Effects of *Inula Britannica* on the Production of Antibodies and Cytokines and on T Cell Differentiation in C57BL/6 Mice Immunized by Ovalbumin

Qing-Hua Song, Takao Kobayashi, Tie Hong and Jong-Chol Cyong*

Department of Bioregulatory Function, Graduate School of Medicine
The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

(Submitted May 25, 2001)

Abstract: In this study, we investigated the effects of *Inula Britannica* on the production of antibodies against ovalbumin, and the differentiation of T cells, in C57BL/6 mice. The oral administration of *Inula Britannica* suppressed IL-4 and IL-6 production in lymphocytes collected from an inguinal lymph node in the immunized leg. On the other hand, the intraperitoneal administration of *Inula Britannica* suppressed IgG1 production, the ratio of IFN-γ⁺IL-4⁻/IFN-γ⁻IL-4⁺ cells and cytokine production of IL-6. It was presumed that the effects of *Inula Britannica* on the production of antibodies were induced by regulation of the balance of Th1 and Th2. Further, IL-4 and IL-6 production by lymphocytes collected from an inguinal lymph node in the immunized leg were suppressed, and therefore production of antibodies was suppressed.

Keywords: *Inula Britannica*; C57BL/6 Mice; Cytokine; B Cell; T Cell; Antibody Production.

Introduction

*Inula Britannica* (IB) is composed of flower heads of several Compositae plants, such as *Inula Britannica* L. and *I. Serrata* BUR. In Kampo medicine, it is classified into a group of medicines for cough due to warmth and humidity, like others such as Pinellia Tuber and Playcodon Root. The effects are the promotion of diuresis and decrease of edema. Research on IB is still scarce. However, it is reported that components extracted from IB have cytotoxicity against human cancer cells (Park and Kim, 1998), and that taraxastery acetate, one component of IB, can protect against experimental hepatitis (Iijima *et al.*, 1995). We
have reported that IB regulated the production of cytokines induced by macrophages, so it 
may suppress the differentiation to Th1, and induce that to Th2. For these reasons, it showed 
preventive effects on experimental acute hepatic injuries (Song et al., 2000; Iijima et al., 1991), and on autoimmune diabetes (Kobayashi et al., submitted) in mice. 

In this study, we used C57BL/6 mice, known as Th1-like mice (Heinz et al., 1989). They were immunized several times, and the effects of IB on primary and secondary immune responses, the balance of Th1/Th2 and the production of cytokines secreted by the lymphocytes in a lymph node were investigated.

Materials and Methods

Experimental Animals

C57BL/6 female mice were obtained from Clea Japan (Tokyo, Japan) at six weeks of age, 
and were provided with commercial pellets (CE2 Clea Japan) through the whole experiment and tap water until the start of the treatment ad libitum.

Crude Drugs

IB was obtained from Uchida Co. (Tokyo, Japan). One hundred grams of the crude herb was boiled with 1000 ml of distilled water until the volume was reduced to 500 ml. The supernatant fluid was filtered and centrifuged. The yield was 15 % of the original herb weight. In the orally administered group IB (IB (p.o.)), the herbal extract was given as drinking water consecutively for five weeks. The concentration of the extract was adjusted to 2.5 g/kg/day as original herb weight (about 375 mg/kg/day as extract). In the intraperitoneally administered group (IB (i.p.)), the herbal extract was given as an intraperitoneal injection of 500 mg/kg/day as original herb weight (about 75 mg/kg/day as extract) once a week for five weeks.

Immunizations

Ovalbumin (OVA) was dissolved in PBS(−) at 0.1 mg/ml. The solution was mixed with incomplete Freund’s adjuvant (FIA) at 1:1 volume, and then was emulsified by repeated passage through a double-hubbed emulsifying needle until a stable emulsion was formed. One week after the administration of the herb extract, a group of six mice were immunized with 20 µg/mouse of OVA (1 µg/mouse) emulsion by injection into the rear footpad (Iijima et al., 1999). The intact mice were injected with emulsion without OVA. Two weeks after the first immunization, 50 µl of blood was collected from the fundus vein, diluted with 200 µl of PBS(−) containing 100 units/ml heparin, and centrifuged. The supernatant was collected and stored at −20°C for assay (primary response). On the next day of bleeding, mice were boosted with an additional OVA as on the first immunization. At two weeks after the second immunization, blood was collected from the trunk, allowed to clot for 1 hour and centrifuged at 1000 × g for 15 minutes at 4°C. Serum was stored at −20°C for assay (secondary response).
Antibodies

Antibodies used in this study were hamster anti-mouse CD3ε IgG (145-2C11), hamster anti-mouse CD28 IgG (37.51), Cy-Chrome (CC)-conjugated rat anti-mouse CD4 IgG2a (RM4-5), fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IFN-γ IgG1 (XMG1.2), R-phycoerythrin (PE)-conjugated rat anti-mouse IL-4 IgG2a (BVD4-1D11), horse radish peroxidase (HRP)-conjugated anti-mouse IgG2a (R15-19) monoclonal antibodies (Pharmingen, San Diego, CA) and peroxidase-conjugated (HRP) rabbit anti-mouse IgG1 (Organon Teknika).

Measurement of Antibody Production in Serum (Song et al., 2000)

Anti-OVA IgG1 and IgG2a antibodies in serum were measured by the ELISA method. The 96-well plates were coated with 4 µg/ml OVA in bicarbonate buffer, pH 9.6, at 4°C. Wells were blocked with 1% BSA (Fraction V, Calbiochem) for 2 hours at room temperature. Diluted serum was applied to the wells and incubated for 2 hours at 37°C. Bound antibodies were detected by incubation with HRP-conjugated anti-IgG1 or HRP-conjugated anti-IgG2a for 1 hour at 37°C. These reactions were developed with 2,2’-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) for 0.5 hours and optical density (OD) at 405 nm was read with a plate reader (BioRad).

Cytokine Expression of Splenic T Lymphocytes

At autopsy, the spleen was immediately removed and pressed with slide glasses in PBS(−). The cell suspension was passed through a #200 metal sieve and washed three times with PBS(−). Spleen cells were resuspended at 5 × 10^6/ml in RPMI1640 medium containing 10% fetal calf serum (FCS, Bioscience), stimulated for 18 hours with anti-CD3 (2 µg/ml) and anti-CD28 antibodies (2 µg/ml) and cultured for the final 6 hours in a medium containing 3 µM monensin (Sigma). The cells were stained with 0.5 µg of Cy-chrome-conjugated anti-CD4, fixed, permeabilized and again stained with 0.1 µg of FITC-conjugated anti-IFN-γ and PE-conjugated anti-IL-4 antibodies by using a cell fixation/permeabilization kit (Cytofix/Cytoperm, Pharmingen) according to the manufacturer’s instructions. Fluorescence-activated cells were analyzed by an EPICS XL flow cytometer (Coulter Cytometry Co., Hialeah, FL). A fluorescence histogram of at least 5000 counts was collected for each sample.

Cytokines Production

Lymphocytes were collected from an inguinal lymph node in the immunized leg. Lymphocytes were prepared as described above, washed and resuspended at 2 × 10^6 cells/ml in RPMI1640 medium containing 10% FCS and stimulated with 10 µg/ml lipopolysaccharide (LPS). After incubation for 36 hours, the supernatant was collected, and IL-6 levels were measured by the ELISA method using an Immunoassay Kit (Bio Source).
Data were analyzed by Student’s t-test to determine significance.

**Results**

**Effects on Spleen Weight**

The spleen weight of the control group, immunized with OVA but without administration of IB, was 4.57 ± 0.16 mg/g body weight, which was significantly increased compared with the intact group, neither immunization with OVA nor without administration of IB, 3.51 ± 0.09 mg/g body weight. The IB (p.o.) group showed 4.06 ± 0.14 mg/g body weight, and the IB (i.p.) group showed 4.17 ± 0.13 mg/g body weight, which were significantly lower increases than those of the control group (Fig.1).

**Effects on Production of IgG Antibody**

The production of IgG1 decreased significantly in the IB (i.p.) group in the primary response, and than was a tendency to decrease in the secondary response. However, no effect was observed in the IB (p.o.) group (Table 1).

**Table 1. Effects of Inula Britannica on Antibody Production in Serum (mean ± SE, n = 6)**

<table>
<thead>
<tr>
<th></th>
<th>Primary Response</th>
<th>Secondary Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG1</td>
<td>IgG2a</td>
</tr>
<tr>
<td>Control</td>
<td>0.10 ± 0.02</td>
<td>0.10 ± 0.06</td>
</tr>
<tr>
<td>IB (p.o.)</td>
<td>0.11 ± 0.03</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>IB (i.p.)</td>
<td>0.05 ± 0.01*</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>

* Significantly different from the control at p < 0.05.
Effects on Production of Cytokines in Spleen Th Cell

The proportions of IFN-γ$$^+$$IL-4$$^-$$ and IFN-γ$$^-$$IL-4$$^+$$ were 1.52 ± 0.17% and 1.45 ± 0.11% in the control group, which were significant increase compared with those in the intact group, 0.98 ± 0.07% and 0.84 ± 0.29%. The ratio of IFN-γ$$^+$$IL-4$$^-$$ / IFN-γ$$^-$$IL-4$$^+$$ was 1.04 ± 0.06 in the control group, and there was a tendency to decrease compared with the intact group, 1.73 ± 0.48 (p = 0.077). IB did not affect the proportion of IFN-γ$$^+$$ IL-4$$^-$$ or IFN-γ$$^-$$IL-4$$^+$$, but the ratio of IFN-γ$$^+$$IL-4$$^-$$ / IFN-γ$$^-$$IL-4$$^+$$ was 1.63 ± 0.23 in the IB (i.p.) group, indicating a suppression compared with the control group (Fig. 2).

![Graph showing cytokine production](image)

Figure 2. Effects of *Inula Britannica* on cytokine production of CD4$$^+$$ T cell stimulated by anti-CD3/CD28 antibody. In the IB (p.o.) group, *Inula Britannica* extract was orally administered at a dose of 375 mg/kg/day as an extract consecutively for five weeks, and in the IB (i.p.) group, it was intraperitoneally administered at a dose of 75 mg/kg/day once a week for five weeks. Each value is the mean ± SE of six mice. *: p < 0.1 or 0.05 vs. Control.

Effects on Cytokine Production in Inguinal Lymph Node Cells Collected From the Immunized Leg

We measured the production of cytokines in lymphocyte from a lymph node stimulated with 1 µg/ml anti-CD3 antibody. The production of IL-4 in the IB (p.o.) (2.78 ± 0.45 pg/ml) and IB (i.p.) groups (1.81 ± 0.54 pg/ml) was significantly reduced compared with that in the control group (5.12 ± 0.85 pg/ml). No significant effects on IFN-γ production or the ratio of IFN-γ/IL-4 were observed (Fig. 3).

Furthermore, we investigated the effects of IB on the production of IL-6 in lymphocytes of a lymph node stimulated with 5 µg/ml LPS. The IB (p.o.) group showed 370 ± 79 pg/ml, a significant reduction compared with 692 ± 119 pg/ml in the control group. A tendency to reduction was observed in the IB (i.p.) group, 336 ± 154 pg/ml (Fig. 4).

Discussion

OVA is known as an intense allergy antigen. The infiltration of subcutaneous and intestinal tissue by eosinophilic granulocytes (Hakugawa *et al.*, 1997), induction of cytokine production (Horino *et al.*, 1997), and effects on the balance of Th cells have been reported after
immunization with OVA (Vinuesa et al., 1997; Horino et al., 1997). Recently, immunological diseases such as atopic dermatitis, allergy, said to be the modern sickness, have shown a tendency to occur more frequently. Decisive therapy methods in modern medicine have not yet been determined, but side effects are serious problems (Toyoshima, 1998; Kurihara et al., 1996). On the other hand, it is reported that Kampo medicines have been useful in the treatment of these diseases (Mori and Koda, 1995; Onda, 1997). IB is believed to promote
the diuresis and decrease edema. For the therapy for atopic dermatitis, it is prescribed that the promotion of diuresis and decrease of edema are indispensable, according to the theory of Kampo medicine. IB is expected to be effective in the therapy of atopic dermatitis. C57BL/6 mice are dominant in Th1 (Heinzel et al., 1989) but immune sensitization by OVA is dominant in Th2 (Lim et al., 1998). To investigate the effects of IB on immune system in Th1-dominant mice, we used the OVA immunized model, and detected antibody production to OVA, the balance of Th1 and Th2, and the cytokines production of spleen cells and lymphocytes of a lymph node. It is thus considered that Th1-dominant mice were used to establish the model of Th2-dominance, and moreover the effects of IB were investigated.

In this study, IB (i.p.) reduced the production of IgG1 in the primary responses in C57BL/6 mice. On the other hand, IB (p.o.) did not affect them. Though it was suggested that IB (p.o.) might be modified by the digestive organs, the efficiency of this treatment was lower than that of IB (i.p.).

IFN-γ and IL-4 regulate the production of antibodies (Croft and Swain, 1991; Nickolaus et al., 1999). IL-4 is known as a B cell growth factor (Punnonen et al., 1997). IFN-γ and IL-4 restrict each other. To confirm whether the suppression by IB of production of antibodies is related to IFN-γ or IL-4, lymphocytes were collected from a lymph node in the immunized leg, and the production of cytokines was measured. It was observed that IB suppressed the production of IL-4 but not of IFN-γ. Because IL-4 promotes the production of IgG1 (Croft and Swain, 1991), it was suggested that the effects of IB were expressed through the suppression of IL-4. On the other hand, IFN-γ is known to be related to the production of IgG2a; IB did not affect IFN-γ, so it cannot affect IgG2a.

IL-6 induces antibody production from B cells (Bonig et al., 1998). It was thought that the decrease of this antibody production was related to IL-6. We measured the production of IL-6 in lymphocytes. It was certain that the production of IL-6 was suppressed by IB both p.o. and i.p. It was considered that these decreases of antibody production were due to a decrease of the inducing factor of antibody production.

IL-4 and IFN-γ are cytokines produced by T cells. The production of antibodies closely relates with T cells (Takatsu, 1997; Yoshino and Sagai, 1999). It was determined that IB suppressed cytokine production in the lymphocytes from a lymph node, so it was expected that IB might also affect the production of cytokines by cells in the spleen. In this experiment, IB did not affect IFN-γ IL-4⁺ or IFN-γ IL-4⁻ cells, but increased the ratio of IFN-γ IL-4⁺/IFN-γ IL-4⁻. It was suggested that IB could promote Th1 dominance, and this corresponded with the results of cytokine production in lymphocytes, but did not correspond with our precious report of the effects of IB on hepatic injuries in mice (Song et al., 2000). The reason may be that the hepatic injuries in the precious report were induced by LPS, a Th1-like model, but the model in this experiment was OVA immunity, a Th2-like model. It was suggested that IB might suppress Th1 when Th1 may be dominant, and suppress Th2 when Th2 is dominant. IB would then have the effect of maintaining the Th balance.

In conclusion, the present study indicates that the effects of IB on the antibodies were due to regulation of the balance of Th1/2. IB also suppressed cytokine production by lymphocytes collected from an inguinal lymph node, especially IL-4 and IL-6, which are closely related to antibody production, and then suppressed the production of antibodies.
The most characteristic feature of Kampo medicines is accordance with the patient’s constitution and symptoms, which governs choice of crude drugs, establishing the prescription. IB showed different actions in mice of a different strain, which proves the theory of “therapy based on Kampo Diagnosis,” an important theory in traditional Kampo medicine. On the other hand, IB is a multicomponent drug, since it is a crude drug. An active component may be potent or weak according to different constitutions. In clinical practice, Kampo medicines must be used with regard to the patient’s constitution and the effect to be expected.

Acknowledgement

We thank Tsumura Co. for this support of this research.

References


