5. ANTI-INFLAMMATORY AND ANTIALLERGIC ACTIVITIES OF PERILLA EXTRACTS

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INTRODUCTION

Many types of cells, including macrophages, produce tumor necrosis factor (TNF). TNF was recently recognized as an important host defense factor that affects not only tumor cells but many kinds of normal cells (Vilcek and Lee, 1991). However, prolonged exposure to TNF might contribute to the wasting of the host which is associated with many chronic disease states (Beutler, 1988). Moreover, acute overproduction of TNF in bacterial infection may induce septic shock leading to acute organ failure and death. Inhibiting overproduction of TNF is therefore an advantageous step toward the suppression of acute and chronic inflammation. This work was undertaken to ascertain whether Perilla extract, which may be a new candidate for an antiinflammatory and antiallergic reagent, can inhibit TNF production and inflammatory states in mice.

MATERIALS AND METHODS

Animals and Cell Line

Males of specific pathogen free strains of C3H/He and ICR mice were used at 6–8 weeks of age. L-929, a transformed fibroblast cell line originally derived from a C3H/He strain mouse, was used for TNF assay.

Culture of Macrophages

Peritoneal exudate cells were obtained from mice 24 hrs after *i.p.* injection of 4 mg glycogen. These cells were suspended in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum. The peritoneal cells were incubated in 96-well microtest plates for 1.5 hr at 37° C and the medium was removed to obtain adherent cells. More than 95% of these adherent cells were macrophages, as determined by Giemsa staining and measurement of uptake of carbon particles. These exudative macrophages were finally cultured in 0.2 ml of medium with or without test samples. The TNF concentration of macrophage supernatant was measured immediately after its collection.

Perilla Extracts

Dried leaves of green types of Perilla (*Perilla frutescens* (L.) Britton var. acuta Kudo forma virides Makino) were mixed with an equal volume of distilled water. This mixture was

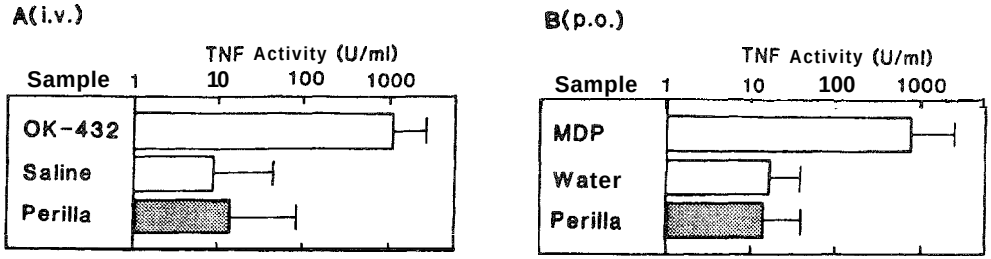

Figure 1 Priming effect of Perilla extracts on TNF production.

homogenized with a polytron and centrifuged to obtain supernatant. The supernatant was orally administered. The extracts were adjusted to pH 7.4 and to an isotonic condition with an osmometer, and then sterilized with a millipore membrane filter to use intravenously. Some of Perilla extracts were kindly provided by Amino Up Chemical Co., Ltd. (Sapporo, Japan).

Measurement of TNF Activity

Production of circulating TNF was measured by the L cell cytotoxicity method. Briefly, TNF activity of serum preparation was measured by in *vitro* 18-hour cyto-toxicity assay in the presence of actinomycin D. Recombinant human TNF was used as a standard TNF preparation. The specificity of TNF in this assay was checked by an antibody-neutralization test using anti-mouse TNF rabbit antibody. In this study, OK-432 (an antitumor reagent from a Gram-positive bacterium, *Streptococcus pyogenes*) was chosen as the TNF-trigger, because of its stable triggering effect and potent activity comparable to LPS, but with no acute toxicity to animals.

INHIBITION OF TNF PRODUCTION BY PERILLA EXTRACTS IN *VIVO*

There are two steps in the process of producing TNF: a priming step (e.g., cytokine or irnmunopotentiator pretreatment) and a triggering step (e.g., bacteria or LPS treatment). We previously reported that a primed state ready for TNF triggering can be obtained by several immunopotentiators and some selected cytokines such as interferons and interleukin-2 (Okutomi and Yamazaki, 1988). We also showed that some vegetable extracts can prime the endogenous production of TNF in *vivo* (Yamazaki et *al.*, 1992). Here, we examined whether Perilla extracts are capable of causing this priming effect for TNF production.

As shown in Figure 1, the TNF activity triggered by OK-432 (3KE) without a priming sample was below 20 U/ml. After priming with MDP (0.5mg) or OK-432 (0.3KE) with triggering by OK-432 (3KE), TNF production was significantly enhanced to about 1000 U/ml. Perilla extracts, however, could not induce the priming effect.

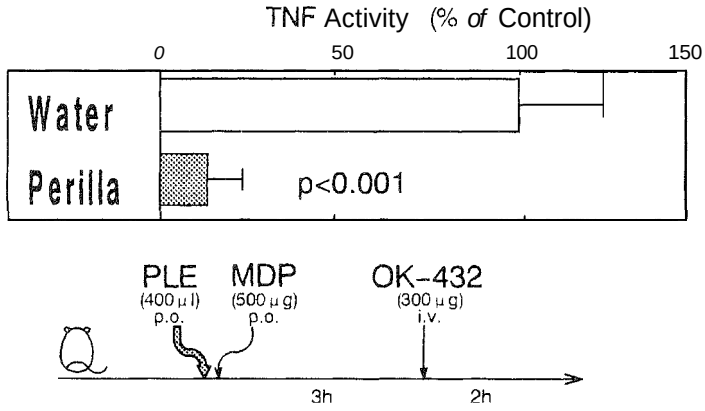

Figure 2 Inhibition of endogeneous TNF production by Perilla.

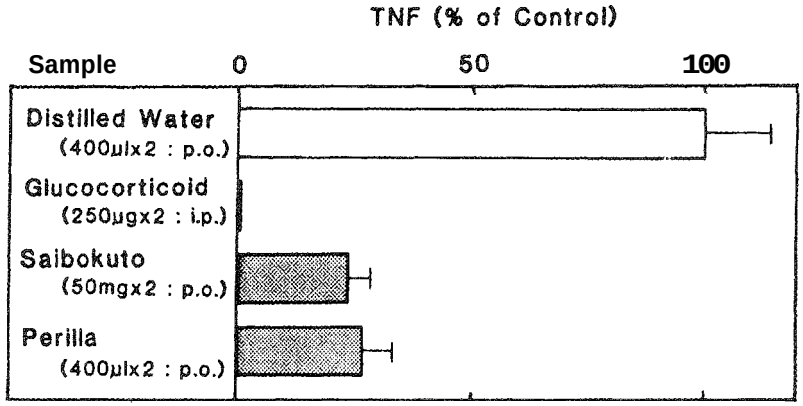

Figure 3 TNF-inhibitory activities of anti-inflammatory reagents.

Next, we examined whether Perilla extracts inhibit the endogenous production of TNF in *vivo*. Mice were administered *p.o.* 0.5 mg of MDP and 3 hrs later were given *i.v.* injection of OK-432. These mice produced 1000-3000 U/ml of TNF. Perilla extracts were administered *p.o.* simultaneously with MDP. As shown in Figure 2, TNF activity was significantly inhibited by *p.o.* administration of green Perilla extracts. Glucocorticoid is a powerful antiinflammatory reagent and saibokuto, a Chinese medicine complex, is also widely used to treat inflammatory disease in Japanese clinics. Figure 3 shows that Perilla extracts have nearly the same activity against TNF production as does saibokuto.

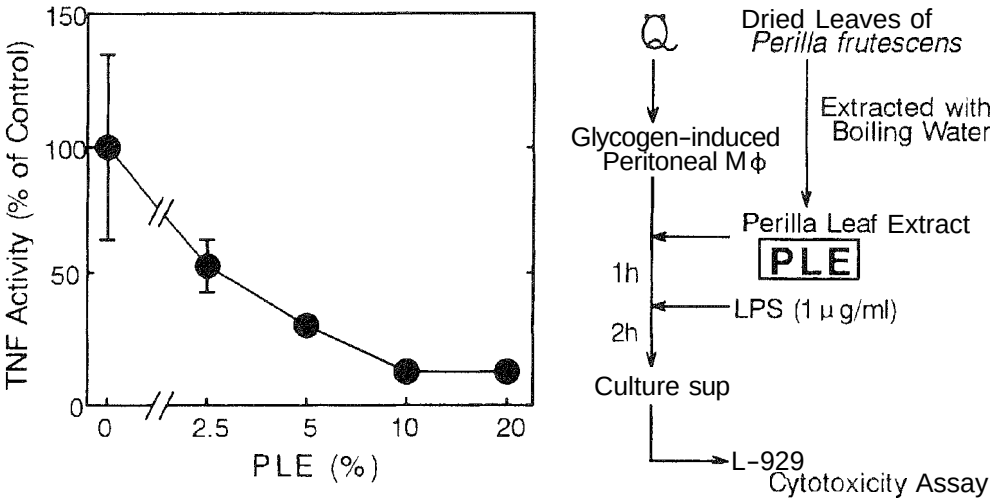

Figure 4 Inhibition of TNF production from macrophages by Perilla

INHIBITORY ACTIVITY OF PERILLA EXTRACTS FOR *IN VITRO* TNF PRODUCTION

Macrophages can release TNF into a culture after appropriate stimulation *in vitro*. We examined whether Perilla extracts can also inhibit TNF production from macrophages *in vitro*. For this, glycogen-induced macrophages were incubated with Perilla extracts for an hour, then LPS-triggered TNF release for 2 hours was measured. As shown in Figure 4, Perilla extracts inhibited TNF-release from macrophages *in vitro* as well as in *vivo*. Pretreatment of macrophages was effective even 10 min after addition of the extracts.

ANTI-INFLAMMATORY ACTIVITIES OF PERILLA EXTRACTS

Certain irritants can induce acute inflammation in mice. Phorbor ester (TPA) induces leukutriene-dependent inflammation and arachidonic acid induces prostaglandin-dependent inflammation. We selected these two typical irritants as acute inflammatory models. Ear swelling of mice was maximally observed 2–4 hrs after painting of these irritants.

Perilla extracts (0.4ml/mouse) were administered *p.o.* **3** hrs and 18 hrs before the painting of 0.25mg of arachidonic acid. As shown in Figure 5, the extracts inhibited arachidonic acid-induced ear swelling of mice. However, their administration after arachidonic acid painting was ineffective. TPA-induced acute inflammation was also inhibited by *p.o.* administration of Perilla extracts (Figure 6), but was not inhibited by their post-treatment. Perilla extracts suppressed two typical acute inflammations.

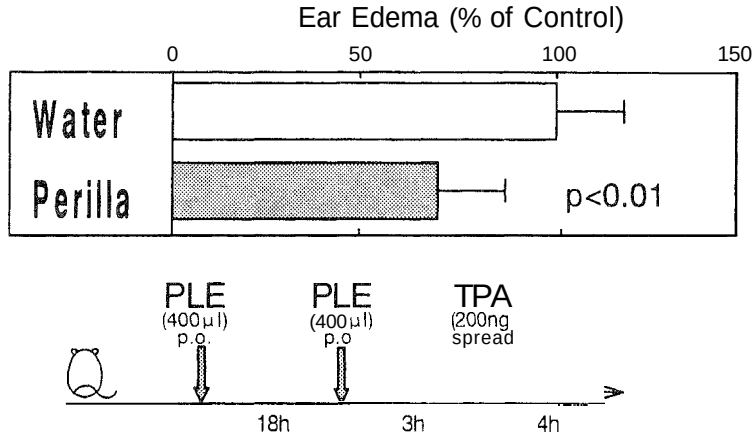

Figure 5 Inhibition of TPA-induced ear swelling by Perilla

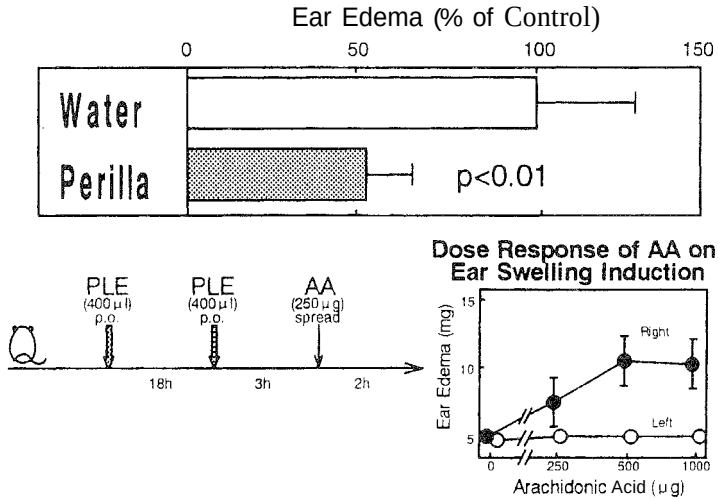

Figure 6 Inhibition of arachidonic acid-induced ear swelling by Perilla

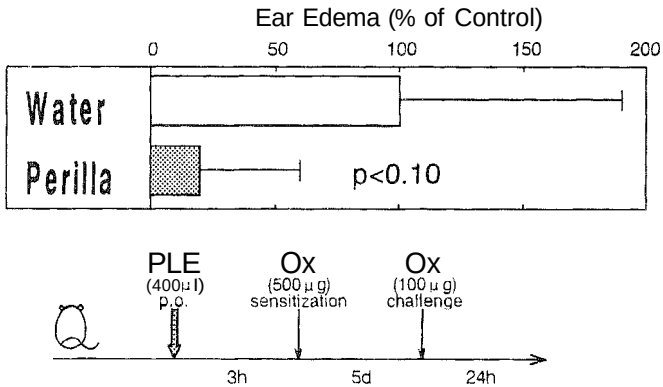

Figure 7 Inhibition of oxazolon-induced ear swelling by Perilla

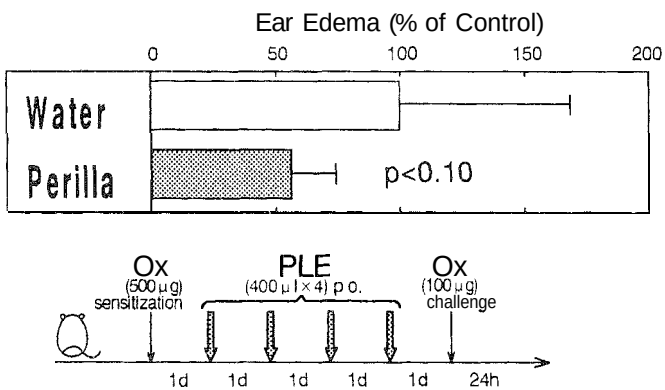

Figure 8 Inhibition of oxazolon-induced ear swelling by Perilla

ANTI-ALLERGIC ACTIVITY OF PERILLA EXTRACTS

Oxazolon induces the type IV allergy. For sensitization, oxazolon was painted onto the abdominal skin of mice and 5 days later challenge-painting was done. Ear swelling was checked 24 hrs after oxazolon challenge. Perilla extracts inhibited oxazolon-induced ear edema when administered before sensitization (Figure 7). Although this delayed type allergy was also suppressed by *p.o.* administration of Perilla extracts prior to challenge (Figure 8), the suppression was not observed after oxazolon challenge.

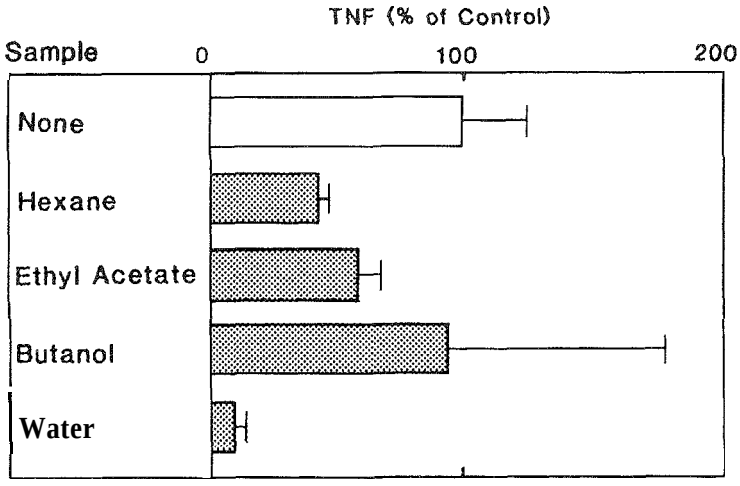

Figure 9 Inhibition of TNF production by solvent-extracted Perilla

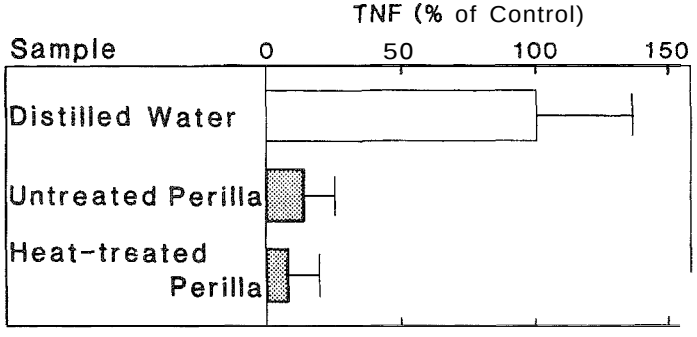

Figure 10 Heat stability of inhibitory effect of Perilla on TNF production

CHARACTERIZATION OF PERILLA EXTRACTS

To identify the active ingredients in Perilla extracts, we first focused on flavor substances of *Perilla frutescens*, which contains various kinds of flavor chemicals, the main perfume substance being perillaldehyde. Perillaldehyde did not inhibit the endogenous production of TNF.

Next, we extracted the active substances in *Perilla frutescens* using several solvents. As shown in Figure 9, the inhibitory activity for TNF production was largely recovered in the water-soluble fraction but not in the fraction of butanol. Both hexane and ethyl acetate fractions showed relatively weak activity. These results suggest that most of the active ingredients in Perilla extracts are hydrophilic rather than hydrophobic substances.

The active substances inhibiting TNF production was heat stable, showing no loss of activity after heating at 100°C for 10 min (Figure 10). The inhibitory activity was restored in the fraction of molecular size below 10,000 daltons. From these results the active factors in *Perilla frutescens* are recognized to be stable and small sized molecular substances.

CONCLUSION

TNF is an important host defense factor that affects many kind of normal cells. (Vilcek and Lee, *1991).* Its overproduction, however, is associated with acute and chronic inflammation and allergy (Beutler, *1988).* Anti-TNF antibody was reported to suppress both types III and IV allergies (Piguet *et al.*,1991; Zhang *et al.*,1992). TNF is produced from many types of cells including mast cells which are key cells in inflammation and allergy (Walsh *et al.*,1991). TNF can also stimulate IgE production. The suppression of TNF production is thus a clue to the causes of inflammation and allergy.

Here, we reported that Perilla extracts inhibited TNF production both in *vivo* and in *vitro.* Oral administration of these extracts suppressed TPA- and arachidonic acid-induced inflammation and oxazolon-induced allergy. The active substances in *Perilla frutescens* may be hydrophilic, stable and small molecular compounds. Perilla extracts may thus be a new candidate for an anti-inflammatory and antiallergic reagent.

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