

Oral Administration of *Astragalus membranaceus* Inhibits the Development of DNFB-Induced Dermatitis in NC/Nga Mice

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Epicutaneously administered chemical antigens like 2,4-dinitrofluorobenzene (DNFB), evoke an atopic dermatitis (AD)-like dermatitis reaction in NC/Nga mice under specific pathogen free (SPF) conditions. *Astragalus membranaceus* (AM), is a popular herbal medicine used to treat allergic diseases in East Asia. In the present study, we examined whether AM suppress AD-like skin lesions in NC/Nga mice treated with DNFB under SPF conditions. Oral administration of AM to DNFB-treated NC/Nga mice was found to inhibit ear thickness increases and the skin lesions induced by DNFB. Moreover, IFN- γ production by CD4⁺ T cells from the lymph nodes of DNFB-treated NC/Nga mice was significantly inhibited by AM treatment, although levels of IL-4 and total IgE in serum were not. Study findings suggest that AM may suppress the development of AD-like dermatitis in DNFB-treated NC/Nga mice by reducing IFN- γ production.

Key words atopic dermatitis; 2,4-dinitrofluorobenzene (DNFB); *Astragalus membranaceus*; NC/Nga mice

Atopic dermatitis (AD) is one of the most common skin diseases, and is characterized by a chronic and relapsing inflammatory dermatitis with immunological disturbance, e.g., hyper-production of total and specific IgE. Patients with AD usually have a personal or familial history of other atopic diseases.¹⁾ Population studies suggest that in most countries AD affects 10–20% of all children at some time during childhood.²⁾ Moreover, AD is a multifactorial disease, and its pathogenesis and etiology are not fully understood.¹⁾

In acute AD lesions, cells expressing T helper 2 (Th2) cytokines such as interleukin-4 (IL-4) and IL-5, are significantly increased, whereas delayed-type allergic reaction and Th1 immune response characterize the pathogenesis of chronic AD.³⁾

Delayed-type hypersensitivity (DTH) reactions, such as, chemical contact allergy, are usually regarded as cell-mediated immune responses, which are frequently attributed to T helper (Th) 1-type cells.⁴⁾ Moreover, dinitrofluorobenzene (DNFB) induced contact hypersensitivity is a T cell-mediated inflammatory skin reaction that is thought to be associated with the activation of type1 helper T cells.⁵⁾

NC/Nga mice are the most extensively studied animal model of AD. The NC/Nga strain originates from Japanese fancy mice, and was established as an inbred strain in 1957. NC/Nga mice have also been reported to develop AD-like eczematous skin lesions when kept in an air-uncontrolled conventional housing but not when maintained under specific pathogen-free (SPF) conditions.²⁾ Clinical symptoms begin with itching, erythema, hemorrhage, scaling, dryness, and alopecia at age 8 weeks. Given the resemblance between these immunological alterations in NC/Nga mice and AD in humans, models based on these mice are of importance for the understanding and treatment of AD.²⁾

The roots of *Astragalus membranaceus* (AM) are one of the most popular health-promoting herbs in East Asia, and have been used for more than 2000 years.⁶⁾ The main constituents of AM roots are polysaccharides, saponins,

flavonoids, amino acids, and trace elements.⁷⁾ Recently AM is reported that it enhanced the levels of IL-4 but reduced level of IFN- γ secretion in TCR (T cell receptor) stimulated T cells.⁸⁾ Additionally, AM in combination with other herbs, Hochu-ekki-to, was used to treat allergic diseases.^{9–11)} Hochu-ekki-to also known to suppressed dermatitis and serum IgE levels in NC/Nga mice.⁹⁾ However, Hochu-ekki-to contain many different constituents, it is difficult to identify the active components. Therefore, in this study, we examined whether AM suppresses AD-like skin lesions in NC/Nga mice.

MATERIALS AND METHODS

Mice Female NC/Nga mice, 8 weeks of age, were obtained from Orient Korea (Gyeonggi, Korea) 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. Experiments conducted in accord with the guidelines issued by the Ethical Committee for Animal Welfare at KyungHee University.

DNFB-Induced Dermatitis Twenty-five microliters of 0.15% DNFB in acetone/olive oil (3 : 1) was applied to each side of right ears and dorsal skin five times with 7 d intervals. Ear thicknesses were measured daily after the first DNFB application. In some experiments, the same volume of DNFB solvent was applied instead of DNFB solution, as a control. Ear thicknesses were measured using a thickness gauge (Digimatic Indicator, Mitsutoyo, Tokyo), and ear thickness increases *versus* baseline were calculated by simple subtraction.

Evaluation of the Inflammation Score The severity of dermatitis on the rostral back of the body was assessed. The evaluated items consisting of Dryness, Crust, keratinization were scored as Table 1.

Drugs Dried root powder of *Astragalus membranaceus* BUNGE (AM) was provided by Won-Kwang University, Iksan,

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Korea. This powder (100 g) was extracted with water, filtered, concentrated *in vacuo*, and lyophilized. This lyophilized AM extract was dissolved in distilled water and administered to mice orally at 100 mg/kg daily as other report.¹²⁾ Prednisolone (Sigma, St. Louis, MO, U.S.A.) was used as a reference drug, and similarly, was dissolved in distilled water and administered orally at 3 mg/kg daily throughout the experiment.

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

Measurement of Cytokine Production *in Vitro* Lymph nodes were excised and CD4⁺ T cells were purified using a MACS separation column (QIAGEN) according to the manufacturer's instructions. The purified CD4⁺ T cells (5×10^5 cells/ml) were stimulated with plate bound anti-CD3 antibody (2C11; 5 μ g/ml) plus a soluble form of purified anti-mouse CD28 antibody (BD Pharmingen) (1 μ g/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.

Serum IgE Measurement Total serum IgE levels were also measured by ELISA. Blood samples were collected 24 h after the fifth application and IgE concentrations in serum were quantified using BD OptEIA™ Set Mouse IgE ELISA kits (BD Pharmingen, CA, U.S.A.). 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, U.S.A.) and maintained overnight at 4 °C. After washing wells with PBS containing 0.05% Tween 20 (washing buffer) 3 times, 200 μ l 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 μ l aliquots of serum samples diluted 30—50 fold with BSA in 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 μ l of streptavidin-horseradish peroxidase-conjugated detection antibody (SAv-HRP) diluted 250-fold with washing buffer was then added, and the plate maintained 1 h at room temperature. After washing 7 times with washing buffer, enzyme reaction was initiated by adding 100 μ l of substrate solution (0.1 M citric acid, 0.2 M Na₂HPO₄, *o*-phenylene diamine and H₂O₂) followed by a period of 30 min at room temperature. The reaction was terminated by adding 50 μ l of 1 M H₂SO₄ to each well, and absorbance at 450 nm was measured immediately using an ELISA reader (EL800) (Bio-Tek bio-tek, VT, U.S.A.). A standard curve was prepared using recombinant mouse IgE diluted with BSA in PBS.

Statistical Analysis Results are expressed as means $\pm$ S.E. (S.E.M.). Significances of changes were evaluated using the Student's *t*-test. Differences between experimental groups were evaluated using analysis of variance. Values of $p < 0.05$ were accepted as significant.

RESULTS

AM Ameliorates AD-Like Skin Lesions in Ear and Dorsal Skins NC/Nga mice have been previously shown to develop AD-like skin lesions after the repeated topical application of DNFB under SPF conditions,⁹⁾ and in the present study NC/Nga mice were used to investigate the preventative effect of AM in this context. Symptom severity in NC/Nga mice was found to increase gradually with time during the 5-week DNFB challenge (Figs. 1a, b). In contrast, oral AM

Table 1. Dermatitis Scores for Rostral Back

	Dryness	Crust	Keratinization
No lesion	0	0	0
Mild inflammation	1	1	1
Moderate inflammation	2	2	2
Severe inflammation	3	3	3
Total score		9	

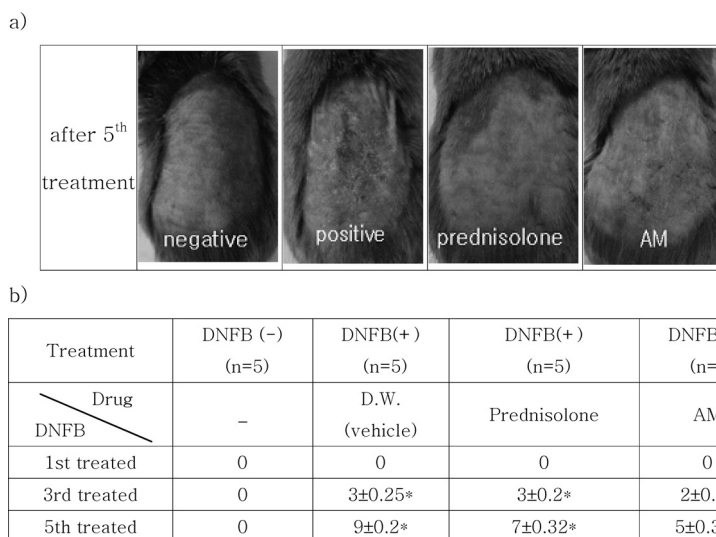


Fig. 1. Comparisons of DNFB-Induced AD-Like Skin Lesions in NC/Nga Mice after AM Administration

This photograph of mouse was taken the after treatment completion (a). Dermatitis scores of rostral back (mean $\pm$ S.E.M.) * $p < 0.05$ compare with DNFB(-) mice (b). Negative; acetone: olive oil (3 : 1) treated, positive; DNFB+D.W. treated, prednisolone; DNFB+prednisolone (3 mg/kg) treated, and AM; DNFB+AM (100 mg/kg) treated. Five animals were allocated to each group.

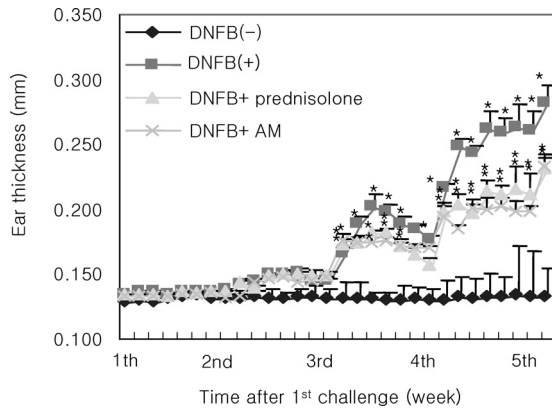


Fig. 2. Change in Ear Thickness Caused by Repeated Topical DNFB Application in NC/Nga Mice

DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB+D.W. treated, DNFB+prednisolone; DNFB+prednisolone (3 mg/kg) treated, and DNFB+AM; DNFB+AM (100 mg/kg). Ear thicknesses were measured daily, after each DNFB application. Values are means $\pm$ S.E.M. of five animals per group. * p < 0.05 compared with DNFB(-) mice.

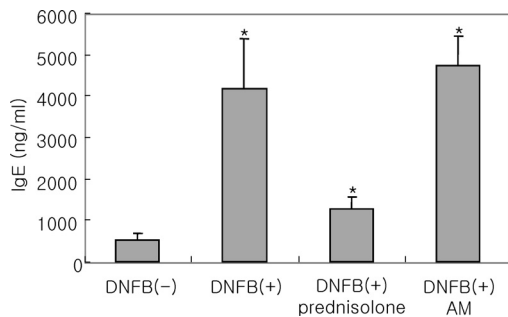


Fig. 3. Serum IgE Levels in NC/Nga Mice

Serum IgE concentrations in the indicated groups were measured 24 h after final administration. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB+D.W. treated, DNFB+prednisolone; DNFB+prednisolone (3 mg/kg) treated, and DNFB+AM; DNFB+AM (100 mg/kg). Data are means $\pm$ S.E.M. (n = 5). * p < 0.05 compared with DNFB(-) group.

(100 mg/kg/d) suppressed AD-like skin lesion development (Figs. 1a, b), and as was expected, prednisolone (3 mg/kg/d) significantly inhibited skin symptoms (Figs. 1a, b)

Ear Swelling Inhibition by AM or Prednisolone Ear swelling is shown in Fig. 2. After the fourth and fifth DNFB applications, apparent biphasic ear swelling was induced. The daily administration of AM or prednisolone significantly inhibited ear swelling (Fig. 2).

AM Did Not Inhibit DNFB-Induced Serum IgE Level Elevation After completing DNFB treatment, serum samples were obtained and total IgE levels were determined by ELISA. Serum IgE levels were markedly increased by DNFB (Fig. 3). Oral AM did not inhibit this DNFB-induced serum IgE increase, whereas prednisolone did (Fig. 3).

AM Inhibited Inflammatory Reaction Histological specimens were prepared from mouse ear lobes obtained 24 h after final treatment. Repeated DNFB-treatment caused potent inflammation, e.g., thickening of the epidermis and inflammatory cell accumulation (Fig. 4). Both AM and prednisolone administration clearly inhibited DNFB-induced inflammation (Fig. 4).

AM Reduced IFN- γ Production But Not IL-4 Production after TCR Stimulation in CD4 T Cells CD4⁺ T cells were prepared from the lymph nodes of mice 24 h final treat-

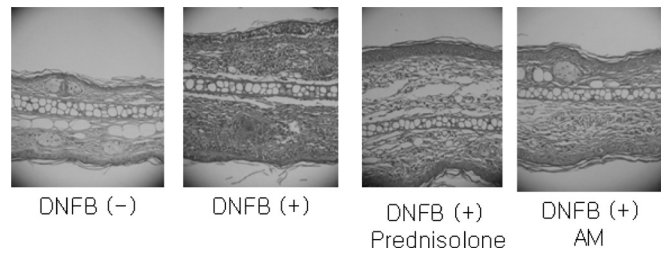


Fig. 4. Histopathologies of Ear Skin Lesions in DNFB-Treated NC/Nga Mice after Treatment

DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB+D.W. treated, DNFB+prednisolone; DNFB+prednisolone (3 mg/kg) treated, and DNFB+AM; DNFB+AM (100 mg/kg). Ear sections were stained with H&E.

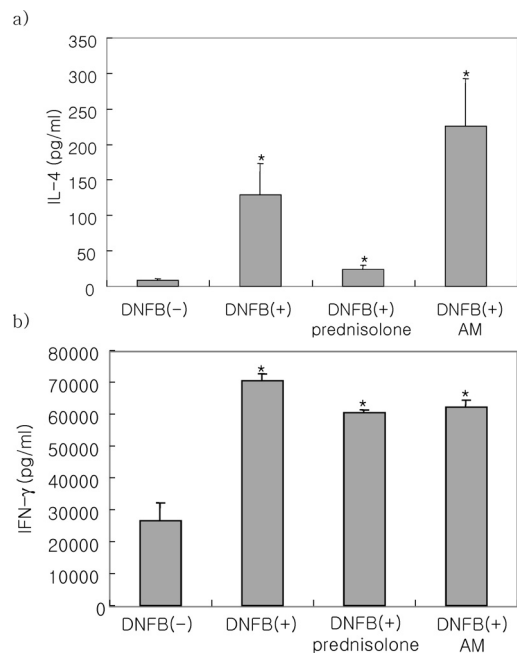


Fig. 5. Productions of IL-4 (a) and IFN- γ (b) by CD4⁺ T Cells from DNFB-Treated NC/Nga Mice

DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB+D.W. treated, DNFB+prednisolone; DNFB+prednisolone (3 mg/kg) treated, and DNFB+AM; DNFB+AM (100 mg/kg). AM (100 mg/kg) was administered orally to NC/Nga mice challenged with DNFB as described in Materials and Methods. Data are means $\pm$ S.E.M. (n = 5). * p < 0.05 compared with DNFB(-) group.

ments and stimulated with anti-CD3 Ab plus anti-CD28 Ab for 60 h. Their abilities to synthesize IL-4 and IFN- γ were assessed by ELISA. AM did not inhibit IL-4 production after CD4⁺ T cell activation whereas prednisolone did. In contrast, AM and prednisolone similarly inhibited the increases in IFN- γ production (Fig. 5).

DISCUSSION

This study shows that AM can reduce the severity of dermatitis-like skin lesions induced by topical DNFB in NC/Nga mice. DNFB-treated NC/Nga mice revealed skin lesions characterized by erythema, edema, excoriation, and scaling, and histologically by massive inflammatory cell infiltration, which are similar to those reported for NC/Nga mice maintained under conventional conditions.²⁾ Moreover, oral AM, like prednisolone, administered to DNFB-treated NC/Nga mice clearly inhibited the development of AD-like

skin lesions (Figs. 1, 2, 4).

The upregulation of total serum IgE is a hallmark of atopic dermatitis. The Th2-type cytokine IL-4 increases the switching of B cells from IgM to IgE. Accordingly, total serum IgE was found to be significantly increased by repeated DNFB treatment in NC/Nga mice (Fig. 3), as was IL-4 production by activated CD4⁺ T cells obtained from these NC/Nga mice (Fig. 5a). However, although the administration of AM significantly reduced the severities of AD-like skin lesions induced by DNFB, it did not inhibit total serum IgE levels or IL-4 production by activated CD4⁺ T cells (Figs. 3, 5a). Recently, it was reported that increased IgE production and subsequent IgE-mediated hypersensitivity reactions may not be responsible for the development of the AD-like skin lesions observed in the NC/Nga mice under conventional conditions,¹³⁾ and thus, further studies are required on this topic.

Recently the Th1-type cytokine IFN- γ was suggested to play a central role in determining the chronicity of AD lesions,^{14,15)} and it has been reported that the majority of allergen-specific, skin infiltrating T cells in chronic AD patients secrete IFN- γ protein.¹⁶⁾ Although the exact pathological role of IFN- γ in the development of skin lesions in AD is not fully understood, recent studies suggest that it is involved.¹⁷⁾ Furthermore it has been demonstrated that chemical antigens stimulate mouse T lymphocyte cytokine secretion. Exposure to a chemical antigen, like DNFB, induces the polarized Th1-type cytokine, IFN- γ .¹⁸⁾ The present study shows that IFN- γ production by lymph node CD4⁺ T cells is increased in DNFB-treated NC/Nga mice and that this is significantly inhibited by AM (Fig. 5b). In addition, when neutralizing anti IFN- γ antibody was administered intraperitoneally to DNFB-treated NC/Nga mice, ear swelling was mildly recovered (preliminary data). Thus, we believe that the AM-mediated amelioration of DNFB induced AD-like skin lesions in NC/Nga mice is IFN- γ dependent.

In conclusion, the oral administration of AM extracts was found to inhibit the development of AD-like skin lesions in NC/Nga mice treated repeatedly with DNFB.

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