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Original Article

Effect of Am-80, a novel retinoid derivative, on contact hypersensitivity caused by repeated applications of hapten in mice

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Abstract

Some retinoids show an anti-inflammatory action through regulation of transcription of various genes. In the present study, the inhibitory effect of 4-((5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl) carbamoyl) benzoic acid (Am-80), a synthetic retinoid, on mouse contact hypersensitivity provoked by repeated applications of 2,4-dinitrofluorobenzene (DNFB) to the ear was investigated. Five-fold applications of DNFB on ears once per week elicited severe contact dermatitis with marked infiltration of inflammatory cells and elevation of anti-dinitrophenyl (DNP)-IgE antibody in the serum. The Am-80 significantly inhibited ear swelling in a dose-dependent manner. In the histopathologic study, infiltration of inflammatory cells was clearly decreased by Am-80. However, Am-80 did not affect the production of DNP-specific IgE antibody both at the transcriptional and post-transcriptional levels. The effects of Am-80 on the transcriptional level of cytokines, interferon (IFN)-γ, interleukin (IL)-1 and IL-4 in cervical lymph nodes were investigated. Marked elevation of mRNA for all cytokines was observed and Am-80 potently inhibited the expression of IFN-γ mRNA, but not IL-1 and IL-4 mRNA. These findings indicated that Am-80 may inhibit the contact dermatitis at the post-sensitization phase by inhibiting IFN-γ production at the transcriptional level in mice.

Key words: 2,4-dinitrofluorobenzene, Am-80, contact dermatitis, cytokine, retinoid.

Introduction

The skin is an important immunologic tissue and acts as the first barrier to protect against invasive factors, including viruses, chemicals and insects. The inflammatory stimulus, which results from exposure to a variety of chemical agents, generates the irritation and itching and can lead to severe contact dermatitis. The contact dermatitis provoked by chemical agents, such as picryl chloride (PC) and 2,4-dinitrofluorobenzene (DNFB), is an inflammatory disorder generated in the local skin. In contact dermatitis, the proliferation of keratinocytes and marked infiltration of inflammatory cells, including neutrophils, monocytes and lymphocytes, to the skin are observed.[1] In addition, there is some evidence to indicate that various kinds of cytokines including interleukin (IL)-4, interferon (IFN)-γ, IL-6 and IL-1 play crucial roles for the initiation and progression of contact dermatitis.[2–5] Among these cytokines, the importance of IFN-γ in the elicitation and progression of contact dermatitis has been verified using IFN-γ-overexpressing mice and IFN-γ receptor-deficient mice.[6,7] Carroll *et al*. have reported that IFN-γ transgenic mice show an augmentation of contact hypersensitivity to topical application of DNFB, compared with wild-type mice.[6] Saulnier *et al*. have reported that dermal infiltration of mononuclear cells

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and epidermal microabscesses induced by the 2,4,6-trinitrochlorobenzene (TNCB) is diminished in IFN-γ receptor-deficient mice.[7] In addition to this evidence, some reports have shown that retinoids regulate transcription of cytokines such as IL-6 and IFN-γ.[8,9] Cippitelli *et al*. have demonstrated that retinoic acid inhibits IFN-γ gene expression through the down-modulation of activation of the IFN-γ promoter.[8]

Retinoid is a potent ligand for retinoic acid receptors (RAR), which belong to the steroid/thyroid receptor superfamily.[10] Three RAR subtypes, including α, β and γ, have been identified.[11] The retinoid-RAR complex elicits a variety of biological activities, such as the regulation of cell growth,[12] differentiation[13] and anti-angiogenesis,[14] through the regulation of transcription of various genes either by activating the expression of genes, including RAR-responsive elements (RARE) in the promoter regions,[15] or by inhibiting expression of some genes by antagonizing AP-1-mediated gene expression.[16]

In a previous study, a novel synthetic retinoid, 4-((5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl) carbamoyl) benzoic acid (Am-80) showed an inhibitory effect on collagen-induced arthritis in mice, which is an autoimmune disease model, by inhibiting the production of pro-inflammatory cytokines at the transcriptional level.[9] Am-80 has approximately 10-fold more potent biological activity than all-trans-retinoic acid by binding to the RARα and RARβ, but not RARγ. Am-80 has superior properties, including stability to light, heat and oxidation and less toxicity and resistance than all-trans-retinoids.

These findings suggest that Am-80 may inhibit contact dermatitis. Previously, a mouse model generating severe contact hypersensitivity[17] was established. In this model, mice receiving repeated paintings of DNFB to their ears showed severe dermatitis, together with marked infiltration of inflammatory cells including neutrophils, mononuclear cells and eosinophils, increased hapten-specific IgE in serum and excess expression of mRNA for IFN-γ and IL-4 in cervical lymph nodes.

In the present study, Am-80 is shown to potently inhibit contact dermatitis induced by repeated applications of DNFB through inhibition of the inflammatory cytokines, especially IFN-γ.

METHODS

Animals

Female C57BL/6 mice of the age of 6 weeks, obtained from Japan SLC Inc. (Hamamatsu, Japan), were housed in plastic cages in an air-conditioned room at 24°C, fed a standard laboratory diet and allowed free access to water. All experiments were carried out following the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animal Science of 1987.[18]

Reagents

The Am-80 was synthesized as described previously.[9] The Am-80 was suspended in 0.5% carboxymethyl cellulose (CMC) solution for oral administration. Monoclonal anti-dinitrophenyl (DNP) IgE antibody (Clone SPE-7; Sigma Chemical Co., St Louis, MO, USA), peroxidase-conjugated streptavidin (Dakopatts a/s, Glostrup, Denmark), bovine serum albumin (BSA for ELISA grade; Sigma Chemical Co.), DNP-BSA (LSL Co. Ltd, Tokyo, Japan), NHS-LC-Biotinylation kit (Pierce Chemical Co., Rockford, IL, USA), substrate kit (Sumitomo Bakelite Co. Ltd, Tokyo, Japan), ISOGEN (Nippon Gene Co. Ltd, Tokyo, Japan), reverse transcriptase polymerase chain reaction (RT-PCR) primer sets (β-actin, IFN-γ, IL-1, IL-4; Stratagene Co. Ltd, La Jolla, CA, USA), 1st-STRANDED TM cDNA Synthesis kit (Clontech Lab. Inc., Palo Alto, CA, USA) and PCR Reagents Kit (Perkin Elmer Japan Co. Ltd, Urayasu, Japan) were purchased from listed suppliers.

Induction of DNFB-induced contact hypersensitivity

Ears of mice were painted with 25 μL 0.15% DNFB in vehicle (acetone:olive oil = 3:1) or vehicle alone applied to each side of the ear once a week. Ear thickness was measured at various time points after applying DNFB using an engineering micrometer (R1-A, Ozaki MFG Co. Ltd, Tokyo, Japan) and is expressed as the increase in thickness from that at time 0. The Am-80 suspended in CMC was orally administered once per day throughout the experiment from the first sensitization with DNFB.

Histopathologic study of skin lesion

Mice ears were removed 24 h after the fifth painting with vehicle or DNFB and fixed with 10% neutral formalin. Ears were then cut into parasagittal slices, dehydrated and embedded in paraffin according to the standard procedures. Paraffin sections were stained with hematoxylin and eosin, then assessed under a light microscope.

Measurement of hapten specific IgE concentration in mice sera

To measure anti-DNP IgE levels in serum, sera were obtained from the mice 24 h after the fifth painting with DNFB. The anti-DNP IgE in mice sera were measured using the enzyme-linked immunosorbent assay (ELISA) described below.

The immunoplate (Maxisorp Nunc-Immuno Plate, Nalge Nunc International, Tokyo, Japan) was coated with monoclonal antimouse IgE antibody and incubated at 4°C overnight. To decrease the non-specific reaction of antibodies, the immunoplate was treated with phosphate-buffered saline (PBS) containing 1% BSA, incubated at room temperature for 1 h and washed three times with PBS containing 0.2% Tween 20 (T-PBS). Monoclonal anti-DNP IgE antibody was sequentially diluted as the standard. Diluted serum samples at a volume of 100 μL were added to each well and the plates were incubated at room temperature for 1 h. After washing with T-PBS, 100 μL diluted biotinylated DNP-BSA was added to each well and incubated at room temperature for 1 h. After extensive washing with T-PBS, 100 μL diluted peroxidase-conjugated streptavidin was added to each well. The enzymatic reaction was stopped by adding 100 μL stop solution after incubation at room temperature for 1 h. The optical density of the reaction mixture was read using an automatic ELISA plate reader (Titertek Multiscan MCC/340, Flow Laboratories Inc.) at 450 nm.

The anti-DNP IgE titers were expressed in ng/mL, based on laboratory generated standards and appropriate commercial standards.

Analysis of mRNA expression of cytokines in mice cervical lymph nodes and ears by RT-PCR

Expression levels of mRNA for cytokines in cervical lymph nodes of mice were assessed by the RT-PCR technique. Using ISOGEN, total RNA was extracted from cervical lymph nodes in mice at 4 h after the fifth treatment with DNFB.

The total RNA (500 ng) was reverse-transcribed with the 1st-STRANDED TM cDNA Synthesis Kit at 42°C for 60 min, 94°C for 5 min and then soaked at 5°C for 5 min. The PCR was performed under standard conditions as follows: (i) 5 min at 94°C for 1 cycle; (ii) 1 min 30 s at 94°C , 1 min 30 s at 60°C and 1 min 30 s at 72°C for 30 or 35 cycles; (iii) 10 min at 72°C for 1 cycle; and (iv) 5 min at 5°C.

Primer sets used for the amplification of first-strand cDNA were as follows: IFN-γ, 5′-TACTGCCACG-GCACAGTCATTGAA-3′, 5′-GCAGCGACTCCTTTTCC-GCTTCCT-3′; IL-1-β, 5′-CAGGATGAGGACATGAG-CACC-3′, 5′-CTCTGCAGACTCAAACTCCAC-3′; IL-4, 5′-ACGGAGATGGATGTGCCAAACGTC-3′, 5′-CGAG-TAATCCATTTGCATGATGC-3′; and β-actin, 5′-GTGG-GCCGCTAGGCACCA-3′, 5′-CGGTTGGCCTTAGG-GTTCAGGGGGG-3′. The PCR products were resolved by electrophoresis and stained with ethidium bromide to reveal the amplified cDNA.

Statistics

Results are expressed as the mean ± SEM. One-way ANOVA/Dunnett's post hoc procedure was used to assess the significance of results. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of Am-80 on the contact dermatitis generated by repeated applications of DNFB

The ears of mice painted with 0.15% DNFB, but not with vehicle alone, provoked severe contact dermatitis in proportion to the number of DNFB applications. Ear swelling was increased at approximately 1 h and reached a peak at 24 h after painting with DNFB (Fig. 1a). The Am-80 was administrated at the dosage of 0.3–3.0 mg/kg per day orally once per day throughout the experiment after the first sensitization with DNFB. The Am-80 significantly inhibited ear swelling 1 h and 24 h after the fifth painting with DNFB, in a dose-dependent manner (Fig. 1b). Throughout this experiment, conspicuous adverse effects, such as loss of weight, were not observed in mice treated with Am-80 (data not shown).

In the histopathologic study, marked infiltration of cells such as neutrophils, lymphocytes and monocytes were elicited in the ears of mice treated with 0.15% DNFB, but not with vehicle alone. In the ears of mice administered Am-80 at the dosage of 3.0 mg/kg per day, infiltration of cells was clearly reduced (Fig. 2).

Effect of Am-80 on the production of anti-DNP IgE at the transcriptional and post-transcriptional levels

We investigated the effect of Am-80 on the production of anti-DNP IgE in sera. The sera were obtained from mice

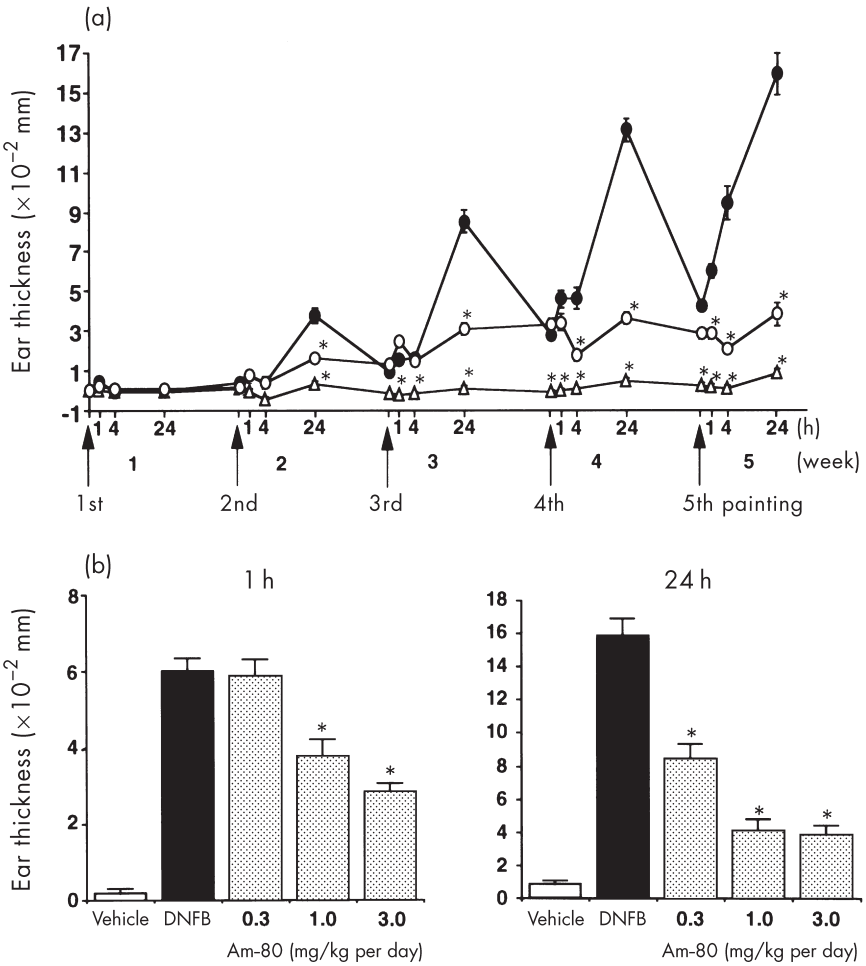

Fig. 1 Effects of 4-((5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl) carbamoyl) benzoic acid (Am-80) in the time course study (a) and dose–response study (b) on the contact dermatitis elicited by repeated applications of 2,4-dinitrofluorobenzene (DNFB). In the dose–response study, the findings after the fifth application of DNFB are shown. The Am-80 was orally administered once per day throughout the experiment from the first sensitization. Results are represented as the mean ± SEM of increased ear thickness from that at time 0 ($n = 6$). (△), vehicle; (●), DNFB; (○) Am-80 3.0 mg/kg per day. *$P < 0.05$ was considered as statistically significant compared with the control group.

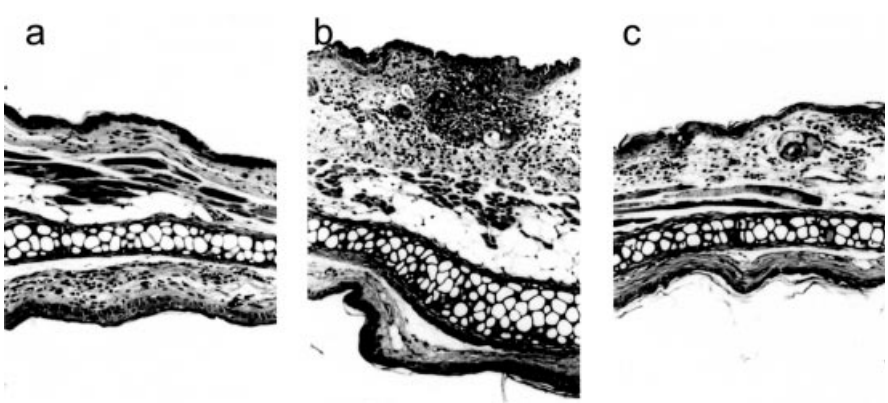

Fig. 2 Effects of 4-((5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl) carbamoyl) benzoic acid (Am-80) on histopathology. At 24 h after the fifth application of 2,4-dinitrofluorobenzene (DNFB), ears of mice were isolated, fixed and stained with hematoxylin and eosin. The Am-80 was administered once per day throughout the experiment from the first application of DNFB. (a) Vehicle, (b) DNFB, (c) DNFB + Am-80 (3.0 mg/kg per day).

24 h after the fifth painting with DNFB. The amounts of anti-DNP IgE were measured using the ELISA technique. The serum level of anti-DNP IgE was significantly elevated in mice treated with 0.15% DNFB, but not with vehicle alone, in proportion to the number of DNFB applications (data not shown). As shown in Fig. 3a, Am-80 did not affect the production of anti-DNP IgE even at the highest dosage (3.0 mg/kg per day, $P = 0.563$). In addition, the effect of Am-80 on the transcriptional level of productive Cε in cervical lymph nodes was examined. Cervical lymph nodes were isolated from mice 4 h after the fifth treatment with DNFB and total

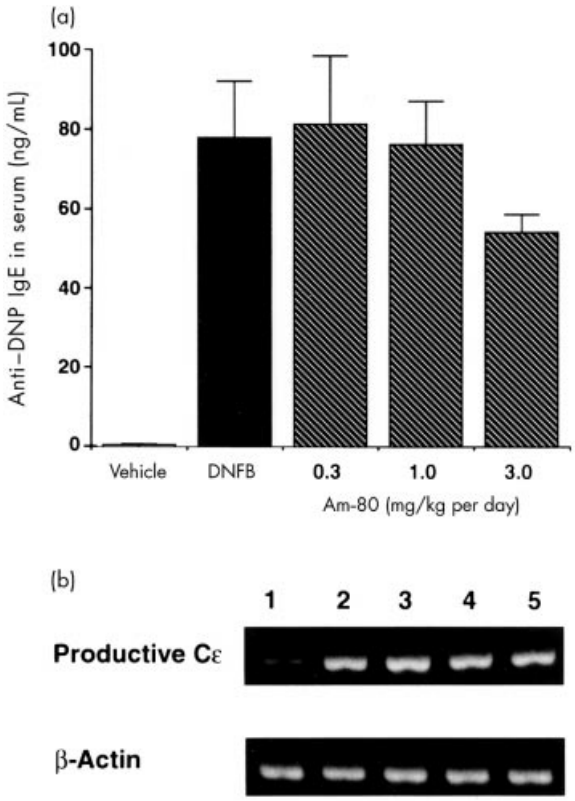

Fig. 3 Effects of 4-((5,6,7,8-tetrahydro-5,5,8,8-tetra-hydro-5,5,8,8-tetra-methyl-2-naphthalenyl) carbamoyl) benzoic acid (Am-80) on serum levels of anti-dinitrophenyl (DNP) IgE. Mice sera were isolated 24 h after the fifth application of 2,4-dinitro-fluorobenzene (DNFB). Quantities of anti-DNP IgE in sera were assessed using the enzyme-linked immunosorbent assay technique. Results are represented as the mean ± SEM of six samples. The Am-80 was administered once per day throughout the experiment from the first application of DNFB. (b) Lane 1, vehicle; lane 2, DNFB; lanes 3–5, DNFB + Am-80 at 0.3 (lane 3), 1.0 (lane 4) or 3.0 (lane 5) mg/kg per day.

RNA was extracted and purified using ISOGEN. The expression level of productive Cε mRNA was assessed using the RT-PCR technique. The Am-80 did not affect the expression level of productive Cε mRNA in cervical lymph nodes (Fig. 3b). These findings suggested that Am-80 did not affect the production of anti-DNP IgE at both the transcriptional and post-transcriptional levels; and that it inhibited the contact dermatitis at the post-sensitization phase.

Effect of Am-80 on expression levels of cytokine mRNA in the cervical lymph nodes of mice

In the preliminary experiment, the expression levels of mRNA for these cytokines in the cervical lymph nodes of DNFB-treated mice reached their peak and plateau 4–8 h after DNFB-sensitization (data not shown). Therefore, the effect of Am-80 on transcription levels of cytokines, including IFN-γ, IL-1 and IL-4, on the cervical lymph nodes of mice at 4 h after the treatment with DNFB was investigated. Expression levels of mRNA for all cytokines was elevated in cervical lymph nodes of mice treated with DNFB compared with mice treated with vehicle alone. The Am-80 obviously diminished expression levels of mRNA for IFN-γ, but not for IL-1 and IL-4, in a dose-dependent manner (Fig. 4).

Discussion

Contact dermatitis is an allergic inflammatory skin disease with marked infiltration of inflammatory cells. In the present study, the effect of Am-80 on mouse contact hypersensitivity due to repeated application of DNFB to ears was investigated. In this model, severe elevation of ear swelling and serum anti-DNP IgE levels were observed. Oral administration of Am-80 once per day showed a potent and significant inhibition of the ear swelling and histopathologic changes in the skin lesion. However, Am-80 did not affect the serum level of anti-DNP IgE and the expression level of productive Cε mRNA in the cervical lymph node. Immunoglobulin E, following the activation of mast cells, plays a crucial role in the ear swelling response at early time points through the release of a variety of chemical mediators, such as histamine, serotonin and leukotrienes. In the present study, although the serum level of anti-DNP IgE was elevated in parallel with the increase of ear swelling, Am-80 inhibited an increase in ear swelling at 1 h after treatment with DNFB, despite no affect on the anti-DNP IgE level. This finding suggests that Am-80 may inhibit the function of mast cells at the post IgE–antigen interaction stage. In addition, Geba *et al*. have reported the importance of serotonin release from platelets during the early phase reaction of contact hypersensitivity.[19] To clarify the mechanism of how Am-80 inhibits the early phase reaction of dermatitis without inhibiting the production of IgE requires further studies.

Much attention has been paid to the role of cytokines in the onset and development of allergic skin disease. Some reports show the roles of IL-4,[2] IFN-γ[3] and IL-6[4] in contact dermatitis. Among them, IFN-γ especially contributes to the initiation and progression of contact hypersensitivity. Interferon-γ, which is generated especially by T helper (Th)1 cells, induces various kinds of biologic events, such as the elicitation of adhesion molecule expression, keratinocyte

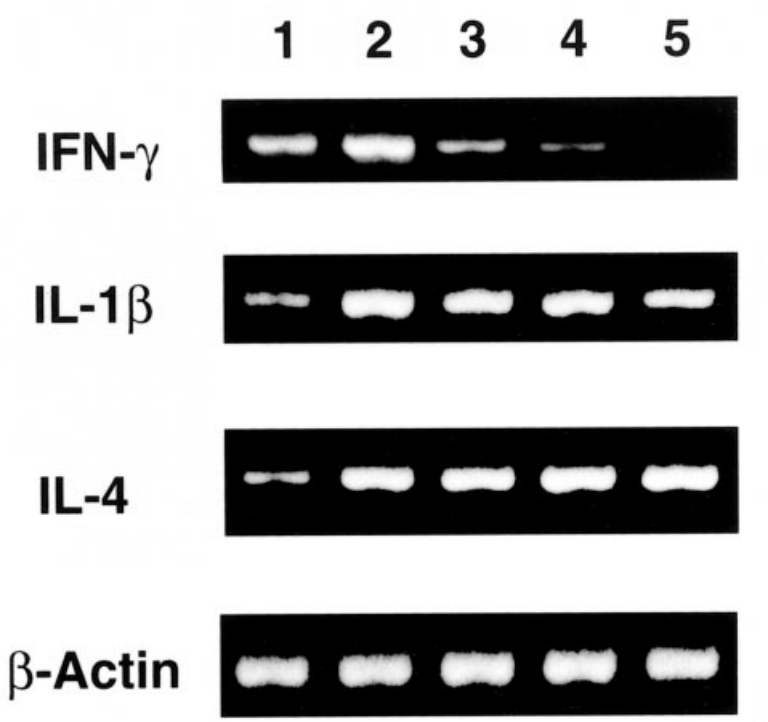

Fig. 4 Effects of 4-((5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl) carbamoyl) benzoic acid (Am-80) on transcriptional levels of interferon (IFN)-γ, interleukin (IL)-1 and IL-4 in the cervical lymph nodes of mice. Lymph nodes were isolated 4 h after the fifth application of 2, 4-dinitrofluorobenzene (DNFB). Expression of mRNA was assessed using the reverse transcriptase polymerase chain reaction technique. The Am-80 was administered once per day throughout the experiment from the first application with DNFB. Lane 1, vehicle; lane 2, DNFB; lanes 3–5, DNFB + Am-80 at 0.3 (lane 3), 1.0 (lane 4) or 3.0 (lane 5) mg/kg per day.

proliferation and down-regulation of Th2-type cytokines.[20] Some studies have also shown the functional role of IFN-γ in contact dermatitis. Carroll *et al*. have reported an augmentation of contact dermatitis in IFN-γ transgenic mice.[6] Furthermore, Saulnier *et al*. have reported an abrogation of dermal infiltration of mononuclear cells and epidermal microabscesses in IFN-γ receptor-deficient mice.[7] In the present study, Am-80 clearly decreased the expression level of mRNA for IFN-γ, but not for IL-1 and IL-4, in cervical lymph nodes of DNFB-sensitized mice. Cippitelli *et al*. have also reported that retinoic acid diminishes the expression level of the IFN-γ gene through down-modulation of the IFN-γ promoter.[8] Their findings support those of the present study. In the histopathologic study of the mouse ear, the marked reduction in inflammatory cell infiltration by Am-80 may have been due to the suppression of adhesion molecules by the inhibition of IFN-γ production. However, in contact hypersensitivity many kinds of cytokines are involved in the initiation and development of the reaction and the cytokine network is complicated. Therefore, further studies will be necessary to clarify this point.

In conclusion, it has been demonstrated that Am-80 potently inhibits the contact dermatitis caused by repeated applications of DNFB, probably through the depression of IFN-γ mRNA. Hence, Am-80 may be useful for treatment of contact dermatitis. For the clinical trial, however, further studies, such as investigating the effect of Am-80 in mice chronically inducing contact hypersensitivity, will be necessary.

References

1 Barker JN. Role of keratinocytes in allergic contact dermatitis. *Contact Dermatitis* 1992; **26**: 145–8.
2 Asada H, Linton J, Katz SI. Cytokine gene expression during the elicitation phase of contact sensitivity: Regulation by endogenous IL-4. *J. Invest. Dermatol.* 1997; **108**: 406–11.
3 Abe M, Kondo T, Xu H, Fairchild RL. Interferon-γ inducible protein (IP-10) expression is mediated by $CD8^+$ T cells and is regulated by $CD4^+$ T cells during the elicitation of contact hypersensitivity. *J. Invest. Dermatol.* 1996; **107**: 360–6.
4 Hope JC, Dearman RJ, Debicki RJ, Kimber I, Hopkins SJ. Interleukin-6 production by draining lymph node cells following primary contact sensitization of mice: Relationship to the proliferative response. *Int. Arch. Allergy Immunol.* 1994; **103**: 378–83.
5 Enk AH, Angelon VC, Udey MC, Katz SI. An essential role for Langerhans cell-derived IL-1B in the induction of primary immune responses in skin. *J. Immunol.* 1993; **150**: 3698–704.
6 Carroll JM, Crompton T, Seery JP, Watt FM. Transgenic mice expressing IFN-γ in the epidermis have eczema, hair hypopigmentation, and hair loss. *J. Invest. Dermatol.* 1997; **108**: 412–22.

7 Saulnier M, Huang S, Aguet M, Ryffel B. Role of interferon-γ in contact hypersensitivity assessed in interferon-γ receptor-deficient mice. *Toxicology* 1995; **102**: 301–12.
8 Cippitelli M, Ye J, Viggiano V *et al*. Retinoic acid-induced transcriptional modulation of the human interferon-γ promoter. *J. Biol. Chem*. 1996; **271**: 26 783–93.
9 Nagai H, Matsuura S, Bouda K *et al*. Effect of Am.-80, a synthetic derivative retinoid, on experimental arthritis in mice. *Pharmacology* 1998; **58**: 101–12.
10 Petkovich M, Brand N, Krust A, Chambon P. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 1987; **330**: 444–5.
11 Mangelsdorf DJ, Ong ES, Dick JA, Evans RM. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 1990; **345**: 224–5.
12 Goodman DS. Vitamin A and retinoids in health and disease. *N. Engl. J. Med*. 1984; **310**: 1023–31.
13 Sporn MB, Roberts A-B. Role of retinoids in differentiation and carcinogenesis. *Cancer Res*. 1983; **43**: 3034–42.
14 Oikawa T, Okayasu I, Ashino H, Morita I, Murota S, Shudo K. Three novel synthetic retinoids, Re 80, Am 580 and Am-80, all exhibit anti-angiogenic activity *in vivo*. *Eur. J. Pharmacol*. 1993; **249**: 113–16.
15 Mangelsdorf DJ, Umesono K, Evans RM. The retinoids: Biology, chemistry and medicine. New York: Raven Press Ltd, 1994; 319–49.
16 Pfahl M, Chytil F. Regulation of metabolism by retinoic acid and its nuclear receptors. *Annu. Rev. Nutr*. 1996; **16**: 257–63.
17 Nagai H, Matsuo A, Hiyama H, Inagaki N, Kawada K. Immunoglobulin E production in mice by means of contact sensitization with a simple chemical, hapten. *J. Allergy Clin. Immunol*. 1997; **100**: S39–44.
18 Japanese Association for Laboratory Animal Science. Guidelines for animal experimentation. *Exp. Anim*. 1987; **36**: 285–8.
19 Geba GP, Ptak W, Anderson GM *et al*. Delayed-type hypersensitivity in mast cell-deficient mice; dependence on platelets for expression of contact sensitivity. *J. Immunol*. 1996; **157**: 557–65.
20 Williams JG, Jurkovich GJ, Maier RV. Interferon-γ. A key immunoregulatory lymphokine. *J. Surg. Res*. 1993; **54**: 79–93.