



Oral administration of *Uncariae rhynchophylla* inhibits the development of DNFB-induced atopic dermatitis-like skin lesions via IFN- γ down-regulation in NC/Nga mice

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ABSTRACT

Ethnopharmacological relevance: *Uncariae rhynchophylla* (UR) is an herb which has blood pressure lowering and anti-inflammatory effects and has been prescribed traditionally to treat stroke and vascular dementia.

Aim of study: In the present study, we examined whether UR suppress Atopic dermatitis (AD)-like skin lesions in NC/Nga mice treated with 2, 4-dinitrofluorobenzene (DNFB) under SPF conditions.

Material and methods: The effect of UR in DNFB-treated NC/Nga mice was determined by measuring the skin symptom severity, levels of serum IgE, and of the amounts of IL-4 and IFN- γ secreted by activated T cells in draining lymph nodes.

Results: Oral administration of UR to DNFB-treated NC/Nga mice was found to inhibit ear thickness increases and the skin lesions induced by DNFB. IFN- γ production by CD4⁺ T cells from the lymph nodes of DNFB-treated NC/Nga mice was significantly inhibited by UR treatment, although levels of IL-4 and total IgE in serum were not.

Conclusion: UR may suppress the development of AD-like dermatitis in DNFB-treated NC/Nga mice by reducing IFN- γ production.

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1. Introduction

Atopic dermatitis (AD) is one of the most common skin diseases and is characterized by a chronic and relapsing inflammatory dermatitis with immunological disturbance, and pruritic and eczematous skin lesions (Leung and Bieber, 2003). The incidence of AD is increasing in industrialized countries and 10–20% of children worldwide are affected by AD (Leung and Bieber, 2003; Shiohara et al., 2004). AD is a multifactorial disease with genetic and environmental components, and therefore its pathogenesis has not been fully defined (Miyauchi-Hashimoto et al., 2005).

NC/Nga mice are the most extensively studied animal model of AD. The NC/Nga strain, derived from Japanese fancy mice, was established as an inbred strain in 1957. These mice sponta-

neously develop AD-like eczematous skin lesions when kept in an air-uncontrolled conventional housing, but not when maintained under specific pathogen-free (SPF) conditions (Shiohara et al., 2004). Given the similarity between the clinical symptoms displayed by NC/Nga mice and AD in humans, models based on these mice are considered to be important for understanding and developing treatments for AD (Shiohara et al., 2004). Although NC/Nga mice can also be used for evaluating effective therapeutic strategies, the low incidence of AD-like lesions, even in these mice when they are maintained under conventional conditions is a drawback (Shiohara et al., 2004). In contrast, chemical hapten administration to these mice produces 100% reproducible AD-like lesions (Shiohara et al., 2004). Additionally, it has been reported that the repeated application of DNFB evokes AD-like skin lesions in NC/Nga mice under SPF conditions (Tomimori et al., 2005; Lee et al., 2007).

T-helper (Th) 2 cytokines, such as, interleukin-4 (IL-4) and IL-5, play important roles in the development of acute AD, whereas delayed-type allergic reactions and Th1 immune responses are responsible for the pathogenesis of chronic AD (Tomimori et al., 2005). Delayed-type hypersensitivity (DTH) reactions, such as, chemical contact allergies, are usually regarded as cell-mediated

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immune responses, which are frequently attributed to Th1-type cells. Moreover, DNFB induced contact hypersensitivity is a T cell-mediated inflammatory skin reaction that is believed to be associated with the activations of type 1 helper T cells (Takeshita et al., 2004).

Uncariae rhynchophylla (UR) is a herb that is prescribed traditionally to treat stroke and vascular dementia. It also is used to relieve headache, dizziness, and tremors resulting from hypertension (Goto et al., 1999). Several studies have described the potent anti-hypertensive and vasodilator functions of UR in animal models of hypertension (Goto et al., 1999, 2000; Shi et al., 2003). Additionally, UR and its constituent, alkaloids have been reported to have a neuroprotective effect on glutamate-induced cell death (Itoh et al., 1999; Goto et al., 2000), Ca²⁺-blocking inhibitory activity (Suk et al., 2002), and anti-oxidative activity (Lin et al., 1995; Hou et al., 2005). UR also has anti-inflammatory activity in LPS stimulated bone marrow derived dendritic cells (our unpublished data).

It is generally accepted that steroid therapy is crucial for the management of AD, but it cannot be used for long periods, and side effects, like rebound phenomenon, occur frequently. In some cases, oriental herbal therapy may be effective at treating AD (Koo and Arain, 1998; Gao et al., 2005). We recently discovered that UR also has anti-inflammatory activity in LPS stimulated bone marrow derived dendritic cells. However, it has not yet been determined whether UR suppresses the development of AD.

In this study, we used the DNFB-treated NC/Nga murine model to examine the inhibitory effect of UR on the development of AD-like skin lesions.

2. Materials and methods

2.1. Mice

Female NC/Nga mice, 8 weeks of age, were obtained from Japan SLC (Shizuoka, Japan) and maintained under SPF conditions. Animals were housed in an air-conditioned animal room at 25 ± 1 °C and a relative humidity (RH) of 40 ± 5%, and fed a laboratory diet and water. The experiments conducted complied with the guidelines issued by the Ethical Committee for Animal Welfare at Kyung Hee University and all procedures were conducted in accordance with the U.S. National Institutes of Health guidelines.

2.2. DNFB-induced dermatitis

25 µL of 0.15% DNFB in acetone/olive oil (3:1) was applied to each side of the right ear and dorsal skin six times with 7-day intervals. Ear thicknesses were measured daily after the first DNFB application. The same volume of acetone/olive oil vehicle was applied instead of DNFB solution to controls. Ear thicknesses were measured using a thickness gauge (Digimatic Indicator, Mitsutoyo, Tokyo).

2.3. Evaluation of inflammation scores

Severities of dermatitis on rostral back skin were assessed by evaluating dryness, crust, and keratinization. The total scores of the skin severity were defined as the sum of the individual score (0, no symptoms; 1, mild; 2, moderate; 3, severe) for each of the following four signs and symptoms: erythema (hemorrhage), edema, excoriation (erosion) and scaling (dryness), on the ear, back regions (Lee et al., 2007).

2.4. Drugs

Dried root powder of UR was purchased from an oriental drug store (Jungdo, Inc., Seoul, Korea). The voucher specimen

(No. E10) was deposited at the Herbarium of College of Oriental Medicine, Kyung Hee University (Seoul, Korea). The dried UR was extracted with boiling water. Briefly, UR (100 g) was boiled in 500 ml of distilled water for 2 h. The residues were removed by filtration, and then the filtrate was evaporated to obtain the desired concentration (2 g dry plants equivalent extract/ml). The liquid was centrifuged at 7000 rpm for 30 min, and the supernatant was lyophilized by freeze-drier to give a powder (yield, 2.35%). This lyophilized UR extract was then dissolved in distilled water and administered to mice orally at 100 mg/kg or 300 mg/kg daily for 6 weeks starting from 1 day after sensitization with DNFB, as previously described (Kim et al., 2004). Prednisolone (Sigma, St. Louis, MO, USA) was used as a reference drug and was also dissolved in distilled water and administered orally at 3 mg/kg daily for 6 weeks from 1 day after sensitization with DNFB.

2.5. Histological analysis

Ear samples were taken 24 h after final DNFB administrations, fixed in 10% buffered formalin, embedded in paraffin, sectioned at 3 µm, and then stained with hematoxylin and eosin (H&E).

2.6. Measurement of cytokine production in vitro

Lymph nodes were excised 24 h after final DNFB administrations and CD4⁺ T cells were purified using a BioMag separation column (QIAGEN, MD, USA) according to the manufacturer's instructions. The purified CD4⁺ T cells (5 × 10⁵ cells/ml) were stimulated with plate bound anti-CD3 antibody (2C11; 5 µg/ml) plus a soluble form of purified anti-mouse CD28 antibody (BD Pharmingen, CA, USA) (1 µg/ml) for 60 h in RPMI-1640 supplemented with 10% FBS, 0.005% of β-mercaptoethanol and 1% of glutamine. The productions of IL-4 and IFN-γ after T cell activation were quantified by ELISA.

2.7. Serum IgE measurement

Total serum IgE levels were also measured by ELISA. Blood samples were collected 24 h after final DNFB administrations and IgE concentrations in serum were quantified using BD OptEIA™ Set Mouse IgE ELISA kits (BD Pharmingen, CA, USA). In brief, a 1:250 dilution of anti-mouse IgE monoclonal antibody in PBS was placed in each well of an immunoplate (Corning 3590 96-well EIA/RIA plate, Corning, NY, USA) and maintained overnight at 4 °C. After washing wells with PBS containing 0.05% Tween 20 (washing buffer) 3 times, 200 µL of PBS containing 1% bovine serum albumin (BSA in PBS) was placed in each well. After 1 h at room temperature, wells were washed three times with washing buffer and 100 µL aliquots of serum samples diluted 30–50 fold with PBS were placed in the wells. After further incubation for 1 h at room temperature, wells were again washed five times with washing buffer, 100 µL of streptavidin-horseradish peroxidase-conjugated detection antibody (SAV-HRP) diluted 250-fold with washing buffer was then added, and the plate was maintained for 1 h at room temperature. After washing 7 times with washing buffer, the enzyme reaction was initiated by adding 100 µL of substrate solution (0.1 M citric acid, 0.2 M Na₂HPO₄, o-phenylene diamine and H₂O₂) and the reaction then was allowed to proceed for 30 min at room temperature. The reaction was terminated by adding 50 µL of 1 M H₂SO₄ to each well, and absorbance at 450 nm was then measured immediately using an ELISA reader (EL800) (Bio-Tek bio-tek, VT, USA). A standard curve was prepared using recombinant mouse IgE diluted with PBS.

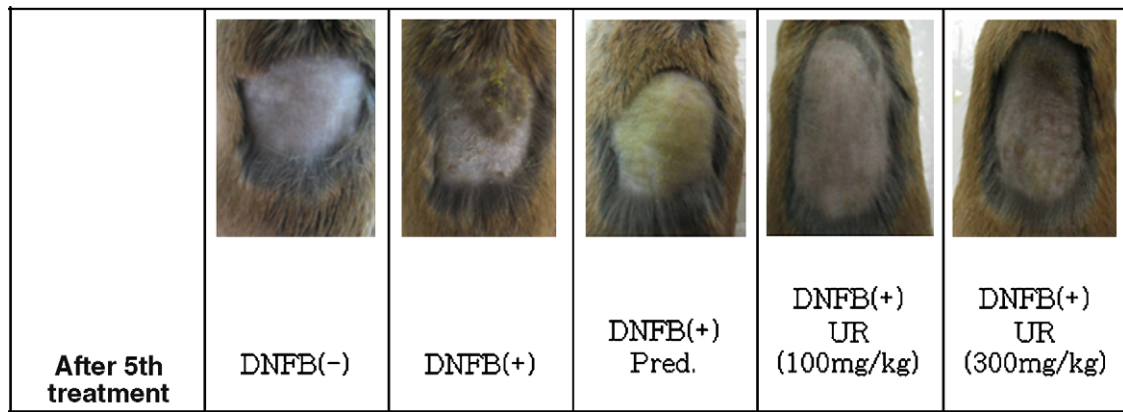


Fig. 1. Comparisons of DNFB-induced AD-like skin lesions in NC/Nga mice after UR administration. This photograph was taken after treatment completion. DNFB(–); acetone:olive oil (3:1) treated, DNFB(+); DNFB treated, DNFB(+) pred.; DNFB + prednisolone (3 mg/kg) treated, and DNFB(+) UR (100 mg/kg or 300 mg/kg); DNFB + UR treated. Five animals were allocated to each group.

2.8. Treatment with anti IFN- γ antibody to DNFB-induced dermatitis

To examine the effect of anti-IFN- γ antibody, either 100 μ g of rat anti-mouse IFN- γ (R4-6A2), or the same amount of rat IgG was administered intraperitoneally twice a week starting from 4th week of the experiment for 2 weeks.

2.9. Statistical analysis

Results are expressed as means $\pm$ S.E. (S.E.M.). Significances of changes were evaluated using the Anova. Differences between experimental groups were evaluated using analysis of variance. Values of $P < 0.05$ were accepted as significant.

3. Results

3.1. UR extracts ameliorated AD-like skin lesions on dorsal skins

Repeated application of DNFB induced AD-like skin lesions on NC/Nga mice (Tomimori et al., 2005; Lee et al., 2007). In accordance with these findings, the severity of symptoms in DNFB treated control NC/Nga mice increased gradually with the number of DNFB challenges (Fig. 1, Table 1). In contrast, oral administration of UR (100 mg/kg or 300 mg/kg) significantly suppressed AD-like skin lesion development as prednisolone (Fig. 1, Table 1).

3.2. Ear swelling was suppressed by UR

After the 2nd DNFB application to ears, ear swelling gradually increased. However, UR administration moderately suppressed this DNFB-induced ear swelling (Fig. 2). Moreover, no significant difference was observed between the 100 mg/kg and 300 mg/kg UR-treated groups and the prednisolone-treated group.

3.3. UR inhibited the infiltration of inflammatory cells into ear lobes

Histological specimens were prepared from ear lobes obtained 2 days after final DNFB treatment. Repeated DNFB-treatment induced severe inflammation of ear lobes, e.g., thickening of the epidermis and inflammatory cell accumulation. Both concentrations of UR and prednisolone administration clearly inhibited DNFB-induced inflammatory cell infiltration (Fig. 3).

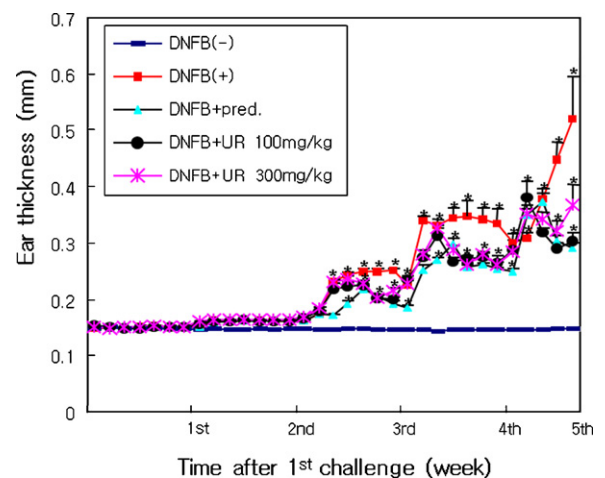


Fig. 2. Changes in ear thickness caused by repeated topical DNFB application in NC/Nga mice. DNFB(–); acetone:olive oil (3:1) treated, DNFB(+); DNFB treated, DNFB(+) pred.; DNFB + prednisolone (3 mg/kg) treated, and DNFB(+) UR (100 mg/kg or 300 mg/kg); DNFB + UR treated. Ear thicknesses were measured daily. Value are means $\pm$ S.E.M. ($n = 5$). * $p < 0.05$ vs. DNFB(–) mice.

Table 1
Dermatitis scores of rostral back (means $\pm$ S.E.M.).

Treatment	DNFB(–) ($n = 5$)	DNFB(+) ($n = 5$)			
		D.W. (vehicle)	Prednisolone	UR (100 mg/kg)	UR (300 mg/kg)
1st treated	0	0	0	0	0
3rd treated	0	$3.4 \pm 0.25^*$	$2.6 \pm 0.25^*$	$1.8 \pm 0.2^*$	$1.6 \pm 0.25^*$
5th treated	0	$8.8 \pm 0.2^*$	$6 \pm 0.45^*$	$4.2 \pm 0.37^*$	$4 \pm 0.32^*$

DNFB(–): acetone:olive oil (3:1) treated; DNFB(+): DNFB treated; DNFB(+) pred.: DNFB + prednisolone (3 mg/kg) treated; DNFB(+) UR (100 mg/kg or 300 mg/kg); DNFB + UR treated. Five animals were allocated to each group.

* $P < 0.05$ vs. DNFB(–) mice.

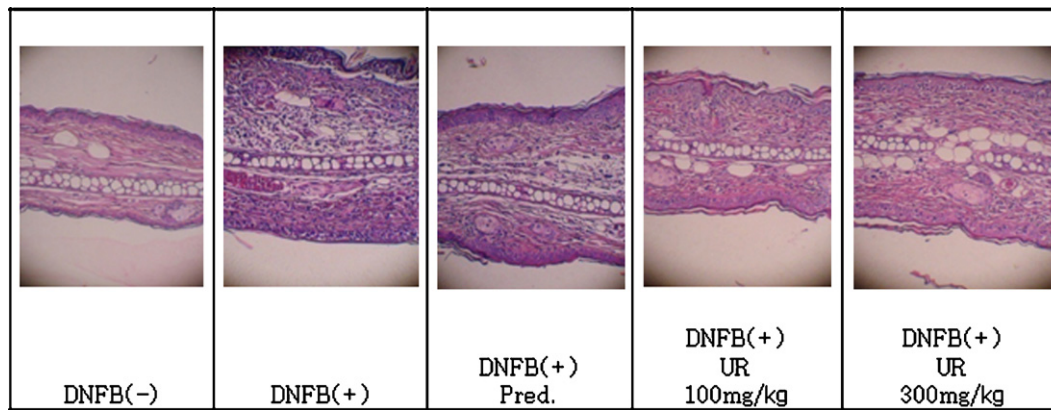


Fig. 3. Histopathologies of the ear lesions in DNFB-treated NC/Nga mice after treatment with UR. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB treated, DNFB(+) pred.; DNFB + prednisolone (3 mg/kg) treated, and DNFB(+) UR (100 mg/kg or 300 mg/kg); DNFB + UR treated. Ear sections were stained with hematoxylin and eosin (H&E).

3.4. UR did not inhibit DNFB-induced serum IgE level elevation

An increase in the level of serum IgE is a hallmark of atopic dermatitis. Other studies have reported that serum IgE levels are elevated in DNFB-treated NC/Nga mice with AD-like skin lesions (Tomimori et al., 2005; Lee et al., 2007). Therefore, we investigated whether UR administration decreased serum IgE levels after DNFB treatment in NC/Nga mice. A significant decrease in serum IgE levels were observed in the prednisolone treated group, but serum IgE levels of UR administration group were similar to those in the DNFB-treated group (Fig. 4), indicating that the UR mediated suppression of DNFB-induced AD-like skin lesions is independent of DNFB-induced serum IgE levels.

3.5. UR reduced IFN- γ production but not IL-4 production after TCR stimulation in CD4⁺ T cells

The cytokines produced by activated CD4⁺ T cells determine their differentiations into Th1 and Th2 cells. Furthermore, Th1/Th2 ratio is related to the overall pattern of cytokine production and to clinical outcome in a variety of pathological states (Singh et al., 1999). To investigate whether UR regulates Th1/Th2 ratio, we stimulated CD4⁺ T cells from the lymph nodes of UR plus DNFB or DNFB treated mice with anti-CD3 antibody plus anti-CD28 antibody for 60 h. Supernatants were then collected and IL-4 and IFN- γ levels were measured by ELISA. It was found that UR administration did not suppress IL-4 production after CD4⁺ T cell activation,

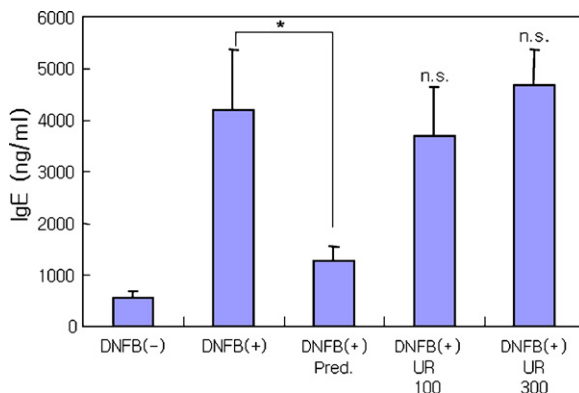


Fig. 4. Serum IgE levels in NC/Nga Mice. Serum IgE concentrations in the indicated groups were measured 24 h after final DNFB treatment. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB treated, DNFB(+) pred.; DNFB + prednisolone (3 mg/kg) treated, and DNFB(+) UR (100 mg/kg or 300 mg/kg); DNFB + UR treated. Data are means $\pm$ S.E.M. ($n = 5$). * $P < 0.05$ DNFB(+) pred. vs. DNFB(+) mice. n.s., $P > 0.05$ DNFB(+) UR vs. DNFB(+) mice.

whereas prednisolone did (Fig. 4a). In contrast, UR and prednisolone similarly suppressed IFN- γ production after CD4⁺ T cell activation (Fig. 4b), indicating that UR suppressed Th1 immune responses.

3.6. Neutralization of IFN- γ reduced ear thickness after DNFB-treatment

As shown Fig. 5, UR significantly decreased IFN- γ secretion levels after CD4⁺ T cell activation. Thus, we investigated whether IFN- γ is critically required for the induction of atopic dermatitis-like skin lesions by DNFB by neutralizing IFN- γ with anti-IFN- γ antibody. Anti-IFN- γ antibody was injected into DNFB-treated NC/Nga mice intraperitoneally twice a week starting from 4th week of the experiment for 2 weeks. Ear edema was found to

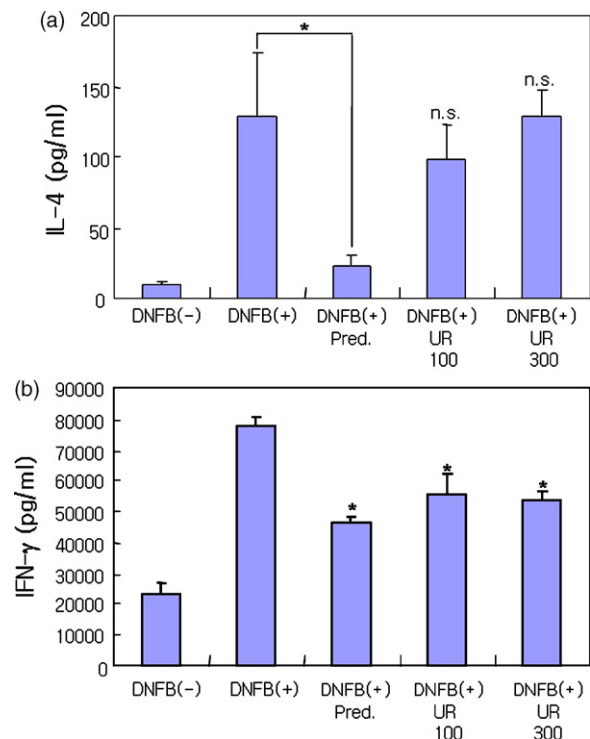


Fig. 5. Productions of IL-4 (a) and IFN- γ (b) by lymph node CD4⁺ T cells from DNFB-Treated NC/Nga mice. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB treated, DNFB(+) pred.; DNFB + prednisolone (3 mg/kg) treated, and DNFB(+) UR (100 mg/kg or 300 mg/kg); DNFB + UR treated. Data are means $\pm$ S.E.M. ($n = 5$). * $P < 0.05$ vs. DNFB(+) mice. n.s., $P > 0.05$ vs. DNFB(+) mice.

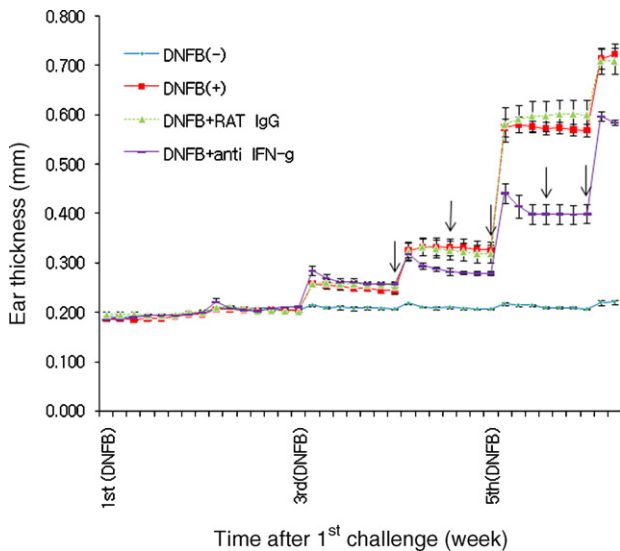


Fig. 6. Effect of anti-IFN- γ antibody on DNFB-induced ear thicknesses. Ear inflammation was induced by administering DNFB topically to ears as described in Section 2. Rat anti-mouse anti-IFN- γ antibody, Rat IgG was administered intraperitoneally twice a week, starting from 4th week of the experiment for 2 weeks. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB treated, DNFB + RAT IgG; DNFB + rat IgG treated, DNFB + anti IFN- γ ; DNFB + anti-IFN- γ treated. Data are means $\pm$ S.E.M. ($n = 5$). The arrows indicate antibody treatments.

be significantly reduced by anti-IFN- γ treatment. In contrast, isotype matched Rat IgG treatment did not affect ear edema (Fig. 6), which indicates that IFN- γ is associated with development of atopic dermatitis-like skin lesions in DNFB-treated NC/Nga mice.

4. Discussion

AD is the most common skin disease and is characterized by erythema, edema, excoriation and scaling (Leung and Bieber, 2003). Furthermore, the incidence of AD has continued to increase over several decades in industrialized countries (Shiohara et al., 2004). In general, topical steroid therapy is crucial for the treatment of AD, but it cannot be administered long term due to its side-effects. Recently several studies have reported that Herbal Oriental medicine therapy may be effective in AD patients (Koo and Arain, 1998; Gao et al., 2005).

In the present study, we found that UR can reduce AD-like skin lesions in DNFB-treated NC/Nga mice, and that this effect of UR is probably due to a reduction in IFN- γ production by activated CD4 $^{+}$ T cells. Increased total serum IgE levels are a hallmark of atopic dermatitis. Furthermore, it is well known that IL-4 (a Th2-type cytokine) class switched IgM secreting B cells to IgE secreting B cells. Accordingly, total serum IgE levels are significantly increased by repeated DNFB treatment. However, UR administration did not reduce these levels (Fig. 4). IL-4 production by CD4 $^{+}$ T cells obtained from the lymph nodes of DNFB treated NC/Nga mice was found to be elevated, and prednisolone almost abrogated DNFB induced IL-4 and IgE production (Figs. 4 and 5a). In contrast, UR reduced the severities of AD-like skin lesions in a manner similar to prednisolone (Fig. 1, Table 1). However, serum IgE levels and IL-4 production by activated CD4 $^{+}$ T cells were not alleviated by UR (Figs. 4 and 5a). Recently, Yagi et al. (2002) reported that increased IgE production and IgE-mediated hypersensitivity reactions may not be responsible for the development of AD-like skin lesions in NC/Nga mice under conventional conditions. Furthermore, it has been reported that repeated exposure to DNFB induces IFN- γ a polarized Th1-type cytokine (Hopkins et al., 2005). In addition,

it is known that IFN- γ (a Th1-type cytokine) might play a role in determining the chronicity of AD lesions (Tomimori et al., 2005). In chronic AD patients, major antigen reactive skin infiltrating T cells secrete IFN- γ (Tomimori et al., 2005). In the present study, IFN- γ production by activated lymph node CD4 $^{+}$ T cells was increased in DNFB-treated NC/Nga mice, and this was significantly inhibited by UR (Fig. 5b). In addition, when neutralizing anti-IFN- γ antibody was injected i.p. into DNFB-treated NC/Nga mice, ear swelling was inhibited in the same manner as after p.o. UR administration (Fig. 6). Thus, we believe that the UR-mediated attenuation of DNFB induced AD-like skin lesions might be IFN- γ dependent. However, further investigations are needed to determine whether the UR-mediated amelioration of DNFB induced AD-like skin lesions is directly related to the down-regulation of IFN- γ secretion by activated CD4 $^{+}$ T cells.

Conclusively, the oral administration UR was found to inhibit the development of AD-like skin lesions in NC/Nga mice repeatedly treated with DNFB via the suppression of Th1 immune response.

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