Oral Administration of a Hop Water Extract Ameliorates the Development of Dermatitis Induced by the Periodical Topical Application of a Mite Antigen in Atopic Dermatitis Model NC/Nga Mice

Shuichi SEGAWA, Hisao KURODA, Takafumi KANEO, and Junji WATARI

Frontier Laboratories of Value Creation, Sapporo Breweries Ltd., 10 Okatohme, Yaizu, Shizuoka 425-0013, Japan

Received October 26, 2007; Accepted December 25, 2007; Online Publication, April 7, 2008 [doi:10.1271/bbb.70695]

We investigated the inhibitory effect of an oral administration of a hop water extract (HWE) on the development of dermatitis by using NC/Nga atopic dermatitis model mice. The induction of allergic dermatitis was conducted by tape-stripping and topical application of a mite antigen (Dermatophagoides farinae) on to the ear once a week for 10 weeks. HWE was orally administered at a dose of 100 or 500 mg/kg. The total immunoglobulin E (IgE) concentration in serum and the ear thickness were periodically examined. Finally, the antigen-specific IgE level in the serum and the production of interleukin (IL)-12 and interferon (IFN)-γ from splenocytes and cervical lymph node cells were measured. The oral administration of HWE significantly inhibited the increase of total IgE production and ear swelling throughout the experimental period. The production of IL-12 was significantly lower in the HWE administered group than in the control group. The results suggest that the intake of HWE may be effective in preventing and alleviating the development of atopic dermatitis-like skin disease.

Key words: hops; allergic disease; atopic dermatitis; mite; immunoglobulin E

Atopic dermatitis (AD) is one of the most common skin diseases and its prevalence is increasing in industrialized countries. This disease is frequently associated with an elevated level of immunoglobulin E (IgE) antibodies against many kinds of allergens.1) IgE antibodies are secreted from IgE-producing plasma cells which are differentiated from B cells.2) IgE production is regulated by several cytokines. Interleukin (IL)-4, which is produced from Th2 cells, as well as activated mast cells and basophils promote the development and expansion of Th2 cells, B cell proliferation, and IgE class switching.3,4) The IgE production induced by IL-4 is inhibited by such Th1-type cytokines as interferon (IFN)-γ and IL-12.5,6) and by immunosuppressive cytokines such as IL-10 and TGF-β.7,8) The cross-linking of IgE intermediated by the binding of a multivalent antigen on the surface of mast cells triggers the release of many chemical mediators such as histamine, leukotrienes and prostaglandins, and of cytokines such as IL-3, IL-4 and IL-5 from these cells.5,10) These chemical mediators and cytokines contribute to the immediate allergic reaction and late-phase inflammatory reaction.9)

NC/Nga mice, an inbred strain established from fancy Japanese mice, develop AD-like skin lesions under conventional care, or upon treatment by repeated challenge with a mite antigen under the specific pathogen-free (SPF) condition.11–14) The induced dermatitis is accompanied by an elevated serum IgE level, increased expression of Th2 cytokines, eosinophil accumulation in the lesions and frequent scratching behavior which are characteristic features of human AD.11)

Such foodstuffs as a persimmon leaf extract and royal jelly have been reported to ameliorate the AD-like skin disease development in NC/Nga mice.15–17) In our previous studies, a hop water extract (HWE) significantly inhibited the histamine release from human basophilic KU812 cells. HWE contained quercetin glycosides such as isoquercitrin, rutin and isoquercitrin malonate, and kaempherol glycosides such as astragaline, kaempherol rutinoside and astragaline malonate. These flavonol glycosides were responsible for the inhibition of histamine release.18) HWE also significantly inhibited antigen-induced nasal rubbing and sneezing in egg albumin-sensitized BALB/c mice.19) Furthermore, in a double blind, placebo-controlled clinical trial, an oral administration of HWE to subjects who had Japanese cedar pollinosis symptoms was effective in alleviating such allergic symptoms.20) However, little is known about the efficacy of HWE to prevent the...
development of AD. We investigated in this study the inhibitory effect of an oral administration of HWE on the development of dermatitis and IgE elevation which were induced by the periodical application of a mite antigen on to a tape-stripped ear by using AD model NC/Nga mice.

Materials and Methods

Mice. Female 4-week-old NC/Nga mice were purchased from Charles River Japan (Kanagawa, Japan). The animals were housed in an air-conditioned room maintained at 23 ± 2°C with a relative humidity of 55 ± 15%. They were given CRF-1 standard laboratory rodent feed (Oriental Yeast, Tokyo, Japan) and water ad libitum. All procedures were performed according to the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animals.

Preparation of the hop water extract. Hop pellets cultivated in the Saaz district of the Czech Republic were used. HWE was prepared as previously described. The concentrations of quercetin and kaempferol aglycones in this HWE, as determined by the acid hydrolysis of flavonol glycosides, were 158 ± 21 μg/g and 186 ± 32 μg/g, respectively.

Induction of allergic dermatitis in the mouse ear. The mice were divided into 4 groups: a positive control, 100 mg/kg of HWE administered group (oral administration of HWE at a dose of 100 mg/kg), 500 mg/kg of HWE administered group (oral administration of HWE at a dose of 500 mg/kg) with the mite antigen challenge (n = 8 per group), and negative control without the mite antigen challenge (n = 5 per group). Allergic dermatitis in NC/Nga mice was induced according to the protocol reported by Gao et al., with a slight modification. Dermatophagoides farinae (mite-Df; LSL, Tokyo, Japan) was used as the antigen. The mite antigen was suspended in phosphate buffered saline (PBS) containing 0.5% Tween 20. Both surfaces of a mouse ear were stripped 3 times by using surgical tape (3M Health Care, St Paul, MN, USA). One hour after the tape-stripping, 30 μl of the 10 mg/ml mite antigen suspension was painted on to each surface of the mouse ear. With the negative control mice, PBS instead of the mite antigen was painted on. Tape-stripping and mite antigen application were performed once a week for 10 weeks. HWE dissolved in distilled water was orally administered with a gastric tube once a day throughout the experimental period at a dose of 100 or 500 mg/5 ml/kg. To the control mice, distilled water was orally administered at a dose of 5 ml/kg. On the day of mite antigen application, HWE was administered 1 h before the antigen application. The ear thickness of the mice was measured before antigen application with a dial thickness gauge (SMD-565, Teclock, Nagano, Japan), and a blood sample was collected from the tail vein. The concentration of total IgE in serum was determined by sandwich ELISA.

Finally, blood samples were collected to measure total IgE and mite-Df-specific IgE in the serum 24 h after the final mite antigen application. Such cytokines as IL-4, IL-12 and IFN-γ, and the production from splenocytes and cervical lymph nodes (CLN) cells obtained from NC/Nga mice were measured. The splenocytes were cultured in an RPMI-1640 medium (Invitrogen Corp., Carlsbad, CA, USA), which contained 10% FBS (Equitech-Bio, Kerrville, TX, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen Corp.), to give 2.5 × 10^6 cells/ml on a 96-well culture plate. CLN cells were prepared from CLN by treating with 1 mg/ml of type I collagenase (Invitrogen Corp.) dissolved in the RPMI-1640 medium, and then cultured in the RPMI-1640 medium, which contained 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin, to give 2.5 × 10^6 cells/ml on a 96-well culture plate. These cells were cultured at 37°C in a 5% CO2 concentration for 3 d. The concentrations of cytokines in the culture supernatant were determined by sandwich ELISA. The schedule of the experiment is indicated in Fig. 1.

ELISA. The concentrations of such cytokines as IL-4, IL-12 and IFN-γ in the culture supernatant, and of IgE and mite-Df-specific IgE in serum were measured by using sandwich ELISA.

Fifty μl of the first antibodies dissolved in a 50 mm sodium carbonate buffer was added to each well of a 96-well microtiter plate and incubated overnight at 4°C. The concentrations of the first antibodies for IL-4, IL-12 and IFN-γ measurement were as follows: 1 μg/ml of the monoclonal anti-mouse IL-4 antibody (R&D Systems, Minneapolis, MN, USA), 1 μg/ml of the purified rat anti-mouse IL-12 (p40/p70) monoclonal antibody (BD Pharmingen, San Diego, CA, USA), and 2 μg/ml of rabbit anti-mouse/rat IFN-γ (Biosource, Camarillo, CA, USA). Each well was washed three times with a washing buffer (PBS containing 0.05% Tween 20). One-hundred μl of 1% BSA in a 50 mM sodium carbonate buffer was added to each well, the plate was incubated for 90 min at 37°C, and then each well was washed five times with the washing buffer. Fifty μl of the culture supernatant or the standard solution was added to each well, the plate was incubated for 90 min at room temperature, and then each well was washed five times with the washing buffer. The following recombinant cytokines were used in this study as standards: recombinant mouse IL-4 (R&D Systems), recombinant mouse IL-12 (R&D Systems) and recombinant murine interferon-γ (Biosource). Fifty μl of the biotinylated second antibody dissolved in the washing buffer containing 1% BSA was added to each well of a 96-well microtiter plate and incubated for 90 min at room temperature. The concentrations of the second antibodies for IL-4, IL-12 and
IFN-γ measurement were as follows: 0.1 µg/ml of the biotinylated anti-mouse IL-4 antibody (R&D Systems), 0.5 µg/ml of the biotinylated rat anti-mouse IL-12 (p40/p70) monoclonal antibody (BD Pharmingen) and 0.1 µg/ml of a mouse anti-rat/mouse IFN-γ biotin conjugate (Biosource). Each well was washed as before. Fifty µl of 0.1 µg/ml of streptavidin-horseradish peroxidase (Biosource) was added to each well, the plate was incubated for 30 min at room temperature in the dark, and then each well was washed as before. A substrate solution of 3,3',5',5'-tetramethylbenzidine (Sigma-Aldrich) was added to each well, and the plate was allowed to develop at room temperature in the dark. The color reaction was stopped by adding 0.5 N H₂SO₄, and the absorbance at 450 nm was measured with a microplate reader (MTP-800 AFC, Hitachi High-Technologies, Tokyo, Japan).

The level of total IgE in the serum was measured with a mouse IgE ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX, USA). The level of the mite-Df-specific IgE titer in the serum was determined by sandwich ELISA. The biotinylated mite-Df extract was used as the second antibody instead of horseradish peroxidase conjugated anti-mouse IgE. The mite-Df-specific IgE titer in the serum was calculated from a comparison with hyperimmunized mouse serum which was obtained from mice that had been intra-peritoneally immunized with 20 µg of the mite-Df extract adsorbed to 2 mg of alum (Sigma-Aldrich) three times (Days 0, 14 and 21). The mite-Df-specific IgE titer of this control serum was arbitrarily taken to be 100 units/ml.

Histological analysis. The ears of the mice which had been induced by repeated topical application with mite-Df were removed on the final day of the experiment. The ear samples were fixed with a 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Osaka, Japan), embedded in paraffin, and then stained with hematoxylin and eosin (HE), or with toluidine blue.

Statistical analysis. Each value is expressed as the mean ± SD. A statistical evaluation of the results for the change in ear thickness and total IgE in the serum was performed by a two-way analysis of variance (ANOVA) followed by Tukey’s HSD test. A statistical evaluation of the results for mite-Df-specific IgE in the serum and for cytokine production from splenocytes and CLN cells was performed by one-way ANOVA followed by Tukey’s HSD test. A probability value of less than 0.05 was considered statistically significant.

Results

Effect of the oral administration of HWE on ear swelling induced by the mite antigen application

Thirty µl of 10 mg/ml of the mite antigen suspension were painted on to each surface of the NC/Nga mouse ear 1 h after tape-stripping. Tape-stripping and mite antigen application were performed once a week for 10 weeks. HWE dissolved in distilled water was orally administered via a gastric tube once a day throughout the experimental period at a dose of 100 or 500 mg/5 ml/kg. Figure 2 shows the changes in ear thickness of the NC/Nga mice. The ear thickness of the mice in the positive control group, to which the mite antigen had been applied, was significantly increased in comparison with that in the negative control group. The increase in ear thickness in the group to which HWE had been orally administered at a dose of 500 mg/kg was significantly lower than the positive control group throughout the experimental period. A histological analysis of the ear skin was performed, and typical photographs of ear specimens which had been stained with HE are shown in Fig. 3. In comparison with the positive control group, the HWE orally administered group showed less ear thickening and hyperplasia of the
effect of HWE on the development of dermatitis in the cells was investigated to clarify the inhibitory mechanism of NC/Nga mice compared with the negative control group. A significant increase in the concentration of mite-Df-specific IgE in the serum of the positive control group was significantly higher than that in the negative control group. On the other hand, there was no difference in the specific IgE concentration in the serum of the positive control group when compared with the negative control group. The oral administration of HWE significantly inhibited the increase in total IgE in serum with time. As shown in Fig. 6, the mite-Df-specific IgE concentration in serum at the end of the experiment was significantly lower than that in the negative control group. A statistical evaluation of the results was performed by two-way ANOVA followed by Tukey’s HSD test. Significantly different from the positive control group at $P < 0.05$.

**Infiltration of mast cells**

Infiltration of mast cells was observed in the mice to which the mite antigen had been applied, while no infiltration of mast cells was apparent in the negative control group.

**Total IgE and mite-Df-specific IgE in serum**

Total IgE concentration in the serum was investigated throughout the experimental period, and the mite-Df-specific IgE concentration in serum at the end of the experiment was also checked. Figure 5 shows the changes in total IgE concentration in the serum. The total IgE level in the serum of the positive control group was significantly increased by the periodical topical application of the mite antigen. On the other hand, total IgE in the serum of the negative control group did not generally increase with time. The oral administration of HWE significantly inhibited this increase of total IgE in the serum with time. As shown in Fig. 6, the mite-Df-specific IgE concentration in the serum of the positive control group was significantly higher than that in the negative control group. On the other hand, there was no significant increase in the concentration of mite-Df-specific IgE in the HWE administered group when compared with the negative control group.

**Cytokine production from splenocytes and CLN cells of NC/Nga mice**

The cytokine production from splenocytes and CLN cells was investigated to clarify the inhibitory mechanism of HWE on the development of dermatitis in the NC/Nga mice. Twenty-four hours after the final mite antigen application, the spleen and CLN were removed, and then splenocytes and CLN cells were cultured for 3 d. The cytokine concentration in the culture supernatant was measured. Table 1 shows the cytokine production from splenocytes and CLN cells. Among the investigated cytokines, IFN-γ production from splenocytes and CLN cells was under the detection limit of our ELISA system. IL-12 production from CLN cells was significantly increased by periodical topical application of the mite antigen. The oral administration of HWE significantly inhibited this increase of IL-12 production in comparison with the positive control group. The IL-4 concentration was at an extremely low level, and the oral administration of HWE did not affect its production.

**Discussion**

Periodical topical application of the mite antigen to the ear of NC/Nga mice caused a significant increase in total IgE and antigen-specific IgE in the serum (Figs. 5 and 6). Ear swelling was also induced by topical application of the mite antigen (Fig. 2). It has been reported that the development of dermatitis in NC/Nga mice is accompanied by an elevated serum IgE level. Therefore, IgE antibodies probably played an important role in the development of AD-like dermatitis in this study. The increase in total IgE concentration in the serum with time was significantly inhibited by the oral administration of HWE. The ear thickness of the HWE-administered mice was less than that of the positive control mice. These results suggest that the oral administration of HWE inhibited the development of AD-like dermatitis induced by topical application of the mite antigen.

IgE antibodies are secreted from IgE-producing plasma cells which are differentiated from B cells. The proliferation and differentiation of B cells into IgE-secreting plasma cells are regulated by several cytokines. IL-4 has been reported to promote B cell proliferation, IgE class switching and then to augment IgE secretion. However, in this study, the IL-4 production from splenocytes and CLN cells was at an extremely low level, and was not affected by the oral administration of HWE. Matsumoto et al. have reported that T cells isolated from NC/Nga mice produced less IL-4, and that B cells isolated from the spleen had high sensitivity to IL-4 and the CD40L signal through the enhanced tyrosine phosphorylation of JAK3. Hyperproduction of IgE in spite of the low IL-4 production from splenocytes in this study, therefore, might have been due to this characteristic of the NC/Nga mice. In this study, IFN-γ production from splenocytes and CLN cells was under the detection limit, although IL-12, which is an important mediator for IFN-γ production, was produced. Matsumoto et al. have reported that NC/
Nga mice showed defective production of IFN-γ when stimulated by IL-12 because of its weak phosphorylation in STAT4. In this present study, IL-12 production from CLN cells was significantly increased by periodical topical application of the mite antigen. The oral administration of HWE significantly inhibited the increase of

---

**Fig. 3.** Histological Features of Skin Lesions Stained with Hematoxylin and Eosin (×100).

Ear sections of the negative control mouse (a), positive control mouse (b), 100 mg/kg HWE-administered mouse (c), and 500 mg/kg HWE-administered mouse (d) were stained with hematoxylin and eosin.

**Fig. 4.** Histological Features of Skin Lesions Stained with Toluidine Blue (×200).

Ear sections of the negative control mouse (a), positive control mouse (b), 100 mg/kg HWE-administered mouse (c), and 500 mg/kg HWE-administered mouse (d) were stained with the toluidine blue.
Hops inhibited the nitric oxide (NO) production and flavonoids and phloroglucinol derivatives contained in hops inhibited the vascular permeability induced by LPS and IFN-γ stimulation in mouse macrophage RAW 264.7 cells. The inhibition of IL-12 production from splenocytes and CLN cells by the oral administration of HWE might have been due to suppression of the inflammatory response caused by macrophage activation. There might be a concern that the decrease of IL-12 production by the oral administration of HWE could cause some adverse side effects. However, in a 90d rat feeding toxicity study, no toxicological findings were apparent by the oral administration of HWE at a dose of 2000 mg/kg (data not shown).

In our previous study, the oral administration of HWE inhibited the vascular permeability induced by compound 48/80 in ICR mice and the histamine release into the serum from ovalbumin-sensitized BALB/c mice which had been induced by an intraperitoneal antigen.

### Table 1. Cytokine Production from Splenocytes and Cervical Lymph Node Cells Isolated from NC/Nga Mice

<table>
<thead>
<tr>
<th></th>
<th>Splenocytes</th>
<th>CLN cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (−)</td>
<td>Control (+)</td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>1.42 ± 0.08</td>
<td>1.25 ± 0.05</td>
</tr>
<tr>
<td>IL-12 (pg/ml)</td>
<td>116.9 ± 13.0</td>
<td>141.9 ± 27.3</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Splenocytes and CLN cells were cultured in an RPMI-1640 medium, which contained 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin, at 37°C in 5% CO₂ concentration for 3d. IL-4, IL-12, and IFN-γ concentrations in the supernatant were determined by sandwich ELISA. Each value represents the mean ± SD, n = 5.

** Significantly different from the positive control group at P < 0.01.

##  Significantly different from the negative control group at P < 0.01.

ND, not detectable.
HWE inhibited the type-I allergic reaction by suppressing the release of such chemical mediators as histamine from the mast cells. It has been reported that the antigen-induced reaction in the skin consists of a bi-phasic response known as the immediate reaction and late reaction. The immediate reaction occurs within 1 h of antigen stimulation and is caused by the activation of mast cells. The late reaction appears 24–48 h after antigen stimulation and is caused by inflammatory cells such as neutrophils, mononuclear cells and eosinophils.26) The late-phase inflammatory reaction plays an important role in the development of chronic skin diseases such as AD.13) Activated mast cells elicit the late-phase reaction through the production of such cytokines as IL-3, IL-4 and IL-5, and such lipid mediators as prostaglandins and leukotrienes. Such flavonoids as luteolin, fisetin, apigenin, kaempherol, and quercetin have been reported to inhibit IL-4 and IL-13 synthesis by human basophils.29) Moreover, quercetin and kaempherol-3, 6-D-galactoside have been reported to suppress cysteinyl leukotrien synthesis through the inhibition of phospholipase A2 and 5-lipoxygenase.30,31) Quercetin glycosides such as isoquercitrin, rutin and isoquercitrin malonate, and kaempherol glycosides such as astragaline, kaempherol rutinoside and astragaline malonate were contained in HWE.18) For these reasons, the oral administration of HWE might have inhibited the development of AD-like dermatitis through the inhibition of mast cell activation caused by cross-linking between the antigen and antigen-specific IgE on the surface. In this study, the increase in total IgE concentration in the serum with time was inhibited by the oral administration of HWE in a dose-dependent manner, although there was no significant difference in IL-12 production from CLN cells between the 100 mg/kg and 500 mg/kg of HWE-administered group. Accordingly, the inhibition of total IgE production by the oral administration of HWE might be attributable to not only the inhibition of IL-12 production, but also to the inhibition of mast cells activation. However, further examinations must be conducted to clarify the precise mechanism by which HWE alleviates this chronic allergic disease.

In conclusion, we investigated the inhibitory effect of an oral administration of HWE on the development of AD-like dermatitis induced by periodical topical application of the mite antigen in NC/Nga mice. The oral administration of HWE significantly inhibited the increase of total IgE in the serum and the ear swelling throughout the experimental period. IL-12 production from CLN was significantly increased by periodical topical application of the mite antigen, and it was significantly inhibited by the oral administration of HWE, although IL-4 and IFN-γ production were not affected.

The results obtained in this study suggest that an intake of HWE may be effective for preventing and alleviating the development of AD-like skin diseases.

References
15) Kotani, M., Matsumoto, M., Fujita, A., Higa, S., Wang, W., Suemura, M., Kishimoto, T., and Tanaka, T.,


