

Immunomodulatory Effect of *Eriobotrya japonica* Seed Extract on Allergic Dermatitis Rats

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Summary We examined the immunomodulatory effect of *Eriobotrya japonica* seed extract (ESE) on rat allergic dermatitis elicited by repeated dinitrofluorobenzene (DNFB) application on the ear. Oral administration of ESE significantly inhibited development of allergic dermatitis based on lower ear thickness and serum immunoglobulin E (IgE) levels. Th1 cytokine interferon- γ (IFN- γ) and interleukin-2 (IL-2), Th2 cytokine interleukin-4 (IL-4) and interleukin-10 (IL-10) in the lesional skin were determined. Oral administration of ESE significantly decreased IL-4 while significantly increasing IL-10 in lesional skin, and the lower levels of IFN- γ and IL-2 were reversed by oral administration of ESE. The infiltration of eosinophils in the lesional skin was decreased by oral administration of ESE. These results suggested that ESE exerts anti-allergic actions by improving the balance of Th1/Th2 in allergic dermatitis.

Key Words *Eriobotrya japonica* seed extract, allergic dermatitis, cytokines, DNFB, eosinophil

Eriobotrya japonica is native to China and Japan and is well known as a kind of delicious fruit. *Eriobotrya japonica* seeds are used as a traditional Chinese herb to treat edema. *Eriobotrya japonica* seeds contain amygdalin (1), unsaturated fatty acids, such as linoleic acid and linolenic acid, and plant sterols, such as β -sitosterol, caffeic acid, chlorogenic acid and various essential amino acids (2).

We previously reported *Eriobotrya japonica* seed extract (ESE) is a strong reactive oxygen species scavenger (2) and effective for prevention and treatment of disorders, such as hepatopathy (3), nephropathy (4), type 2 diabetes (5), mucositis (6), allergic contact dermatitis (7), and inhibiting mast cell activation (8).

Itchiness and edema are symptoms that influence the quality of life of patients with atopic dermatitis. Most patients with atopic dermatitis have peripheral blood eosinophilia and increased serum immunoglobulin (Ig) E concentrations. The deviation of the immune response toward either the Th1 or Th2 pathway can elicit a number of pathologic conditions, such as Th2 deviation in allergic reactions and Th1 deviation in organ-specific autoimmunity. Human atopic dermatitis has a strong immune-mediate component and is marked by increased activity levels of Th2 cells and their cytokine, interleukin-4 (IL-4), with fewer immunomodulatory Th1 cells and their cytokines IL-2 and

gamma-interferon (IFN- γ).

In this study, the allergic dermatitis rat animal model was validated, and the immunomodulatory effect of ESE on the late phase of allergic dermatitis was examined.

MATERIALS AND METHODS

Preparation of extract. *Eriobotrya japonica* seed extract was prepared as previously described (7, 8). In brief, *Eriobotrya japonica* seeds were collected in Wakayama and Kochi Prefectures and dried in sunlight. *Eriobotrya japonica* seeds (1 kg) were powdered in a blender equipped with a refrigerator at 1,000 rpm, and extracted by stirring with a mixer at 300 rpm for 1 wk after being dissolved in 70% ethanol. After filtration, the supernatant was collected and evaporated under vacuum to afford 130 g of dried extract. The dried extract was diluted in 1,000 mL water and the diluted solution was stored in a refrigerator until being administered to animals.

Animals. Male 6-wk-old Sprague-Dawley rats were purchased from Japan SLC, Inc., Japan. The animals were kept in a specific pathogen-free animal facility that maintained a temperature of 19–25°C, humidity of 30–70% and a 12 h day/night cycle, and were given access to pellet food and water. The experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Sensitization and elicitation procedure. The rats were divided into three groups of seven animals each: The

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control group were sensitized with 1 mL saline solution containing 10 μ g dinitrophenyl (DNP)-Ovalbumin (OVA) (DNP-OVA, Sigma Chemical Co., St Louis, MO, USA) and 1 mg $\text{Al}(\text{OH})_3$ (Sigma) gel, given as an intraperitoneal injection (i.p.) on days 0, 7, 14. Twenty-one days following the first sensitization, animals were challenged by applying 100 μ L of 0.5% 2,4-dinitrofluorobenzene (DNFB, Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution in acetone : olive oil (4 : 1) to both sides of the right ear (50 μ L on the dorsal side and 50 μ L on the ventral side) 2 times with a 3-d interval. The ESE group were treated the same as the control group rats but received ESE (10 mL/d rat) in drinking water from 2 d before the first challenge to the end of the experiment. The vehicle group was sham-sensitized and received only 1 mg/mL $\text{Al}(\text{OH})_3$ suspended in 1 mL saline (Fig. 1).

Ear thickness was measured 72 h after each challenge using a dial thickness gauge (Ozaki Seisakusho, Tokyo, Japan) under light ether anesthesia. Changes in ear thickness were expressed as mean ear thickness $\pm$ SE.

Measurement of serum IgE level. After 72 h of the second DNFB challenge, blood samples were obtained and centrifuged at $3,000\times g$ for 30 min and stored at -80°C until assay. Total serum IgE was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Inc). The serum samples (100 μ L 10 to 50-fold diluted serum) were applied to each well. The total serum IgE concentration in each well was estimated using a standard sample from the manufacturer's kit.

Measurement of cytokines. Amounts of cytokines in skin lesions were determined by ELISA. Skin biopsies were cut into small pieces and homogenized vigorously with a 30-fold volume of PBS containing 0.05% Tween-20, 100 μ g/mL, and aprotinin 2 μ g/mL. The homogenates were frozen in nitrogen, thawed in a 37°C water

bath, sonicated for 10 s, and centrifuged for 10 min at $12,000\times g$. After the centrifugation, the supernatant was subjected to ELISAs in accordance with the protocol provided by the manufacturer. Briefly, 100 μ L homogenized supernatants for IL-4, IL-10, and IFN- γ (R&D Systems) were incubated with the monoclonal capture antibody for 2 h at room temperature and then washed five times. Polyclonal conjugated antibody was added again and incubated for 2 h. It was then washed and the substrate solution added for 30 min. The optical density at 450 nm was measured in an ELISA microplate reader.

Demonstration of eosinophil infiltrate in the skin by immunohistochemistry. In some experiments, ears were excised at various times after the second challenge. Samples were fixed with 10% formalin for 1 h and embedded in paraffin, and 4 μ m fresh sections were deparaffinized in xylene, and rehydrated through graded concentrations of ethanol. Eosinophils were stained using an alkaline phosphatase antialkaline phosphatase method as described previously (9).

The myelin basic protein (MBP) immunostaining to detect eosinophils in tissue was performed. Slides were incubated with a murine monoclonal IgG1 antibody against the eosinophilic granule protein MBP (clone BMK-13, MONOSAN, Netherlands) overnight at room temperature in a humid chamber, and with an unconjugated rabbit anti-mouse immunoglobulin secondary antibody. Specific binding was revealed after 1 h incubation with biotinylated goat anti-mouse IgG Ab (MP Biomedicals, LLC, USA). After 30 min incubation with alkaline phosphatase conjugated avidin (Rockland Immunochemicals, Inc., USA), the slides were developed with alkaline phosphatase substrate (naphthol phosphate, AppliChem), supplemented with Fast Red (Sigma Chemical Co.) as a chromogen, and counterstained with hematoxylin. Individual inflammatory cell types were counted in 4–8 high-power fields (HPFs) at $\times 200$ and expressed as cells per HPF, with mean and SE calculated.

Statistical analysis. Descriptive data are expressed as mean $\pm$ SE. Groups were compared by a one-way ANOVA with post hoc analyses for pairwise comparisons (ANOVA) and linearity. Statistical significance was inferred at a two-sided value of $p < 0.05$ (t -test).

RESULTS

The effects of ESE on ear swelling

The ear swelling change in DNFB-induced rat ear dermatitis is shown in Fig. 2. DNFB applied to the ear of sensitized mice caused immediate type dermatitis with ear thickness increasing after 15 min. In order to investigate the effect of ESE on the late phase of immediate type response of allergic dermatitis, the ear thickness was determined at 72 h after each DNFB challenge. The time course of ear swelling response showed that administration of ESE suppressed the ear swelling of immediate response and alleviated the ear thickness by 15.2% in the first challenge and 32.5% in the second challenge, respectively, compared with the control

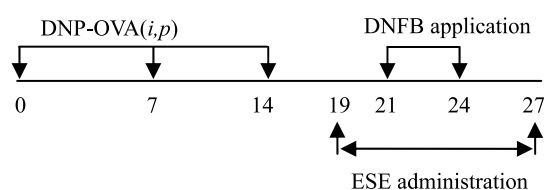


Fig. 1. Sensitization and elicitation procedure. Control group were sensitized with 1 mL saline solution containing 10 μ g dinitrophenyl (DNP)-Ovalbumin (OVA) and 1 mg $\text{Al}(\text{OH})_3$ gel, given as an intraperitoneal injection (i.p.) on days 0, 7, 14. Twenty-one days following first sensitization, animals were challenged by applying 100 μ L of 0.5% 2,4-dinitrofluorobenzene (DNFB) solution in acetone : olive oil (4 : 1) to both sides of the right ear (50 μ L on the dorsal side and 50 μ L on the ventral side) 2 times with 3-d interval. The ESE group were treated the same as the control group rats but received ESE (10 mL/d rat) in drinking water from 2 d before the first challenge to the end of the experiment. The vehicle group was sham-sensitized and received only 1 mg/mL $\text{Al}(\text{OH})_3$ suspended in 1 mL saline.

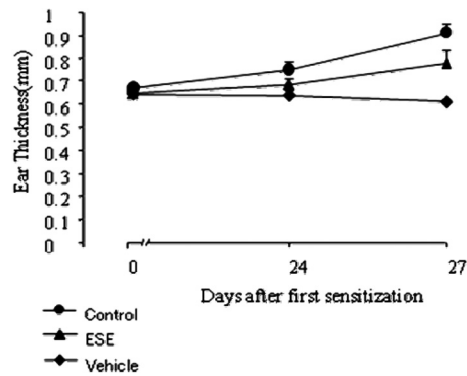


Fig. 2. The effects of ESE on ear swelling. Rats were sensitized with 1 mL saline solution containing DNP-OVA ($10 \mu\text{g}$) and $\text{Al}(\text{OH})_3$ (1 mg), given as an intraperitoneal injection on days 0, 7, 14. Twenty-one days following sensitization, rats were challenged with 100 μL acetone/olive oil (4 : 1) containing 0.5% DNFB to the two sides of the right ear at 3-d intervals two times. Ear thickness was measured at 72 h after DNFB challenge. Values are expressed as the mean $\pm$ SE of seven animals. * $p < 0.05$, compared with the control value (Student's t -test).

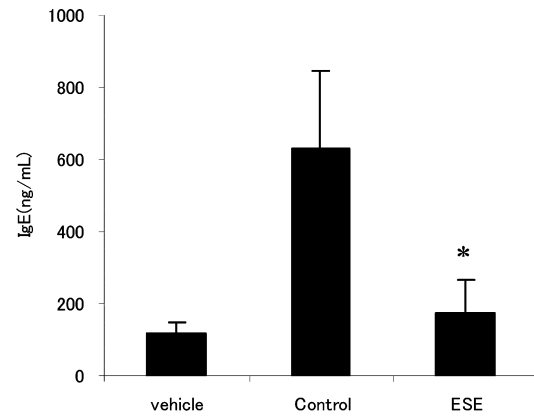


Fig. 3. The effects of ESE on serum IgE level. Rats were sensitized with 1 mL saline solution containing DNP-OVA ($10 \mu\text{g}$) and $\text{Al}(\text{OH})_3$ (1 mg), given as an intraperitoneal injection on days 0, 7, 14. Twenty-one days following sensitization, rats were challenged with 100 μL acetone/olive oil (4 : 1) containing 0.5% DNFB to the two sides of the right ear at 3-d intervals two times. After 72 h of the second DNFB challenge, blood was obtained and serum IgE level was determined with ELISA. Values are expressed as the mean $\pm$ SE of seven animals. * $p < 0.05$, compared with the control value (Student's t -test).

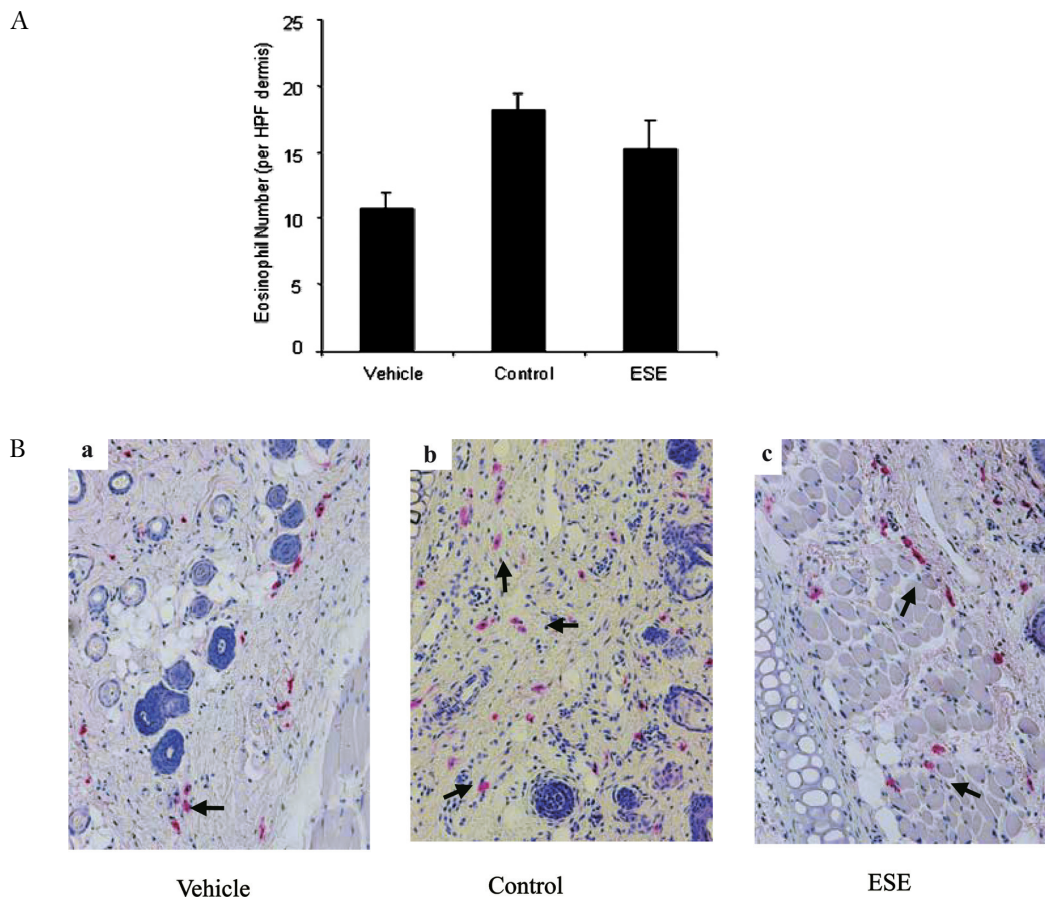


Fig. 4. A: The effects of ESE on eosinophil number in lesional skin. Rats were sensitized with 1 mL saline solution containing DNP-OVA ($10 \mu\text{g}$) and $\text{Al}(\text{OH})_3$ (1 mg), given as an intraperitoneal injection on days 0, 7, 14. Twenty-one days following sensitization, rats were challenged with 100 μL acetone/olive oil (4 : 1) containing 0.5% DNFB to the two sides of the right ear at 3-d intervals two times. After 72 h of the second challenge, ears were excised and subjected to immunohistology. Individual inflammatory cell types were counted in 4–8 high-power fields (HPFs) at $\times 200$ and expressed as cells per HPF, with mean $\pm$ SE calculated. B: The representation of immunohistology staining of eosinophils in lesional skin. (a) Vehicle; less infiltration of eosinophils was observed. (b) Control; infiltration of eosinophils was observed. (c) ESE; ESE inhibited eosinophil infiltration when orally administered beginning 2 d before the first DNFB challenge. Magnification: $\times 200$.

Table. 1. Effects of *Eriobotrya japonica* seed extract on cytokines in lesional skin.

	Vehicle	Control	ESE
IL-10 (pg/mg protein)	40.7±1.5	44.1±4.0	66.8±8.0*
IL-4 (pg/mg protein)	1,579.7±481.8	3,378.9±785.4	1,223.9±199.4*
IFN- γ (pg/mg protein)	2,985.1±308.4	2,660.0±102.	3,255.4±218.8
IL-2 (pg/mg protein)	985.4±61.1	917.9±37.7	958.4±62.6

Rats were sensitized with 1 mL saline solution containing DNP-OVA (10 μ g) and Al(OH)₃ (1 mg), given as an intraperitoneal injection on days 0, 7, 14. Twenty-one days following sensitization, rats were challenged with 100 μ L acetone/olive oil (4 : 1) containing 0.5% DNFB to the two sides of the right ear at 3-d intervals two times. After 72 h of the second challenge, ears were excised and subjected to cytokine determination. Values are expressed as the mean±SE of seven animals.

* p <0.05, compared with the control value (Student's t -test).

group.

The effects of ESE on serum IgE level

After 72 h of the second DNFB challenge, blood was obtained and centrifuged at 5,000 rpm for 30 min; the serum IgE level was determined with ELISA. Serum IgE level in the ESE group (172.5±96.1 ng/mL) decreased significantly compared to the control group (629.5±217.0 ng/mL) (p <0.05) (Fig. 3).

The effects of ESE on the levels of IL-10, IL-4, IL-2 and IFN- γ

After 72 h of the second DNFB challenge, skin biopsies were homogenized and centrifuged at 12,000× g for 10 min and subjected to ELISA for IL-10 and IL-4. ESE administration significantly increased the levels of IL-10 in ESE-treated group compared with the vehicle group and control group, but no difference between the vehicle group and control group was found. ESE administration significantly inhibited the levels of IL-4 in the ESE-treated group compared with the control group (Table 1). The levels of IFN- γ and IL-2 protein in dermatitis tissue showed a similar pattern. Although the IFN- γ levels of the control group decreased in comparison with the vehicle group, ESE administration increased IFN- γ production; the production of IL-2 in the ESE group was also slightly higher than that of the control group (Table 1).

The effects of ESE on the infiltration of eosinophil in lesional skin

To identify the inflammatory cells infiltrating the dermis of lesional skin, eosinophils were stained by the immunostain technique using antibodies against MBP. Orally administering ESE decreased the infiltration of eosinophils (Fig. 4).

DISCUSSION

In a previous report, we clarified the main components of ESE and investigated the antioxidative action (2, 6); we also clarified that ESE was useful for the improvement of allergic diseases (7, 8) and nephropathy (4) in a rat model, but immunomodulatory effects on allergy diseases are still unclear.

In previous study, rats were sensitized with DNFB application on the abdomen. Allergic dermatitis was elicited by repeated application of DNFB five times. Ear swelling appeared as an immediate response followed by a late phase response (7).

We are also interested in an allergic dermatitis model procedure in a previous report (10). In this study, SD rats were actively sensitized with dinitrophenol (DNP)-ovalbumin (OVA) intraperitoneally. Serum IgE levels increased with DNP-OVA immunized at this stage (data not shown). After the DNFB challenge, ear swelling appeared as an immediate response followed by a late phase response from the first challenge.

In order to explain the improving effects of ESE on the contact dermatitis, the serum immunoglobulin E and cytokines in lesional skin were measured. The immunoglobulin E level was significantly inhibited by oral ESE administration, and a vast majority of patients with atopic dermatitis exhibit hyperproduction of immunoglobulin E, particularly during disease onset or flare. Immunoglobulin E-dependent late-phase reactions may influence the chronic inflammation response in atopic dermatitis (11). The allergic dermatitis improving function may be established as one of the effects of ESE on allergic dermatitis.

ESE administration significantly reduced the levels of the anti-inflammatory cytokine IL-4, and increased the production of the anti-inflammatory IL-10 in lesional skin. It also has been reported that IL-10 is a natural suppressant of irritant and of chronic hypersensitivity responses, and hence limits immune-mediated damage in the skin. Mouse models with IL-10 deficiencies display exaggerated chronic hypersensitivity responses and more extensive immunopathologic cutaneous damage (12–15). Intradermal administration of IL-10 before challenge can inhibit the elicitation stage of allergic contact dermatitis. It is interesting that we got a similar result in a rat model. These observations and our results suggest that therapeutic manipulation of IL-10 production could be a part that ESE plays in the treatment of allergic contact dermatitis.

It is noteworthy that the IL-4 concentration in inflamed tissue was significantly reduced by oral administration of ESE, whereas no significant changes in IFN- γ concentration were observed. Evidence exists that the Th1 and Th2 types of immune response are reciprocally regulated in vivo (16–18). IFN- γ , which is mainly produced by Th1 cells, especially inhibits the proliferation of Th2 cells and their IL-4 production (19, 20). These results suggest that the inhibitory effect of ESE on IL-4 production was not due to Th1 activation,

but it is possible that oral administration of ESE might directly inhibit IL-4 production, and increase IL-10 production.

The infiltration of eosinophils in the lesional skin in atopic dermatitis has been well established. A correlation between eosinophilia and the degree of spongiosis has been noticed in acute dermatitis or acute exacerbations of chronic atopic dermatitis (21). This suggests that the immunomodulatory effects of ESE on alleviating the symptom of the allergic dermatitis may be through inhibiting the eosinophil infiltration into the lesional skin.

In summary, the suppression of the hypersensitivity reaction together with reduction of IgE and IL-4 production and increase of IL-10 may explain our findings that ESE may serve as a dietary supplementation for the immunomodulation of allergic disease. Further study for other cytokines from lymphocytes and antigen-presenting cells will enhance our understanding the mechanism of the anti-allergic activities of ESE.

Acknowledgments

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REFERENCES

- 1) Gray DO, Fowden L. 1972. Isolation of 4-methylene-proline from *Eriobotrya japonica*. *Phytochemistry* **11**: 745–750.
- 2) Yokota J, Takuma D, Hamada A, Onogawa M, Yoshioka S, Kusunose M, Miyamura M, Kyotani S, Nishioka Y. 2006. Scavenging of reactive oxygen species by *Eriobotrya japonica* seed extract. *Biol Pharm Bull* **29**: 467–471.
- 3) Nishioka Y, Yoshioka S, Kusunose M, Cui T, Hamada A, Ono M, Miyamura M, Kyotani S. 2002. Effects of extract derived from *Eriobotrya japonica* on liver function improvement in rats. *Biol Pharm Bull* **25**: 1053–1057.
- 4) Hamada A, Yoshioka S, Takuma D, Yokota J, Cui T, Kusunose M, Miyamura M, Kyotani S, Nishioka Y. 2004. The effect of *Eriobotrya japonica* seed extract on oxidative stress in adriamycin-induced nephropathy in rats. *Biol Pharm Bull* **27**: 1961–1964.
- 5) Tanaka K, Nishizono S, Makino N, Tamaru S, Terai O, Ikeda I. 2008. Hypoglycemic activity of *Eriobotrya japonica* seeds in type 2 diabetic rats and mice. *Biosci Biotechnol Biochem* **72**: 686–693.
- 6) Takuma D, Sun G, Yokota J, Hamada A, Onogawa M, Yoshioka S, Kusunose M, Miyamura M, Kyotani S, Nishioka Y. 2008. Effect of *Eriobotrya japonica* seed extract on 5-fluorouracil-induced mucositis in hamsters. *Biol Pharm Bull* **31**: 250–254.
- 7) Sun G, Zhang Y, Takuma D, Onogawa M, Yokota J, Hamada A, Yoshioka S, Kusunose M, Miyamura M, Kyotani S, Nishioka Y. 2007. Effect of orally administered *Eriobotrya japonica* seed extract on allergic contact dermatitis in rats. *J Pharm Pharmacol* **59**: 1405–1412.
- 8) Onogawa M, Sun G, Takuma D, Hamada A, Yokota J, Yoshioka S, Kusunose M, Miyamura M, Kyotani S, Nishioka Y. 2009. Animal studies supporting the inhibition of mast cell activation by *Eriobotrya japonica* seed extract. *J Pharm Pharmacol* **61**: 237–241.
- 9) Frew JA. 1988. The relationship between infiltrating CD4+ lymphocytes, activated eosinophils, and the magnitude of the allergen-induced late phase cutaneous reaction in man. *J Immunol* **141**: 4158–4164.
- 10) Tahara E, Satoh T, Watanabe C, Shimada Y, Itoh T, Nagai H, Terasawa K, Saiki I. 1999. A third-phase cutaneous (very late phase) response after elicitation with dinitrofluorobenzene in passively or actively sensitized mice. *Allergol Int* **48**: 265–273.
- 11) Abramovitis W. 2005. Atopic dermatitis. *J Am Acad Dermatol* **53**: s86–93.
- 12) Berg DJ, Leach MW, Kuhn R, Rajewsky K, Muller W, Davidson NJ, Rennick D. 1995. Interleukin 10 but not interleukin 4 is a natural suppressant of cutaneous inflammatory responses. *J Exp Med* **182**: 99–108.
- 13) Kondo S, Pastore S, Shivji GM, McKenzie RC, Sauder DN. 1994. Characterization of epidermal cytokine profiles in sensitization and elicitation phases of allergic contact dermatitis as well as irritant contact dermatitis in mouse skin. *Lymphokine Cytokine Res* **13**: 367–375.
- 14) Schwarz A, Grabbe S, Riemann H, Aragane Y, Simon M, Manon S, Andrade S, Luger TA, Zlotnik A, Schwarz T. 1994. In vivo effects of interleukin-10 on contact hypersensitivity and delayed-type hypersensitivity reactions. *J Invest Dermatol* **103**: 211–216.
- 15) Ferguson TA, Dube P, Griffith TS. 1994. Regulation of contact hypersensitivity by interleukin 10. *J Exp Med* **179**: 1597–1604.
- 16) Heinzel FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. 1989. Reciprocal expression of interferon γ or IL-4 during the resolution or progression of murine leishmaniasis: evidence for distinct helper T cell subsets. *J Exp Med* **169**: 59–72.
- 17) Romagnani S. 1991. Human Th1 and Th2 subsets: doubt no more. *Immunol Today* **12**: 256–257.
- 18) Mosmann TR, Coffman RL. 1987. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* **7**: 145–173.
- 19) Gajewski TF, Schell SR, Nau G, Fitch FW. 1989. Regulation of T cell activation: differences among T-cell subsets. *Immunol Rev* **111**: 79–110.
- 20) Stout RD, Bottomly K. 1989. Antigen-specific activation of effector macrophages by IFN- γ producing (Th1) T cell clones. Failure of IL-4-producing (Th2) T cell clones to activate effector function in macrophages. *J Immunol* **142**: 760–765.
- 21) Kiehl P, Falkenberg K, Vogelbruch M, Kapp A. 2001. Tissue eosinophilia in acute and chronic dermatitis: a morphometric approach using quantitative image analysis of immunostaining. *Br J Dermatol* **145**: 720–729.