

DA-9601 Decreases Immediate-Type Allergic Reaction and Tumor Necrosis Factor- α Production

Hyo-Hyun Park,^a Sug-Hyun Kim,^{a,b} Soyoung Lee,^a Jin-Young Moon,^b Won-Hwan Park,^b Cheorl-Ho Kim,^c Jae Myung Chung,^d Tae-Young Oh,^e Chang-Duk Jun,^f and Sang-Hyun Kim^{*,a}

^aDepartment of Pharmacology, School of Medicine, Kyungpook National University, 2-101 Dong-in-dong, Jung-gu, Daegu 700-422, Korea, ^bCardiovascular Medical Research Center and Dept. of AM-Pointology & Diagnostics, Dongguk University, 707 Suk-jang-dong, Kyung-ju, Kyungpook 780-714, Korea, ^cDepartment of Biological Science, SungKyunKwan University, 300 Chon-chon-dong, Jang-an-gu, Suwon 440-746, Korea, ^dDepartment of Emergency Medicine, Kyungpook National University Hospital, 2-101 Dong-in-dong, Jung-gu, Daegu 700-422, Korea, ^eDong-A Pharmaceutical Research Institute, 47-5 Sang-gal-dong, Giheung-gu, Yongin 449-905, Korea, and ^fDepartment of Life Science, Gwangju Institute of Science and Technology, 1 Oryong-dong, Buk-gu, Gwangju 500-712, Korea

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The immediate-type allergic reaction is involved in many allergic diseases such as asthma, allergic rhinitis, and sinusitis. The discovery of drugs for the treatment of immediate-type allergic disease is a very important subject in human health. The formulated ethanol extract of *Artemisia asiatica* Nakai (DA-9601) has been reported to have anti-oxidative and anti-inflammatory activities. In this report, we investigated the effect of DA-9601 on the immediate-type allergic reaction and studied its possible mechanisms of action, focusing on the mast cell-mediated allergic reaction. DA-9601 inhibited compound 48/80-induced systemic anaphylactic reactions and serum histamine release in mice. DA-9601 decreased the IgE-mediated passive cutaneous anaphylaxis, the model of local allergic reaction *in vivo*. DA-9601 dose-dependently reduced histamine release from mast cells activated by compound 48/80 or IgE. Furthermore, DA-9601 decreased the gene expression and production of tumor necrosis factor (TNF)- α in phorbol 12-myristate 13-acetate plus A23187-stimulated mast cells. These findings provide evidence that DA-9601 could be a candidate as an anti-allergic agent.

Key words — immediate-type allergic reaction, DA-9601, mast cells, tumor necrosis factor- α

INTRODUCTION

Immediate-type allergic reaction (anaphylactic allergic reaction) is a life threatening syndrome induced by the sudden systemic release of inflammatory mediators, such as histamine, heparin, lipid-derived mediators and various cytokines from mast cells. Mast cells play an important role in a wide variety of biological responses. They are critical effector cells in the development of many physiologic changes during allergic and anaphylactic responses.^{1,2)}

Mast cell activation by both IgE-dependent and IgE-independent stimuli, bring about the process of degranulation that results in the fusion of the cyto-

plasmic membranes with the plasma membrane. This is accompanied by both the fast external release of granule-associated stored mediators as well as by the generation and release of newly generated mediators, such as products of arachidonic acid metabolism and, at later times, by the production and release of an array of cytokines.^{2,3)} Among the pre-formed and newly synthesized inflammatory substances released upon degranulation of mast cells, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity.⁴⁾ Mast cell degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells.^{5,6)} The synthetic compound 48/80 is known to be one of the most potent secretagogues. An appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylaxis.^{7,8)} It has been established that the anti-IgE antibody in-

*To whom correspondence should be addressed: Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu 700-422, Korea. Tel: +82-53-420-4838; Fax: +82-53-423-4838; E-mail: shkim72@knu.ac.kr

duces passive cutaneous anaphylaxis (PCA) as a typical model for the mast cell-dependent immediate-type allergic reaction.⁹⁾

Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases and several proinflammatory and chemotactic cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-4, IL-8, IL-13 and transforming growth factor (TGF)- β .¹⁰⁻¹²⁾ Therefore, modulation of secretion of these cytokines from mast cells can provide a useful therapeutic strategy for allergic inflammatory disease.

DA-9601 is a formulated ethanol extract of *Artemisia asiatica* (*A. asiatica*) Nakai. DA-9601 has been developed as an anti-gastritis agent and has been reported as having anti-oxidative and anti-inflammatory effects.¹³⁻¹⁶⁾ From the chemical analysis, DA-9601 contains mainly eupatilin and other flavonoids. The effects of DA-9601 on experimentally induced gastrointestinal and hepatic lesions are mediated through its antioxidant actions. From the *in vivo* studies, intragastric administration of DA-9601 at 10, 30 or 100 mg/kg for 3 days attenuates carbon tetrachloride-induced liver damage through restoring hepatic glutathione levels in rats.¹⁷⁾ DA-9601 (180 or 360 mg, 3 times a day) is currently in phase III clinical trial for patients with acute and chronic gastritis. Because immediate-type allergic reaction mediated by mast cells is closely related to inflammatory processes, DA-9601, with anti-inflammatory activity, is anticipated to have anti-allergic activity. To evaluate the anti-allergic effects of DA-9601, we studied the effect of DA-9601 on the compound 48/80-induced systemic reaction, IgE-mediated local allergic reaction, and histamine release from mast cells. In addition, the effect of DA-9601 on phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-induced gene expression and production of TNF- α in a human mast cell line (HMC-1) cells was also investigated.

MATERIALS AND METHODS

Reagents and Cell Culture — The standardized ethanol extract (lot 98-LO1) of *A. asiatica*, prepared according to the published procedure,¹⁷⁾ was supplied from Dong-A Pharmaceutical (Kyunggi-do, Korea). The content of eupatilin, a pharmacologically active ingredient of *A. asiatica*, was 2.25%(w/w), as determined by HPLC. Compound

48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), o-phthalaldehyde, PMA, calcium ionophore A23187, and metrizamide were purchased from Sigma (St. Louis, MO, U.S.A.). The HMC-1 was grown in Iscove's media (Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine at 37°C in 5% CO₂. The passage ranging 4–8 of HMC-1 cells was used throughout the study. **Animals** — The original stock of male ICR mice and male Sprague-Dawley rats were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea). The animals were housed 5 per cage in a laminar air flow room maintained under a temperature of 22 ± 2°C and relative humidity of 55 ± 5% throughout the study. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Compound 48/80-Induced Systemic Reaction

— Mice were given an intraperitoneal injection of 8 mg/kg, body weight (BW) of the mast cell degranulator, compound 48/80. DA-9601 was intraperitoneally administered 1 hr before the injection of compound 48/80 (*n* = 10/group). Mortality was monitored for 1 hr after induction of anaphylactic shock. After the mortality test, blood was obtained from the heart of each mouse to measure serum histamine content.

Passive Cutaneous Anaphylaxis (PCA) Reaction

— An IgE-dependent cutaneous reaction was examined as previously described.⁷⁾ PCA reaction was generated by sensitizing skin with an intradermal injection of anti-DNP IgE followed 48 hr later with an injection of DNP-HSA into the mouse tail vein. The mice were injected intradermally with 0.5 μ g of anti-DNP IgE. After 48 hr, each mouse was received an injection of 1 μ g of DNP-HSA containing 4% Evans blue (1 : 4) *via* the tail vein. Thirty minutes after the challenge, the mice were killed and the dorsal skin was removed for measurement of the pigment area. The amount of dye was determined colorimetrically after extraction with 1 ml of 1 M KOH and 9 ml of a mixture of acetone and phosphoric acid (5 : 13) based on the previous report.⁸⁾ The intensity of absorbance was measured at 620 nm in a spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan).

Preparation of Rat Peritoneal Mast Cells (RPMC)

— RPMCs were isolated as previously de-

scribed.¹⁸⁾ In brief, the peritoneal cells were collected and suspended in Tyrode buffer (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% bovine serum albumin), layered on 2 ml of metrizamide (22.5%, w/v), and centrifuged for 15 min at 400 *g*. The cells that remained at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue exclusion.

Histamine Assay — Histamine content from serum and RPMC were measured by the o-phthalaldehyde spectrofluorometric procedure.¹⁹⁾ The blood from the mice was centrifuged at 400 *g* for 10 min and the serum was withdrawn to measure histamine content. RPMC were preincubated with DA-9601, and then incubated for 10 min with compound 48/80 (5 μ g/ml). RPMC suspensions were also sensitized with anti-DNP IgE (10 μ g/ml) for 16 hr. The cells were preincubated with DA-9601 at 37°C for 10 min prior to the challenge with DNP-HSA (1 μ g/ml). The cells were separated from the released histamine by centrifugation at 400 *g* for 5 min at 4°C.

Lactate Dehydrogenase Release — Both released and total lactate dehydrogenase (LDH) concentrations were determined as described previously.²⁰⁾ For the total LDH determination, the cells were lysed by adding 1 μ l of Triton X-100 (1% final concentration) and incubated for 30 min in the incubator at 37°C. Samples were transferred to plate containing 100 μ l of 4.6 mM pyruvic acid in 0.1 M potassium phosphate buffer (pH 7.5). β -NADH (reduced nicotinamide adenine dinucleotide) in 0.1 M potassium phosphate buffer (pH 7.5) was added, mixed, and the absorbance read kinetically using a PowerWave_x Microplate Scanning spectrophotometer (Bio-Tek Instrument, INC, Winooski, VT, U.S.A.). The activity of LDH was normalized to the volume, and the released LDH activity was expressed as a percentage of total cellular LDH.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) — The total cellular RNA was isolated from the cells (1 \times 10⁶/well in 24-well plate) after stimulation of PMA (20 nM) plus A23187 (1 μ M) with or without DA-9601 for 2 hr using a TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's protocol. The first strand complementary DNA

(cDNA) was synthesized using the Superscript II reverse transcriptase (Life Technologies). RT-PCR was used to analyze the expression of mRNA for TNF- α and β -actin (internal control). The conditions for the reverse transcription and PCR steps were similar to those previously described.¹⁸⁾ The primer sets were chosen by the Primer 3 program (Whithead Institute, Cambridge, MA, U.S.A.). The cycle number was optimized in order to ensure product accumulation in the exponential range. The amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide, documented using a Kodak DC 290 digital camera, and digitized with UN-SCAN-IT software (Silk Scientific, Orem, UT, U.S.A.). The band intensity was normalized to that of β -actin in the same sample.

Enzyme-Linked Immunosorbent Assay (ELISA) — HMC-1 cells were sensitized with PMA (20 nM) plus A23187 (1 μ M) for 8 hr with or without DA-9601. The ELISA was performed by coating 96-well plates with 6.25 ng/well of monoclonal antibody with specificity for TNF- α . Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 and twice with PBS alone. For the standard curve, rTNF- α was added to the serum which was previously determined to be negative to endogenous TNF- α . After exposure to the medium, the assay plates were exposed sequentially to biotinylated 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) tablet substrates. Optical density was read within 10 min of the addition of the substrate with a 405 nm filter.

Western Blot Analysis — HMC-1 cells (3 \times 10⁶ in a 6-well plate) were washed with PBS and resuspended in lysis buffer. Samples of protein were electrophoresed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as previously described,²¹⁾ and then transferred to nitrocellulose membrane. The TNF- α was assayed using anti-TNF- α antibody (R&D Systems Inc. Minneapolis, MN, U.S.A.). Immunodetection was done using enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, U.S.A.).

Statistical Analysis — Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, U.S.A.). Treatment effects were analyzed using one way analysis of variance followed by Duncan's Multiple Range test. A value of $p < 0.05$ was used to indicate significant differences.

RESULTS

DA-9601 Inhibits Compound 48/80-Induced Systemic Reaction

To determine the effect of DA-9601 on allergic reaction, an *in vivo* model of systemic reaction was used. Compound 48/80 (8 mg/kg BW) was used to induce a systemic fatal allergic reaction. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 hr, after which the mortality rate was determined. As shown in Table 1, injection of compound 48/80 to mice induced fatal shock in 100% of animals. When DA-9601 was intraperitoneally administered at concentrations ranging from 0.001 to 1 mg/kg BW for 1 hr, the mortality with compound 48/80 was dose-dependently reduced.

DA-9601 Decreases Compound 48/80-Induced Serum Histamine Release

The effect of DA-9601 on compound 48/80-induced serum histamine release was investigated. DA-9601 was given at concentrations ranging from 0.001 to 1 mg/kg BW 1 hr before ($n = 10/\text{group}$) compound 48/80 injection. After 30 min of compound 48/80 injection, blood was withdrawn from the mice and the histamine content was measured. DA-9601 dose-dependently decreased compound 48/80-induced serum histamine release (Fig. 1).

DA-9601 Inhibits IgE-Mediated PCA Reaction

PCA is one of the most important *in vivo* models of anaphylaxis in a local allergic reaction.²²⁾ Local extravasation is induced by a local injection of anti-DNP IgE followed by an intravenous antigenic challenge. Anti-DNP IgE was injected into the right dorsal skin sites. As a control, the left dorsal skin site of these mice was injected with saline alone. After 48 hr, all animals were injected intravenously with DNP-HSA plus Evans blue dye. DA-9601 was administered intraperitoneally 1 hr prior to the challenge with antigen. The administration of DA-9601 (0.001–1 mg/kg, BW) showed a dose-dependent inhibition in the PCA reaction (Fig. 2).

DA-9601 Reduces Compound 48/80-Induced or IgE-Mediated Histamine Release from RPMC

The inhibitory effect of DA-9601 on compound 48/80-induced or anti-DNP IgE-mediated histamine release from RPMC is shown in Fig. 3. DA-9601 dose-dependently inhibited compound 48/80-induced or IgE-mediated histamine release at concen-

Table 1. Effect of DA-9601 on Compound 48/80-Induced Systemic Anaphylaxis

Dose (mg/kg, BW)	Compound 48/80 (8 mg/kg)	Mortality (%)
None (saline)	+	100
DA-9601 0.001	+	100
0.01	+	80
0.1	+	60
1	+	0
1	–	0

Groups of mice ($n = 10/\text{group}$) were intraperitoneally pretreated with 200 μl of saline or DA-9601 at various doses 1 hr before the intraperitoneal injection of compound 48/80. Mortality (%) within 1 hr following compound 48/80 injection is represented as the number of dead mice \times 100/total number of experimental mice. BW: body weight.

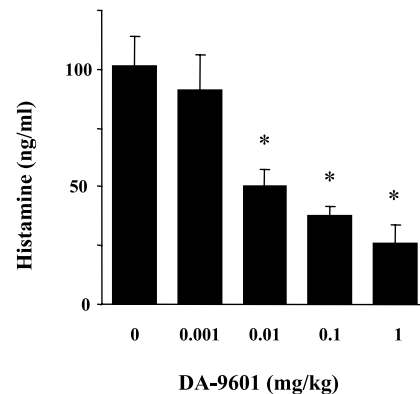


Fig. 1. Effect of DA-9601 on Compound 48/80-Induced Serum Histamine Release

Groups of mice ($n = 10/\text{group}$) were intraperitoneally pretreated with 200 μl saline or DA-9601. DA-9601 was given at various doses 1 hr before the compound 48/80 injection. The compound 48/80 (8 mg/kg, BW) was intraperitoneally given to the group of mice. After the mortality test, blood was obtained from the heart of each mouse to measure serum histamine content. Each data represents the mean \pm SEM of three independent experiments. * $p < 0.05$: significantly different from the saline value.

trations of 10–100 $\mu\text{g}/\text{ml}$.

DA-9601 Inhibits Gene Expression and Production of TNF- α from HMC-1 Cells

TNF- α is one of the most important proinflammatory cytokines. Therefore, we tested the effect of DA-9601 on the gene expression and production of TNF- α by RT-PCR, Western blotting, and ELISA, respectively. As shown in Fig. 4A, the gene expression of TNF- α was increased by stimulation of PMA plus A23187. Pretreatment of DA-9601 (10 and 100 $\mu\text{g}/\text{ml}$) inhibited PMA plus A23187-induced gene expression of TNF- α . Pretreatment of DA-9601

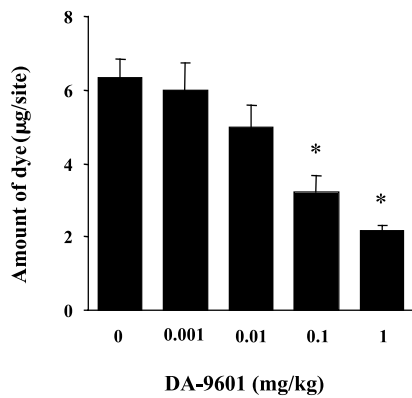


Fig. 2. Effect of DA-9601 on the 48 hr PCA

DA-9601 was intraperitoneally administered 1 hr prior to the challenge with antigen. Each data represents the mean \pm SEM of three independent experiments. * $p < 0.05$: significantly different from the saline value.

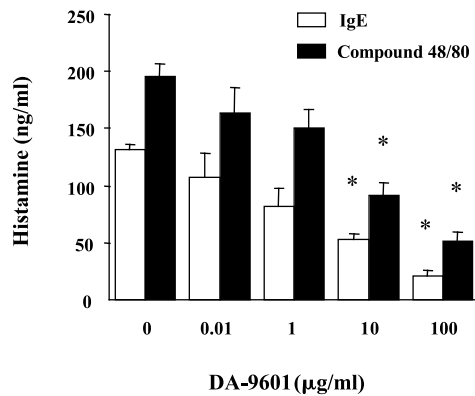


Fig. 3. Effect of DA-9601 on Compound 48/80- or IgE-Induced Histamine Release from RPMC

The cells (2×10^5 cells/ml) were preincubated with DA-9601 at 37°C for 10 min prior to incubation with either compound 48/80 (naïve RPMC) or DNP-HAS (anti-DNP IgE sensitized RPMC). Each data represents the mean \pm SEM of three independent experiments. * $p < 0.05$: significantly different from the saline value.

also decreased PMA plus A23187-induced production of TNF- α (Fig. 4B). Culture supernatants were assayed for TNF- α levels using ELISA. As shown in Fig. 4C, DA-9601 decreased the secretion of PMA plus A23187-induced TNF- α . The concentration of DA-9601 used in the experiment did not interfere with the viability of cells as assessed by LDH assay (Fig. 5).

DISCUSSION

Immediate-type allergic reaction (anaphylactic allergic reaction) is a life threatening syndrome in-

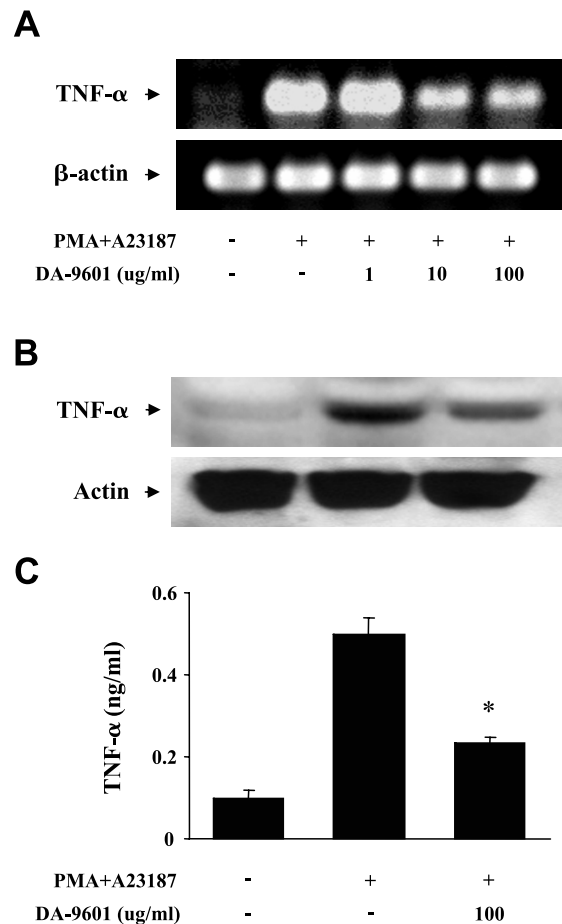


Fig. 4. Inhibitory Effect of DA-9601 on Gene Expression and Production of TNF- α in HMC-1 Cells

HMC-1 cells were treated with DA-9601 for 30 min before being stimulated with PMA (20 nM) plus A23187 (1 μ M). Extraction and analysis of mRNA and protein performed as described in MATERIALS AND METHODS. A) The gene expression of TNF- α was analyzed by RT-PCR. Band intensity for each gene expressions was normalized against β -actin. B) The production of TNF- α was analyzed by Western blot. Actin was used as a loading control. C) TNF- α level in supernatant was measured using ELISA and represented as the mean \pm SEM of two independent experiments. *Significantly different from the PMA plus A23187 value at $p < 0.05$.

duced by the sudden systemic release of inflammatory mediators, such as histamine, heparin, lipid-derived mediators and various cytokines from mast cells. Mast cells are located throughout the human body, and upon allergen exposure, they are stimulated *via* the IgE receptor.¹⁾ The results of this study demonstrated that DA-9601 has anti-allergic properties. Pretreatment with DA-9601 profoundly inhibited compound 48/80-induced systemic reaction and IgE-mediated local allergic reaction. DA-9601 inhibited compound 48/80-induced or IgE-mediated histamine release from RPMC. These results indicate that mast cell-mediated immediate-type aller-

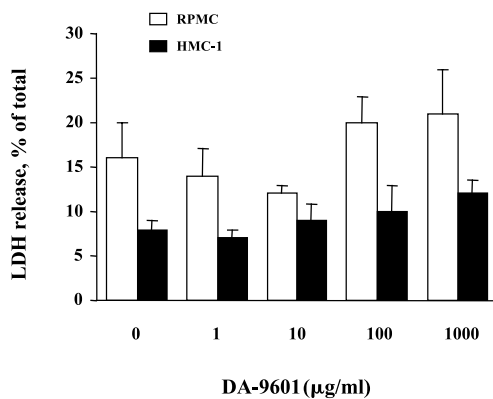


Fig. 5. Cytotoxicity of DA-9601 to RPMC and HMC-1 Cells

RPMC (2×10^4 /well in 96-well plate) and HMC-1 (4×10^4) were treated with various concentration of DA-9601 for 24 hr. The LDH release was expressed as a percentage of total cellular LDH. Results from a representative experiment are expressed as mean \pm SEM of three independent experiments.

gic reactions are inhibited by DA-9601. It is known that stimulation of mast cells with compound 48/80 or IgE initiates the activation of a signal transduction pathway which leads to histamine release. Several studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins.²³⁾ The evidence indicates that the protein is Gi-like and that the activation is inhibited by benzalkonium chloride.²⁴⁾ Tasaka *et al.*,²⁵⁾ reported that compound 48/80 increases the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. This result indicates that the increase in membrane permeability may be an essential trigger for the release of the mediator from mast cells. In this sense, anti-allergic agents having a membrane-stabilizing action may be desirable. DA-9601 might act on the lipid bilayer membrane thus preventing the prevention of the perturbation being induced by compound 48/80.

PCA is one of the most important *in vivo* models of anaphylaxis in local allergic reactions. The mice administered with DA-9601 were protected from IgE-mediated PCA. This finding suggests that DA-9601 might be useful in the treatment of allergic skin reactions.

Mast cell-derived cytokines, especially TNF- α have a critical biological activity in the allergic reaction. It has been reported that mast cells are a principal source of TNF- α in human dermis, and degranulation of mast cells in the dermal endothelium is abrogated by the anti-TNF- α antibody.²⁶⁾ This report may indicate that a decrease in TNF- α is a one of the key indicators of reduced allergic symptoms.

The HMC-1 cell line is useful for studying cytokine activation pathways.²⁷⁾ DA-9601 inhibited the gene expression and production of TNF- α in PMA plus A23187-stimulated HMC-1 cells. This result may suggest that one possible pathway of the anti-allergic effect of DA-9601 results from the reduction of TNF- α release from mast cells. The effect of DA-9601 on TNF- α production by mast cells *in vivo* and the relative importance of mast cells as a source of TNF- α during inflammatory and immune responses are important areas for future studies. In conclusion, the results obtained in the present study provide evidence that DA-9601 might contribute to the treatment of mast cell-mediated allergic diseases.

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