ENHANCEMENT OF CELL PROLIFERATION AND CYTOKINES PRODUCTION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS BY EXTRACTS FROM BLOOD-ENRICHING DANG-GUI DECOCTION

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“Blood-enriching Dang-Gui Decoction” (BDGD), comprised of Angelica sinensis (Oliv.) Diels and Astragalus membranaccus (Fisch) Bge and used for improvement health in traditional Chinese medicine, was selected for immunopharmacological activity test. Effects of aqueous extracts of BDGD, A. sinensis, and A. membranaccus on human peripheral blood mononuclear cells (PBMC) proliferation were determined by tritiated thymidine uptake, respectively. Aqueous extracts from all three preparations significantly enhanced the proliferation of PBMC induced by phytohemagglutinin (PHA) with an EC₅₀ of 106.3 ± 5.8, 91.3 ± 2.5, and 23.8 ± 4.0 µg/ml, respectively. Furthermore, all extracts dose-dependently enhanced interleukin-2 (IL-2), interleukin-3 (IL-3), and interferon-γ (IFN-γ) production in PHA-stimulated PBMC, suggesting that pharmacological activities of these drugs may be mediated by regulating the production of cytokines in PBMC. These results indicate that agents with immunomodulatory effects are contained in BDGD.

Key Words: Blood-enriching Dang-Gui Decoction, Angelica sinensis, Astragalus membranaccus, PBMC, Proliferation, IL-2.

INTRODUCTION

“Blood-enriching Dang-Gui Decoction” (BDGD), a traditional Chinese formulation comprising Angelica
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*A. sinensis* (Oliv.) Diels and *Astragalus membranaceus* (Fisch) Bge, is applied for stimulating red blood cell production and enhancing cardiovascular function. A. *sinensis* belonging to the Umbelliferae family has been demonstrated to produce natural products with various biological activities: (1) Ferulic acid contained in *A. sinensis* exerts an antiinflammatory effect in the inflammatory pathology.  

(2) The Dang Gui injection prepared from *A. sinensis* increases IL-2 production in mouse spleen mononuclear cells stimulated with ConA.  

(3) Polysaccharides isolated from *A. sinensis* show strong antitumor activity.  

The pharmacological activities of *A. membranaceus* include: (1) It markedly stimulates mouse spleen cells to proliferate and induces macrophages to produce interleukin-6 (IL-6) and tumor necrosis factor (TNF). (2) Treatment with *A. membranaceus* increases albumin synthesis in the liver of nephrotic rat.  

(3) *A. membranaceus* inhibits coxakie virus replication in myocardial tissue of mice. (4) Extracts of *A. membranaceus* suppress the growth of renal cell carcinoma. (5) *A. membranaceus* extracts are able to enhance antibody production in mice. (6) *A. membranaceus* is an effective immune modulator, capable of potentiating in vitro the antitumor activity of lymphokine activated killer cells. Although BDGD has been generally used in traditional Chinese medicine for a long time, there has been a relative scarcity of definitive evidence to prove its pharmacological activity. In the present study, BDGD was selected for immunomodulatory activity determination.

The central event in generation of immune responses is the activation and clonal expansion of T cells. Interaction of T cells with antigens or phytohemagglutinin (PHA) initiates a cascade of biochemical events and gene expression that induces resting T cells to enter the cell cycle, proliferate and differentiate. It has been demonstrated in many previous studies with T cells that a series of genes such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) are pivotal in the growth of T lymphocytes induced by antigens or PHA. Interleukin-3 (IL-3) is a multifunctional cytokine, particularly affecting haematopoietic lineages. Thus, growth modulators or other external events that affect the T cell proliferation are likely to act by controlling the expression or function of the products of these genes. However, the immune responses to invasive organisms, if sufficiently intense or inappropriately prolonged, could paradoxically aggravate the injury or even causing death. The use of immunomodulatory medications must therefore be discreet. Regulation of T lymphocyte activation and proliferation and cytokine production has been shown to be one of actions of immunomodulatory drugs.

In order to prove the immunomodulatory effect of BDGD, human peripheral blood mononuclear cells (PBMC) containing T lymphocytes, were used as target cells. Effects of the aqueous extracts obtained from BDGD, *A. sinensis*, and *A. membranaceus* on PBMC proliferation and cytokines production induced by PHA were examined.

**MATERIALS AND METHODS**

*The source of A. sinensis and A. membranaceus*
Both *A. sinensis* and *A. membranaceous* were purchased from Chinese medicine shops in Taipei and identified by Dr. Te-Feng Tsai. Those voucher specimens have been deposited in the herbarium of the National Research Institute of Chinese Medicine.

**Preparation of BDGD crude extracts**

Both *A. sinensis* and *A. membranaceous* were ground into powder and dried at 45 °C to 50 °C. 100 gm of *A. sinensis* and *A. membranaceous* were extracted with boiling water for 40 min, respectively (600 ml × 2). Furthermore, BDGD was prepared as *A. sinensis* and *A. membranaceous* mixed at 1:5 ratio (w/w) and extracted with boiling water for 40 min (600 ml × 2). The infusions for *A. sinensis*, *A. membranaceous*, or BDGD were filtered through filter paper to remove insoluble materials and then supernatants were lyophilized. The obtained preparations were defined as AS (*A. sinensis* aqueous extracts), AM (*A. membranaceous* aqueous extracts) and BDGD (Blood-enriching Dang-Gui Decoction aqueous extracts). They were dissolved by phosphate buffer saline (PBS) and stored at 4 °C until for use.

**Preparation of PBMC**

Ten healthy male subjects (25 to 35 yr, mean age 29 yr) were chosen for this investigation. Heparinized human peripheral blood (20 ml) were obtained from healthy donors. PBMC was isolated by the Ficoll-Hypaque gradient density method as described previously. The 20 ml peripheral blood was centrifuged at 2000 rpm, 4 °C for 10 min to remove the plasma. Blood cells were diluted with PBS buffer then centrifuged in a Ficoll-Hypaque discontinuous gradient at 1500 rpm for 30 min. The PBMC layers were collected and washed with cold distilled water and 10X Hanks’ buffer saline solution (HBSS) to remove red blood cells. The cells were resuspended to a concentration of 2 × 10^6 cells/ml in RPMI-1640 medium supplemented with 2% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin.

**Lymphoproliferation test**

The lymphoproliferation test was modified from previously described. The density of PBMC was adjusted to 2 × 10^6 cells/ml before use. 100 µl of cell suspension was applied into each well of a 96-well flat-bottomed plate (Nunc 167008, Nunclon, Raskilde, Denmark) with or without 1 µg/ml PHA (Sigma). The extracts from *A. sinensis*, *A. membranaceous*, or BDGD were added to the cells at varying concentrations, respectively. The plates were incubated in 5% CO₂-air humidified atmosphere at 37 °C for 3 days. Subsequently, tritiated thymidine (1 µCi/well, NEN) was added into each well. After a 16 hr incubation, the cells were harvested on glass fiber filters by an automatic harvester (Dynatech, Multimash 2000, Billingshurst, U.K.). Radioactivity in the filters was measured by a scintillation counting. The activity of each extract on PBMC proliferation was calculated by the following formula:

\[
\text{Activity (\%)} = \frac{\text{Experiment group (CPM)} - \text{Control Group (CPM)}}{\text{Control Group (CPM)}} \times 100
\]
Control group (CPM)
**Determination of IL-2, IL-3, and IFN-γ production**

PBMC (2 × 10^5 cells/well) were cultured with PHA alone or in combination with varying concentrations of *A. sinensis*, *A. membranaccus*, or BDGD crude extracts for 3 days, respectively. The cell supernatants were then collected and assayed for IL-2, IL-3 and IFN-γ concentrations by the enzyme immunoassays (EIA; R&D systems, Minneapolis, U.S.A.). No detectable cross-reactivity with other cytokines has been reported for the EIA kits used.

**Statistical analysis**

Data were presented as Mean±SD, and the differences between groups were assessed with student’s *t* test.

**RESULTS**

**The aqueous extracts of BDGD, *A. sinensis*, and *A. membranaccus***

Altogether, aqueous extracts AS, AM, and BDGD were obtained from *A. sinensis*, *A. membranaccus*, and “Blood-enriching Dang-Gui Decoction”, respectively. These extracts are listed in Table 1 and recovery rate of each extract is also described. These extracts were used for the biological function assays.

**Effects on PBMC proliferation**

To study the effects on PBMC cell proliferation, resting cells or cells activated with PHA were treated with various concentrations of BDGD aqueous extracts and cell proliferation was determined by tritiated thymidine uptake. To elucidate whether *A. sinensis* or *A. membranaccus* played important roles in biological functions of BDGD, the effects of AS and AM on PBMC proliferation were also determined. As shown in Fig. 1, treatment with PHA for 3 days, stimulated cell proliferation by about 32 fold (483 ± 13.4 vs. 15299 ± 276.5 CPM, *P* < 0.0001), as reflected by the increase in tritiated thymidine uptake. Furthermore, the enhancement effects of BDGD, AS, and AM on the resting or activated cells were concentration dependent. At 12.5 µg/ml, the enhancement percentages of BDGD, AS, and AM were 9.4 ± 3.0%, 17.7 ± 3.9%, and 35.2 ± 1.6% on PBMC proliferation activated with PHA, respectively. The corresponding degrees of enhancement for 50 µg/ml were 27.1 ± 2.7%, 27.2 ± 2.0%, and 73.3 ± 0.6%, while that for 200 µg/ml were 96.6 ± 5.2%, 74.4 ± 4.1%, and 123.8 ± 2.2%. Moreover, the EC_{50} of BDGD, AS, and AM on activated PBMC proliferation are shown in Table 2. Comparison with other extracts, AM had the highest enhancement activity on HMNC proliferation.

**Effects on IL-2, IL-3, and IFN-γ production in activated PBMC**

To study whether enhancement of activated PBMC proliferation was related to cytokines production, the cells...
were incubated with or without BDGD, AS, and AM for 3 days. Supernatants were then collected, and the production

![Graph showing effects of BDGD, AS, and AM on PBMC proliferation.](image)

**Table 1. Aqueous extracts from “Blood-enriching Dan-Gui Decoction” included in the study.**

<table>
<thead>
<tr>
<th>Aqueous Extracts</th>
<th>Recovery Rate (%)</th>
<th>Weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. sinensis (AS)</td>
<td>24.79</td>
<td>24.79</td>
</tr>
<tr>
<td><em>A. membranaccus</em> (AM)</td>
<td>19.08</td>
<td>19.08</td>
</tr>
<tr>
<td>Blood-enriching Dang-Gui Decoction (BDGD)</td>
<td>15.56</td>
<td>14.00</td>
</tr>
</tbody>
</table>

of IL-2, IL-3, and IFN-γ was assayed by EIA. As shown in Fig. 2, treatment with PHA for 3 days, stimulated cell to produce IL-2 by about 15 fold (1.64 ± 2.1 vs. 25.2 ± 4.3 pg/ml, P < 0.001), as reflected by the increase in concentration. Enhancement effects of BDGD, AS, and AM on the resting or activated cells were concentration dependent. At 12.5 µg/ml, the enhancement percentages of BDGD, AS and AM were 107 ± 20.7%, 107 ± 19.9%, and 277 ± 17.1% on IL-2 production in PBMC activated with PHA, respectively. The corresponding degrees of
enhancement for 50 µg/ml were 135 ± 24.0%, 220 ± 35.1%, and 334 ± 23.5%, while that for 200 µg/ml were 929 ±

Fig. 2. The IL-2 production in PBMC cultures treated with BDGD, AS, or AM.
PBMC (2×10⁵/well) were treated by 0, 12.5, 25, 50, 100, and 200 µg/ml of (A) BDGD, (B) AS, or (C) AM with or without PHA (1 µg/ml) for 3 days. Then the cell supernatants were collected and IL-2 concentration was determined by EIA. Each point is the mean of three independent experiments.

<table>
<thead>
<tr>
<th>Biological Activity</th>
<th>AS</th>
<th>AM</th>
<th>BDGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC Proliferation</td>
<td>91.3 ± 2.5</td>
<td>23.8 ± 4.0</td>
<td>106.3 ± 5.8</td>
</tr>
<tr>
<td>IL-2 Production</td>
<td>8.0 ± 2.2</td>
<td>2.0 ± 0.8</td>
<td>12.0 ± 3.6</td>
</tr>
<tr>
<td>IL-3 Production</td>
<td>20.0 ± 1.6</td>
<td>31.3 ± 5.8</td>
<td>15.8 ± 2.7</td>
</tr>
<tr>
<td>IFN-γ Production</td>
<td>12.0 ± 3.0</td>
<td>4.0 ± 1.8</td>
<td>60.0 ± 6.8</td>
</tr>
</tbody>
</table>

PBMC (2 × 10⁵ cells/well) were cultured with PHA alone or in combination with varying concentration of AS, AM, or BDGD for 3 days. The cell supernatants were then collected and assayed for IL-2, IL-3, and IFN-γ concentrations by the enzyme immunoassay. The cell proliferation was determined by the tritiated thymidine uptake method. The EC₅₀ represents concentrations required to achieve 50% enhancement of cell proliferation or cytokines production.
Dang-Gui as an immunomodulatory agent

Fig. 3. Effects of BDGD, AS, and AM on IFN-γ production in PBMC cultures.

PBMC (2×10^5/well) were treated by 0, 12.5, 25, 50, 100, and 200 µg/ml of (A) BDGD, (B) AS, or (C) AM with or without PHA (1 µg/ml) for 3 days. Then the cell supernatants were collected and IFN-γ concentration was determined by EIA. Each point is the mean of three independent experiments.

Furthermore, as shown in Fig. 3, treatment with PHA for 3 days, stimulated cell to produce IFN-γ by about 55 fold (0.0 ± 2.6 vs. 55.0 ± 6.0 pg/ml, P < 0.0001). The IFN-γ production in activated PBMC was enhanced by BDGD, AS and AM. Enhancement effects of BDGD, AS and AM on IFN-γ production in the resting or activated cells were concentration dependent. Similarly, the IL-3 production in activated PBMC was stimulated by BDGD, AS and AM (Fig. 4). At 12.5 µg/ml, the enhancement percentages of BDGD, AS and AM were 18.5 ± 8.7%, 8.2 ± 3.3%, and 11.1 ± 1.1% on IL-3 production in PBMC activated with PHA, respectively. The corresponding degrees of enhancement for 50 µg/ml were 182.1 ± 14.0%, 137.3 ± 7.5%, and 65.2 ± 2.7%, while that for 200 µg/ml were 451.7 ± 28.5%, 226.8 ± 33.3%, and 227.8 ± 26.4%. The EC_{50} of BDGD, AS and AM on IL-2, IL-3, and IFN-γ productions in PBMC are shown in Table 2. Comparison with other extracts, AM had the lowest EC_{50} on IL-2 (8.0 ± 2.2 vs. 2.0 ± 0.8 µg/ml, P < 0.05; 12.0 ± 3.6 vs. 2.0 ± 0.8, P < 0.01) and IFN-γ (12.0 ± 3.0 vs. 4.0 ± 1.8 µg/ml, P < 0.05; 60.0 ± 6.8 vs. 4.0 ± 1.8 µg/ml, P < 0.001) production in PBMC. However, IL-3
Fig. 4: The IL-3 production in PBMC cultures treated with BDGD, AS, or AM.

PBMC (2×10^5/well) were treated by 0, 12.5, 25, 50, 100, and 200 µg/ml of (A) BDGD, (B) AS, or (C) AM with or without PHA (1 µg/ml) for 3 days. Then the cell supernatants were collected and IL-3 concentration was determined by EIA. Each point is the mean of three independent experiments.

Production in PBMC was more sensitive to BDGD treatment (20.0 ± 1.6 vs. 15.8 ± 0.8 µg/ml, P < 0.05; 31.3 ± 5.8 vs. 15.8 ± 0.8 µg/ml, P < 0.05).

DISCUSSION

In the present study, BDGD commonly used in traditional Chinese medicine for improvement health was extracted by water. The aqueous extracts of BDGD, *A. sinensis*, and *A. membranaccus* were subjected to biological activity assay. The results indicated that all three aqueous extracts enhanced cell proliferation in PBMC activated by PHA. The IL-2, IL-3, and IFN-γ production in activated PBMC were increased by BDGD, *A. sinensis*, or *A. membranaccus* treatment. We predict that immunomodulatory agents are present in BDGD. It has been demonstrated in previous studies with BDGD that BDGD has myocardial protection functions. This is the first report of immunomodulatory functions on human lymphocytes identified in BDGD.
PHA is a mitogen for T lymphocytes. It binds to N-acetylgalactosamine glycoproteins expressed on the surface of T cells and activates the cells to proliferate. Thus, T cells were major proliferating cells in PBMC activated with PHA. As we know, interaction of T cells with antigens or mitogens initiates a cascade of genes expression such as IL-2 and IFN-γ mRNA that induces the resting T cells to enter the cell cycle (G0 to G1 transition) and culminates in expression of the high affinity receptor for IL-2 and secretion of IL-2. In response to IL-2, the activated T cells progress through the cell cycle, proliferation and differentiating into memory cells or effector cells. Our results indicated that the aqueous extracts from BDGD stimulated IL-2 and IFN-γ production in PBMC cultures. We hypothesize that action mechanisms of BDGD on PBMC proliferation may have involved the regulation of IL-2 and IFN-γ production in the cell cultures. IL-3 is also called as a multiple colony stimulating factor (multi-CSF) and secreted by activated T cells. BDGD is used for enrichment red blood cell production in traditional Chinese medicine. The results indicated that aqueous extracts of BDGD increased IL-3 production in PBMC cultures. Comparison with other extracts, BDGD had the highest activity on IL-3 production. Thus, these results correlated with its putative pharmacological activities. We suggest that BDGD stimulating red blood cell growth may through stimulating T lymphocytes to secret IL-3. On the other hand, the cell proliferation and IL-2 and IFN-γ production of T lymphocytes play important roles in bacterial and viral infection. In the present study, we found that aqueous extracts from BDGD, A. sinensis, or A. membranaccus enhanced PBMC proliferation and IL-2 and IFN-γ production. It suggests that immune stimulators may be included in the BDGD, A. sinensis, and A. membranaccus.

The data indicated that A. sinensis and A. membranaccus augmented cell proliferation and IL-2, IL-3, and IFN-γ productions in PBMC cultures. These results correlated with previous studies that the extracts from A. sinensis and A. membranaccus are mitogens for murine and human lymphocytes in vitro. A. membranaccus possesses a strong immune potentiating activity in vivo. However, if sufficiently intense or inappropriately prolonged of immune responses, could paradoxically aggravate the injury or even causing diseases such as tissue inflammation and allergy and death. The Chinese herbs are applied for treatment these diseases. As we know that IFN-γ play an important role in inflammatory responses. Comparison with EC50, IFN-γ production in PBMC was more sensitive to A. sinensis treatment and BDGD had the highest EC50. Although A. membranaccus is a strong stimulator for IFN-γ production, ferulic acid contained in A. sinensis has an antiinflammatory effect. We suggest that BDGD can regulate immune responses in a reasonable strength and then improve human health. Plans are underway for the detection of IL-2, IL-3, and IFN-γ mRNAs expression in PBMC treated with BDGD, A. sinensis, or A. membranaccus. The isolation of pure principles and their mechanisms of action are subjected for further study.

ACKNOWLEDGEMENT

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REFERENCES


當歸補血湯抽出物促進人類周邊血單核細胞之細胞增殖與細胞激素產生

當歸補血湯 (Blood-enriching Dang-Gui Decoction) 是由當歸 (Angelica sinensis) 與黃耆 (Astragalus membranaceus) 所組成，在傳統中醫上用於促進人體健康，在此研究中我們將之用於免疫藥理活性分析。我們由當歸補血湯、當歸與黃耆中各別取得水抽出物，將之分別加入人類周邊血單核細胞 (Human peripheral blood mononuclear cells) 中，以放射線氚-胸嘧啶吸收法 (3H-Thymidine Uptake) 分析細胞增殖的情形，結果顯示當歸補血湯、當歸與黃耆各別水抽出物皆能促進植物凝集素 (Phytohemagglutinin) 所引起之細胞增殖，它們促進細胞百分之五十增殖之濃度 (EC\textsubscript{50}) 分別為 106.3 ± 5.8 µg/ml, 91.3 ± 2.5 µg/ml 及 23.8 ± 4.0 µg/ml。當歸補血湯、當歸與黃耆其促進人類單核細胞增殖之作用機轉可能與調控第二介白質 (Interleukin-2; IL-2)、第三介白質 (Interleukin-3; IL-3) 及丙種干擾素 (Interferon-γ; IFN-γ) 產生有關，因為當歸補血湯、當歸與黃耆各別水抽出物皆能促進植物凝集素 (Phytohemagglutinin; PHA) 所引起之第二介白質、第三介白質與丙種干擾素在人類周邊血單核細胞中之產生，並呈一劑量性反應。因此我們認為，在當歸補血湯中含有免疫調控因子 (Immunomodulatory agents)。

關鍵詞：當歸補血湯，當歸，黃耆，人類周邊血單核細胞，增殖，第二介白質。