*, 1988) and increases antibody secretion and helper T-cell activity in normal and immunosuppressed mice (Zhao *et al.*, 1990). Rhubarb root possesses both antibacterial (Zhang *et al.*, 1992) and antiviral (Wang *et al.*, 1996) activity. In addition, extracts from isatis root have been demonstrated to increase white blood cell and peripheral blood mononuclear cell (PBMC) counts in mice (Xu and Lu, 1991).

Tempering these laboratory findings, however, is the reality that single-herb therapies often produce levels of systemic toxicity unacceptable for a long-term therapeutic agent. For example, used in isolation, *Astragalus* root causes oral ulcers and irritability. Also, both rhubarb root and isatis root are widely known to produce stomach cramps and diarrhea. As a result of this dilemma, much of traditional Chinese pharmacology is concerned with the judicious mixture of herbal ingredients to minimize residual toxicity while preserving or enhancing efficacy. Bing de ling is a product of this philosophy, designed to deliver the immunologic stimuli of its component herbs while minimizing the side effects associated with their use in isolation.

In this study, we utilized a murine model to examine the effects of the bing de ling formula on the mammalian immune system. After administering bing de ling for 3 to 5 days alongside herbal or water controls or both, significant elevation of interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ) secretion and stimulation of macrophage, natural killer (NK) and LAK cell activity was detected. These results provide evidence that bing de ling effects a general cellular immunologic stimulation similar or superior to those achieved in single-herb studies and identify bing de ling as a promising candidate for further laboratory and clinical studies.

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## MATERIALS AND METHODS

### Herbal medicine

Bing de ling solution consisted of Astragalus root (*Astragalus membranaceus*), rhubarb root (*Rheum palmatum*), white atractylodes (*Atractylodes macrocephala*), isatis root (*Isatis tinctoria*), scutillaria root (*Scutellaria baicalensis*), dogberry (*Cornus officinalis*), and shield fern root (*Dryopteris eras-rhizoma*) at a concentration of 0.121 g/ml of water. Anti-alcohol formula, our herbal control solution, was formulated with pueraria root, pueraria flower, costus root, and magnolia flower 0.121 g/ml.

### Animals

Experiments were conducted with female BALB/c mice, 6 to 8 weeks of age. Mice were purchased from the National Cancer Institute, Bethesda, MD, and housed in the animal facility at H. Lee Moffitt Cancer Center & Research Institute. Both control fluids (water and anti-alcohol herbal formula) and bing de ling were administered in 100- $\mu$ l dosages twice daily via gastric lavage. Dosages were selected to correspond with those used in clinical practice at The Center for Traditional Chinese Medicine.

### Cell lines

Mouse melanoma line B16-F10, mouse lymphoma line YAC-1, and mouse myeloma line J558 were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and were cultured according to ATCC instructions.

### Enzyme-linked immunosorbant assay

Single-cell suspensions of splenocytes from individual mice fed bing de ling, water, or the anti-alcohol herbal control were prepared as previously described (Tan *et al.*, 1996). Splenocytes ( $5 \times 10^6$ ) were cultured in 24-well plates with anti-CD3 antibody (1  $\mu$ g/ml) for 48 h. Supernatant liquids were collected and assayed using OptEIA™ IL-2 and OptEIA™ IFN- $\gamma$  ELISA sets

from PharMingen (San Diego, CA). Medium-bind EIA plates (Corning Corporation, Acton, MA) were coated with anti-IL-2 or anti-IFN- $\gamma$  antibody overnight and blocked with PBS with 10% fetal bovine serum (FBS) for 30 min. Serial dilutions of IL-2 and IFN- $\gamma$  recombinant protein standards and samples were incubated for 2 h, followed by application of biotinylated anti-IL-2 or anti-IFN- $\gamma$  antibody for 1 h and streptavidin-alkaline phosphatase (1:3000) for 30 min. After addition of TMB™ substrate (DAKO Corp, Carpinteria, CA), plates were developed for 10 to 30 min, stopped by 1 N H<sub>2</sub>SO<sub>4</sub>, and read within 15 min at 450 nm (Tan *et al.*, 1996).

### NK and LAK cell generation and cytotoxicity assay

For NK cell preparation, whole blood samples from mice fed bing de ling or control liquids were collected via cardiac puncture into heparinized tubes. Peripheral blood lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation, washed twice, and resuspended in 10% FBS before assaying. To generate LAK cells, single-cell suspensions of splenocytes from the two groups of mice were adjusted to  $2 \times 10^6$  cell/ml and cultured for 3 to 4 days in RPMI medium containing  $5 \times 10^{-5}$  M 2-mercaptoethanol, rIL-2 (500 to 1000 U/ml) (Cetus Corporation, Emeryville, CA), 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1% Minimal Essential Medium, 0.1 mM nonessential amino acids, and penicillin-streptomycin 100 U/ml (Grimm *et al.*, 1982).

Cytotoxicity <sup>51</sup>Cr release assay was performed as previously described (Tan *et al.*, 1996) using YAC-1 and B16 cells as targets for NK and LAK effector cells, respectively. Briefly, target cells were incubated in 100  $\mu$ l (100  $\mu$ Ci) of <sup>51</sup>Cr in 100  $\mu$ l of FBS for 1 h at 37°C. Effector cells were added to 100  $\mu$ l of target cells ( $1 \times 10^5$  cells/ml) in effector:target ratios of 100:1, 50:1, 25:1, and 12.5:1 in a 96-well plate and incubated for 4 h at 37°C. Spontaneous release was set by adding 100  $\mu$ l of medium and 100  $\mu$ l of radiolabeled target cells, while maximum release was set by adding 90  $\mu$ l of medium and 10  $\mu$ l of Triton X-100 and 100  $\mu$ l of radiolabeled target cells. After 4 h of incubation, the upper 100  $\mu$ l of medium from each well was removed and the

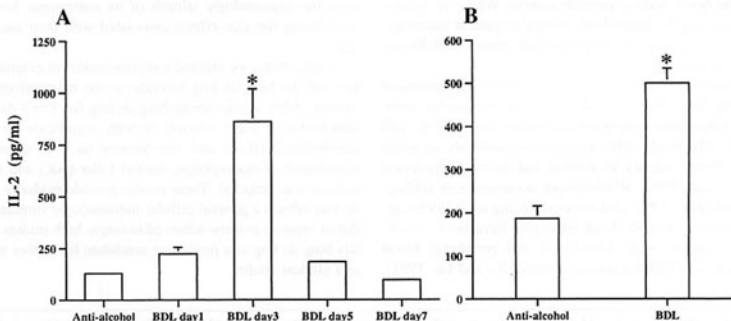
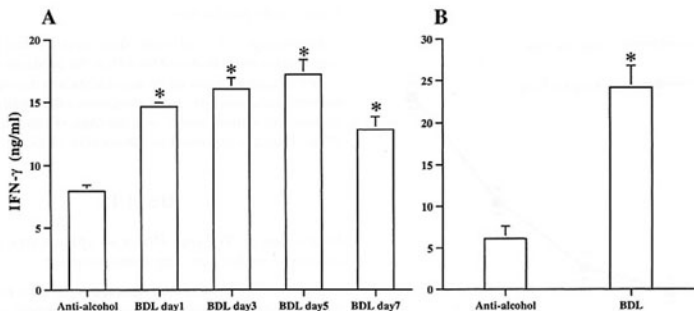


FIG. 1. Production of IL-2 is increased in the splenocytes of mice receiving bing de ling. (A) Mice were fed either control formula or bing de ling until days indicated. (B) Because IL-2 production peaks at day 3, an additional experiment was performed to confirm IL-2 production at this time. Data are presented as means; lines indicate SD. For each group, N = 3, \*P < 0.05 vs. control.



**FIG. 2.** Bing de ling feeding induces production of IFN- $\gamma$  in splenocytes. (A) Splenocytes were prepared from mice fed with either control diet or bing de ling on days as indicated. (B) Production of IFN- $\gamma$  on day 3 by splenocytes as a result of bing de ling feeding was confirmed by an independent experiment. For each group,  $N = 3$ . \* $P < 0.05$  vs. control.

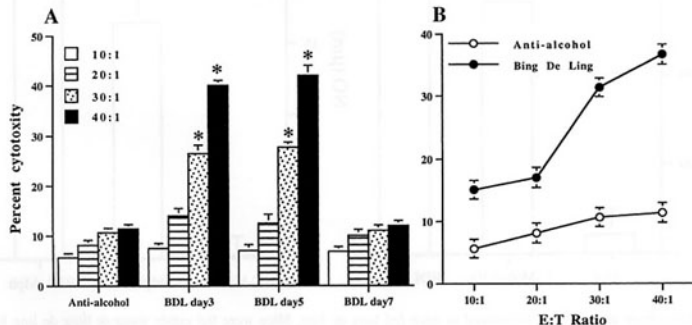
radioactivity measured. The percentage of cytotoxicity was calculated using the formula:  $(\text{cpm effect cells} - \text{cpm spontaneous release}) / (\text{cpm maximum release} - \text{spontaneous release}) \times 100$ .

#### Peritoneal macrophage isolation

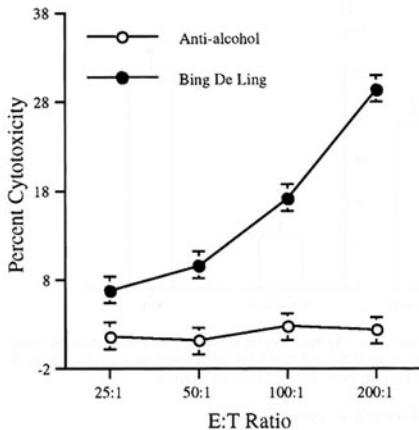
Peritoneal macrophages (Mph) were obtained by washing the peritoneal cavities of bing de ling-treated and control mice with serum-free culture medium. The percentage of immune cells (Mph, lymphocytes, granulocytes) in the peritoneal cell population was estimated by morphologic criteria with the use of Giemsa staining. To enrich Mph, peritoneal cells were seeded in 96-well plates, and nonadherent cells were vigorously washed out after 2 h of adhesion in serum-free RPMI to remove all non-Mph cells. Usually, approximately half of the initial peritoneal cells remained, more than 90% of which were Mph. In some experiments, Mph were activated with bacterial lipopolysaccharide (LPS, 5  $\mu\text{g/ml}$ ), a nonspecific activator.

#### Macrophage cytotoxicity tests

The antitumor cytostatic activity (CSA) of Mph was determined by inhibiting  $^3\text{H}$ -thymidine ( $^3\text{H}$ -TdR) incorporation into DNA by J558 myeloma cells. Tumor cells ( $2 \times 10^4$ /well) were cocultured for 48 h with Mph ( $1 \times 10^5$ /well) in the presence or absence of LPS (5  $\mu\text{g/ml}$ ) or alone as a control in quadruplicate in 96-well plates in 200  $\mu\text{l}$  of 10% FBS-RPMI. DNA synthesis was estimated by pulsing the cells with  $^3\text{H}$ -TdR (0.25  $\mu\text{Ci}$ /well) during the last 4 h of incubation. Then cells were transferred to glass fiber filters by an automated cell harvester (Tomtec, Hamden CT), and  $^3\text{H}$ -TdR incorporation was determined by a liquid scintillation beta-counter (Pharmacia Wallac, Finland). The percentage of cytostasis was calculated as  $(\text{cpm}_c - \text{cpm}_{\text{EX}}) / \text{cpm}_c \times 100$ , where  $\text{cpm}_c$  represents  $^3\text{H}$ -TdR incorporation by tumor cells incubated in medium alone and  $\text{cpm}_{\text{EX}}$  represents  $^3\text{H}$ -TdR incorporation by tumor cells in contact with Mph.



**FIG. 3.** Bing de ling administration induces NK cell cytotoxic activity. Data shown are percentage of cytotoxicity in the PBMCs from mice after feeding either control formula or bing de ling for 3 days. Data are presented as means of % cytotoxicity  $\pm$  SD. For each group,  $N = 3$ . The NK cell cytotoxicity on day 5 was reproduced in an additional experiment. E = effector cells; T = target cells (YAC-1). \* $P < 0.05$  vs. control.



**FIG. 4.** Oral feeding of bing de ling stimulates LAK activity. Percentage of LAK cell cytotoxicity was derived from mice fed for 3 days with either control herbal formula or bing de ling. Results are expressed as means of % cytotoxicity  $\pm$  SD. For each group,  $N = 3$ . Data were confirmed by three additional experiments. E = effector cells; T = target cells (B16).

#### Nitric oxide production

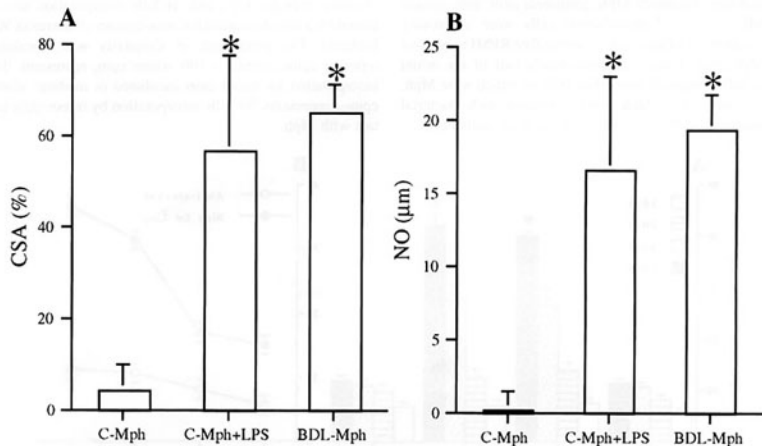
Macrophages ( $1 \times 10^5$ /well) from bing de ling-treated or control mice were incubated for 48 h in the presence or absence of LPS ( $5 \mu\text{g/ml}$ ). Nitric oxide accumulation in the supernatants medium was quantified spectrophotometrically using the Griess reagent and sodium nitrite as a standard (Draper and Hibbs, 1988). Data are expressed as micromoles of nitrite produced.

## RESULTS

#### Production of IL-2 and INF- $\gamma$ by splenocytes is increased by bing de ling administration

Interleukin-2 is a cytokine secreted by T cells to stimulate T-cell proliferation and facilitate the development of cell-mediated immunity (Mosmann and Sad, 1996). The secretion of IL-2 is therefore a valuable indicator of immunologic upregulation. Our results revealed that IL-2 production by anti-CD3 antibody-stimulated splenocytes obtained from bing de ling-treated mice was significantly greater than that by splenocytes from control mice 3 days after bing de ling feeding (Fig. 1).

Secreted by NK cells and CD4, and CD8 T lymphocytes, IFN- $\gamma$  activates Mph and is associated with the development of strong cellular immunity, both of which play an important role in fighting viral infection and in antitumor effects (Mosmann and Sad, 1996; Young and Hardy, 1995). Our results indicated that anti-CD3 antibody-stimulated splenocytes from mice treated with bing de ling secreted more IFN- $\gamma$  than did anti-CD3 antibody-stimulated splenocytes from control anti-alcohol-treated mice (Fig. 2).



**FIG. 5.** Macrophage activities are stimulated in mice fed bing de ling. Mice were fed either water or bing de ling for 5 days. (A) Macrophage cytostatic activity (CSA) in mice fed bing de ling is elevated and similar to that of macrophages from control mice stimulated with LPS. Data are presented as the means of % CSA; lines indicate SD. (B) Nitric oxide production is also increased in macrophages derived from mice given bing de ling. C-Mph = control macrophages; C-Mph+LPS = control macrophages stimulated with LPS; MDL-Mph = bing de ling-fed mouse macrophages. For each group,  $N = 3$ . These experiments were repeated twice with similar results. \* $P < 0.05$  vs. control.

### Bing de ling administration stimulates both NK and LAK cell cytotoxicity

Natural killer cells are granular, non-T, non-B lymphocytes critical in early immune responses to viruses and other intracellular pathogens, as well as antibody-dependent cell-mediated cytotoxicity (See *et al.*, 1997; Harshan and Gangadharam, 1991). The LAK cells, a derivative of NK cells, bolster these defenses by killing several non-NK-sensitive cell types, including some types of tumor cells (Grimm *et al.*, 1982). To evaluate the change in cytotoxicity of NK and LAK cells caused by bing de ling treatment *in vivo*, we examined the cytolytic activity of these effector cells against YAC-1 lymphoma and B16 melanoma cells, respectively. The  $^{51}\text{Cr}$  release assay revealed elevated lysis of tumor target cells by both NK and LAK cells obtained from bing de ling-treated mice in comparison with those isolated from control mice (Figs. 3 and 4).

### Macrophage activity is stimulated in mice given bing de ling

Peritoneal macrophages are terminally differentiated cells important in the maintenance of innate immunity, in early non-adaptive steps of host defense, and as effector cells in humoral and cell-mediated immunity (Williams *et al.*, 1999). Macrophages activated by microbial products and cytokines can exert cytotoxic activity against infecting organisms and malignant cells. One of the mechanisms of Mph cytotoxicity is the secretion of nitric oxide capable of inhibiting replication of viruses (Lin *et al.*, 1997) and mitochondrial respiration and DNA synthesis in tumor cells (Stuehr and Nathan, 1989). Therefore, we examined *in vitro* both the antitumor activity of peritoneal Mph obtained from bing de ling-treated mice against J558 myeloma cells and nitric oxide production. Resident Mph isolated from control mice treated with water did not exert significant cytostatic activity against J558 myeloma cells. Under the same conditions of experiment, however, resident Mph from the peritoneal cavities of bing de ling-treated mice significantly suppressed DNA synthesis in J558 target cells, comparable to the effects of Mph from control mice additionally activated *in vitro* with LPS (Fig. 5A). The cytostatic effect of Mph on J558 cells induced by administration of bing de ling correlated strongly with nitric oxide production (Fig. 5B).

## DISCUSSION

This study suggests that bing de ling potentiates upregulation of immune activity in a murine model. Our results reveal a twofold to fourfold elevation of *ex vivo* IL-2 and IFN- $\gamma$  production. Induction of IFN- $\gamma$  at such levels has been shown to correlate with IL-12 gene therapy-induced antitumor immune responses (Martinotti *et al.*, 1995; Tan *et al.*, 1996). Bing de ling treatment has also stimulated antitumor cytotoxic activity of NK cells, LAK cells, and Mph obtained from bing de ling-treated mice. As these results encompass both primary killing of tumor cells and production of secondary cytokine messengers, stimulation of the network of immunologic responses is implied. These data are consistent with the favorable testimonials of patients who have used bing de ling.

Our results are also particularly interesting when analyzed

alongside bing de ling's reported efficacy against viral disorders. Natural killer cells are critical in controlling viral replication during early phases of infection and in killing virally infected cells before specific CD8 T cells have been activated (See *et al.*, 1997). Moreover, the IFN- $\gamma$  secreted by NK cells is an important activator of macrophages, which in turn reinforce early NK-mediated and later T-cell-mediated antiviral activity. Numerous previous reports have suggested that various Chinese medicinal herbs stimulate immune responses in patients (see review by Sinclair, 1998). Activation of NK or LAK cells or induction of IL-2 and IFN- $\gamma$  correlates with improved clinical outcomes (Horie *et al.*, 1994; Chu *et al.*, 1994; Cao *et al.*, 1994; Liao *et al.*, 1995). These previous findings and our results with bing de ling imply that bing de ling can also modulate immune responses and thus improve clinical outcomes of patients with disorders that can benefit from immunologic stimulation.

Several aspects of bing de ling therapy require further investigation. Most significantly, the mechanisms of bing de ling's immunologic stimulation have yet to be elucidated. Indeed, the functional mechanisms of its individual components, which are likely prerequisite to any understanding of the synergies that may exist within the formula, are unknown (Chu *et al.*, 1988; Ma, 1991). Furthermore, controlled clinical trials would not only allow study of bing de ling's function in healthy patients but also test its value as a novel therapy for viral infections, cancer, and other disorders that may respond to immunologic stimulation.

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