Effects of *Citrus unshiu* Powder on the Cytokine Balance in Peripheral Blood Mononuclear Cells of Patients with Seasonal Allergic Rhinitis to Pollen

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We evaluated the effects of a 50% methanol extract of *Citrus unshiu* powder (MEC) on cytokines in peripheral blood mononuclear cells (PBMCs) obtained from patients with seasonal allergic rhinitis to cedar pollen. The levels of cytokines, such as TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, and GM-CSF, produced by pollen-stimulated PBMC were measured. We found that MEC suppressed pollen-induced TNF-α release and increased IFN-γ release from PBMCs. The results suggest that *Citrus unshiu* powder has an immunomodulatory effect *in vitro* and that its use could improve seasonal allergic rhinitis symptoms.

Key words: anti-allergic effect; rhinitis to pollen; *Citrus unshiu*; cytokine; PBMC

More than 25% of the population in industrialized countries suffers from IgE-mediated (type I) allergic symptoms.¹ The disease occurs as the result of a breakdown in the mechanisms that control responses to innocuous environmental antigens (allergens). Pollen from the Japanese cedar (*Cryptomeria japonica*) is one of the main causes of allergy in spring in Japan; more than 10% of the population suffers from pollinosis caused by exposure to this pollen.²,³ Two major allergens, Cry j 1 and Cry j 2, have been isolated from this pollen,²,³ and both Cry j 1 and Cry j 2 are thought to be important in the pathogenesis of Japanese cedar pollinosis.⁴ To treat pollinosis effectively, the patient’s management should be directed not only towards rapid and adequate relief from clinical symptoms, but also towards a sustained improvement in daily living. Even though conventional therapies for allergic symptoms are advancing steadily, natural products, such as phytochemicals and herbal extracts, have been widely used by consumers as alternatives to prescription drugs, even though definitive clinical evidence of their efficacy is lacking.

Recently, citrus flavonoids have been reported to possess various pharmacologic effects, such as anti-allergic, anti-inflammatory effects and even anti-cancer activity.⁵–⁷ Our group has confirmed that hesperetin, hesperidin aglycone, inhibited IgE-mediated stimulation of rat basophilic leukemia cell line RBL-2H3, and that *Citrus unshiu*, one of the most popular citrus fruits, suppressed IgE-mediated stimulation of the basophils of patients with seasonal allergic rhinitis (SAR) *in vitro*.⁸ The aim of the current study was to evaluate the effects of *Citrus unshiu* powder on the cytokine balance in freshly prepared peripheral blood mononuclear cells (PBMC) obtained from patients with SAR to Japanese cedar pollen. The levels of cytokines, such as TNF-α, IFN-γ, and IL-4, that are produced by pollen-stimulated PBMC were measured. Since peripheral serum cytokine levels are known to be changeable even with pollen exposure, we developed a method of evaluating cytokine production in pollen-stimulated PBMC.

Fruits (*Citrus unshiu*) were squeezed for orange juice making by a standard method, and the residue was powdered at Nitto Fuji Milling Co. (Shizuoka, Japan). The composition of the powder was as follows: water 8.0%, minerals 2.5%, proteins 7.9%, fat 3.0%, fibers 10.1%, citric acid 4.8 g, carbohydrate (mainly fructose) approximately 70%. A 50% methanol extract of the *Citrus unshiu* powder (MEC) was used in assays, and
the MEC concentrations were expressed as the material powder equivalent.

Whole blood samples (approximately 8 ml) were donated by 15 patients with SAR to Japanese cedar pollen. The diagnosis of pollen allergy was made on the basis of an episode of allergic reaction, supported by laboratory evidence, such as IgE-ELISA. Written informed consent was obtained from all donors. Initially, the PBMCs of four patients (nos. 1–4) were stimulated with Japanese cedar (Cryptomeria japonica) pollen extract (Hayashibara Biochemical Laboratories, Okayama) with or without MEC. PBMCs were isolated from whole blood samples by density centrifugation in lymphocyte separation medium (ICN Biomedicals, Aurora, OH). The PBMCs were washed and resuspended in RPMI1640 medium (Invitrogen, Boston, MA) containing 10% human AB serum (Takara Bio, Shiga), 3 mM glutamine (Invitrogen), 50 µM 2-mercaptoethanol (Kanto Chemical, Tokyo), 50 U/ml penicillin (Invitrogen) and 50 µg/ml streptomycin (Invitrogen). The PBMCs (1 × 10^6/well) were cultured in a 96-well cell culture plate (Corning Coster, Wilkes Barre, PA) for 24 h at 37 °C under a humidified 5% CO2 atmosphere with or without pollen extract (0.2 or 2 µg/ml) and with MEC (400 µg/ml). Cultures of PBMCs alone were included as a negative control. After culture, the plate was centrifuged at 300 g for 10 min, and cytokines (TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, and GM-CSF) released to the media were examined due to its antitumor activity; 9) it is believed to play a role in many immunologic and inflammatory reactions. 10,11) Hence, it was not unexpected that the addition of pollen to the PBMCs triggered TNF-α production (Fig. 1). It has also been reported that treatment with anti-TNF-α antibody can inhibit IgE-mediated allergy. 12) Food components can have a similar effect; for example, in mice, a perilla leaf extract suppressed overproduction of TNF-α and subsequently had anti-inflammatory and anti-allergic activities. 13) Given these data, it is thought that MEC, which has the same activity, would also be effective for suppressing the allergic reaction in vivo.

In order to enhance PBMC cytokine production, we also stimulated the PBMCs of the other 11 patients (nos. 5–15) with pollen extract at increasing concentration (20 µg/ml) and for a longer time (72 h). Under these conditions, we documented the higher release of certain cytokines, such as IFN-γ and IL-4, from the pollen-stimulated PBMCs on preliminary micro-bead assay. The concentrations of IFN-γ and IL-4 were measured by sandwich ELISA (DuoSet Sandwich ELISA, R&D Systems, Minneapolis, MN) to confirm the results obtained by the micro-bead analyses. (Table 1). It was found that the pollen extract suppressed IFN-γ release from the patients’ PBMCs, but did not affect IL-4 production (Table 1, B). Low allergen-induced IFN-γ secretion from the patients’ PBMCs was also observed by Contreras et al. 14) Of particular note in our study, we found that MEC markedly enhanced IFN-γ release from pollen-stimulated PBMCs (Table 1, C). Therefore, it appears that MEC had an immunomodulatory effect in vitro.

The IFN-γ/IL-4 ratio, an indicator of Th1/Th2 balance, was examined. The ratio dropped dramatically (p < 0.0001) with the addition of pollen extract (Fig. 2, B). MEC, by enhancing IFN-γ production, increased (p < 0.001) this ratio (Fig. 2, C) without changing the IL-4 level. On the other hand, Higa et al. 15) recently reported that fisetin, one of the flavonols, inhibited the expression of Th2 cytokines, such as IL-4, IL-5, and IL-13, via the NFAT (nuclear factor of activated T cells) pathway in human basophilic cell line KU812 cells stimulated with calcium ionophore and/or

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**Fig. 1.** Effects of MEC on Pollen-Induced TNF-α Release from SAR Patients’ PBMCs.

Japanese cedar pollen extract (0.2 or 2 µg/ml) was added to PBMCs of four patients (nos. 1–4) with or without MEC. The subsequent release of TNF-α in the supernatant was quantified by the micro-bead method by the Bio-Plex Suspension Array System. Data are expressed as means ± standard errors. *p < 0.05*.
Table 1. Effects of MEC on Concentrations of IFN-γ and IL-4 in Pollen-Stimulated PBMCs

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>5</td>
<td>14.7</td>
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<td>25.3</td>
<td>6.5</td>
</tr>
<tr>
<td>7</td>
<td>15.3</td>
<td>nd</td>
</tr>
<tr>
<td>8</td>
<td>16.5</td>
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</tr>
<tr>
<td>9</td>
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</tr>
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<td>10</td>
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<td>5.9</td>
</tr>
<tr>
<td>11</td>
<td>21.2</td>
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<td>6.5</td>
</tr>
<tr>
<td>15</td>
<td>18.8</td>
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A, non-stimulated PBMCs (control)
B, pollen-stimulated PBMCs without MEC
C, pollen-stimulated PBMCs with MEC
*nd, not detected.

Fig. 2. Effects of MEC on the IFN-γ/IL-4 Ratio in Pollen-Stimulated PBMCs.
Japanese cedar pollen extract (20 μg/ml) was added to PBMCs of 11 patients (nos. 5–15) with or without MEC. The subsequent release of IFN-γ and IL-4 in the supernatant was quantified by sandwich ELISA. The lines and closed circles indicate individual patient data. A–C, see Table 1.

In conclusion, of the nine cytokines tested that were produced by PBMCs of SAR patients, TNF-α and IFN-γ were found to respond well to pollen stimulation. MEC, an extract of Citrus unshiu powder, suppressed TNF-α production and increased the IFN-γ/IL-4 ratio, an indicator of the Th1/Th2 balance, in pollen-stimulated PBMCs of the SAR patients. These results imply that the powder has an immunomodulatory effect and that its use could improve SAR symptoms. A pilot clinical study of the use of Citrus unshiu powder for SAR prevention is underway.

References


