Inhibitory Mechanisms of Glycoprotein Fraction Derived from *Miscanthus sinensis* for the Immediate Phase Response of an IgE-Mediated Cutaneous Reaction

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We investigated the inhibitory effect of the glycoprotein fraction (fraction 2) extracted from *Miscanthus sinensis* Andersson (M. sinensis) on biphasic cutaneous reactions in mice passively sensitized with IgE. Biphasic skin reactions with peak responses at 1 (IPR, immediate phase reaction) and 24 h (LPR, late phase reaction) were caused by passive sensitization with an anti-dinitrophenol IgE monoclonal antibody (anti-DNP IgE mAb) followed by an epicutaneous challenge of 0.1% dinitrofluorobenzene (DNFB) in 100% ethanol. Intraperitoneal injection of fraction 2 before the DNFB challenge significantly inhibited the biphasic ear swelling response in passively sensitized mice in a dose-dependent manner (1–30 mg/kg). We also found that fraction 2 was effective at inhibiting the vascular permeability in mouse ear induced by an injection of compound 48/80, histamine or serotonin. In addition, fraction 2 inhibited scratching behavior as well as ear edema observed within 2 h after DNFB challenge. Marked inhibition was observed in both passively sensitized and non-sensitized mice. The locomotor activity of mice was also reduced by the administration of fraction 2 as well as by diphenhydramine. These results suggest that the inhibitory effect of glycoprotein fraction 2 of *M. sinensis* on an IgE-mediated allergic inflammatory reaction is due to the protection of mediator-induced vascular permeability and that in addition to the inhibition of an inflammatory reaction, a sedative action is responsible for the inhibition of allergy-induced scratching responses.

**Key words** *Miscanthus sinensis*; IgE-mediated skin reaction; scratching; vascular permeability; sedation

It is well known that symptoms of allergic inflammation such as bronchial asthma, allergic rhinitis and atopic dermatitis involve an immediate phase reaction (IPR), which is acute reactions of the permeability and broncho contraction induced by degranulation of mast cells, and late phase reaction (LPR), which is a chronic inflammatory reaction, accompanied by the infiltration of inflammatory cells to the lesion site and the induction of various chemical mediators and cytokines. In atopic dermatitis, the condition of chronic disease worsens with a repeat of acute responses to environmental factors. Therefore, the inhibition of IPR is an important step in the treatment of allergic inflammation.

In a search for new anti-allergic agents, we have investigated the effect of several plant materials and herbal medicines on murine IgE-mediated cutaneous dermatitis. In this model, passive sensitization with a murine monoclonal IgE antibody specific for the dinitrophenyl group (anti-DNP IgE mAb) followed by a challenge of dinitrofluorobenzene (DNFB) to mouse ears induces a biphasic cutaneous reaction with IPR and LPR at 1 and 24 h after the antigen challenge, respectively. We have recently reported that the glycoprotein fraction (fraction 2) of the non-dialysate fraction from a water extract of *Miscanthus sinensis* Andersson (M. sinensis), a corn-related plant species, suppressed ear swelling with IgE-mediated biphasic cutaneous reactions.

Pruritus is an unpleasant symptom of atopic dermatitis, as well as other cutaneous diseases, and it accompanies several visceral disorders such as chronic renal failure. Pruritus produces scratching, worsening the condition of atopic patients. Therefore, improvement of pruritus is important in the therapy for atopic dermatitis. However, the physiological and pathological mechanisms of pruritus and how pruritus relates to immunological mechanisms remain unknown because of the lack of a reliable animal model. We have reported that scratching behavior with the hind paws, induced by subcutaneous injections of pruritogenic agents such as compound 48/80 and substance P into therostal back in mice, was likely an itch-associated behavior.

In the present study, we have investigated the inhibitory mechanisms of fraction 2 of *M. sinensis* on the IPR of the IgE-mediated biphasic skin reactions in mice, as well as the effect on scratching behavior after DNFB challenge.

**MATERIALS AND METHODS**

**Mice** Specific pathogen-free female BALB/c mice (6 weeks old) were purchased from SLC, Hamamatsu, Japan, and maintained in the Laboratory for Animal Experiments, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University. This study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

**Antigens and Chemicals** DNFB was purchased from Nacalai Tesque, Kyoto, Japan, and was dissolved in 100% ethanol. Prednisolone 21-acetate (Sigma Chemical Co., St. Louis, MO, U.S.A.) and amlexanox (kindly provided by Takeda Chemical Industries, Ltd., Osaka, Japan) were suspended in 0.5% methylcellulose solution. Diphenhydramine and methylsergide were purchased from Sigma Chemical Co.

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St. Louis, dissolved in sterile saline and administered intraperitoneally (i.p.) 24 h, orally 1 h or i.p. 30 min prior to either the antigen challenge, injection of stimulant or observation of scratching and locomotor activity, respectively.

**Plant Materials** We have already reported the procedures for extracting and fractionating the spikelets of *M. sinensis*. Voucher samples (TMPW 339 [TMPW]) have been deposited in the Museum of Materia Medica, Analytical Research Center for Ethnomedicines, Toyama Medical and Pharmaceutical University, Toyama, Japan. Fraction 2 of the extract of *M. sinensis* (63.4% protein and 30.8% sugar composed mainly of rhamnose, glucose and ribose) was diluted in saline, then administered i.p. 2 h prior to the antigen challenge, agent injection or observation of scratching and locomotor activity.

**Anti-DNP IgE Preparation** An anti-DNP monoclonal antibody-producing cell line (EC1) was cultured in 10 ml of an equal volume mixture of RPMI-1640 and Dulbecco’s modified Eagle medium, minimum essential medium with high glucose supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Life Technologies, Inc., Grand Island, NY) and 2 mK glutamine until reaching a confluent state. The supernatant was harvested, centrifuged at 400×g and stored at −80°C until use. The IgE antibody titer was estimated to be 1:1024 by a heterologous passive cutaneous anaphylaxis in rats injected intravenously with DNP-bovine serum albumin as an antigen.

**Induction of Skin Reactions in Mouse Ears** BALB/c mice were passively sensitized by i.v. injection of 1 ml of anti-DNP IgE mAb 24 h before DNFBJ challenge. Skin reactions were elicited by applying 10 μl of 0.1% DNFBJ in 100% ethanol to each side of each ear of both the sensitized and non-sensitized mice. The ear swelling was evaluated by measuring ear thickness using a dial thickness gauge (G-1A type, Peacock, Ozaki MFG., Co., Ltd., Osaka, Japan), immediately before and at appropriate times after the challenge. The results were expressed as the average ear swelling (increase in ear thickness, μm)±S.D. of three mice.

**Measurement of Vascular Permeability** Mice (5–8 mice/group) were injected with 25 μl of saline, compound 48/80 (4 mg/ml, Sigma Chemical Co., St. Louis, MO, U.S.A.), histamine (200 μμ, Wakko Pure Chemical Industries, Ltd., Osaka, Japan) or serotonin (80 μμ, 5-hydroxytryptamine, Sigma Chemical Co., St. Louis, MO, U.S.A.) into their ears 20 min after the i.v. injection of 0.5 ml of 0.5% Evans blue in saline. Mice were sacrificed by cutting the carotid 20 min after each stimulation, and pairs of ears were dissolved with 0.7 ml of 1 N KOH solution in a stoppered tube at 37°C overnight, then 9.3 ml of a mixture of 0.6 N H2PO4 solution and acetone (5:13) was added. After vigorous shaking, the precipitate was removed by centrifugation at 2000 rpm for 10 min and the amount of blue dye in the supernatant was spectrophotometrically measured at 620 nm. The vascular permeability was calculated by comparison with that in mice which received the injection of Evans blue alone. The results represent average permeability (OD, optical density)±S.D.

**Scratching Behavioral Experiments** For scratching behavioral examination, 4 passively sensitized mice/group were put into an acrylic cage (13×9×30 cm) for about 2 h for acclimation before the experiment. Immediately after the DNFBJ challenge, they were put back into the same cage and, for the observation of scratching, behavior was recorded using an 8 mm video camera (CCD-TRV60, Sony, Tokyo, Japan) under unmanipulated conditions. Scratching of the challenged site with the hind paws was counted. Each mouse was used for only one experiment. The mice generally scratched several times for about 1 s and a series of these behaviors was counted as one incident of scratching.

**Locomotive Behavioral Experiments** For locomotive behavioral examination, 4 untreated mice/group were put into the acrylic cage. Two hours after the acclimation, locomotion was observed using a sensor for measurement of small animal locomotion (NS-AS01, Neuroscience, Tokyo, Japan) equipped with a system printer (NSP-008, Neuroscience, Tokyo, Japan) under unmanipulated conditions. Locomotor activity was evaluated as the number of passages under the sensor in 1 h. Each mouse was used for only one experiment.

**Statistical Analysis** The statistical significance of differences between the groups was determined by applying Mann-Whitney’s U-test for the experiments of ear swelling and vascular permeability, and two-way repeated-measures ANOVA for behavioral experiments. The results shown in the Figs. and Table are representative of more than 3 experiments.

**RESULTS**

**Effect of Fraction 2 of *M. sinensis* on IgE-Mediated Biphasic Skin Reactions** In the model of the IgE-mediated skin reaction, DNFBJ dissolved in an acetone–olive oil (3:1) mixed solution was used as the challenging antigen and applied to the ears in mice passively sensitized with anti-DNP IgE mAb. The exposure of this mixed solution alone (without DNFBJ) did not elicit ear swelling in sensitized and nonsensitized mice, but nonspecific wiping and scratching behaviors were frequently observed. Considering the scratching behavioral examination in the following experiment, to avoid nonspecific behavior, DNFBJ was dissolved in 100% ethanol in place of the acetone–olive oil mixed solution. As shown in Fig. 1, the challenge with DNFBJ in sensitized mice caused a biphasic skin reaction with peaks (IPR and LPR) at 1 and 24 h, while only ear swelling at 1 h after the DNFBJ challenge was observed in non-sensitized mice. This finding was consistent with previous reports using acetone–olive oil mixed
solution. The application of ethanol alone to the ear of non-sensitized mice did not produce ear swelling without wiping and scratching behavior. In this model, the i.p. administration of fraction 2 of *M. sinensis* 2 h before the challenge of 0.1% DNFB in ethanol in passively sensitized BALB/c mice resulted in the marked inhibition of ear swelling in both IPR and LPR in a dose-dependent manner (Table 1).

Table 1. Effect of Fraction 2 on Ear Swelling of IgE-Mediated Biphasic Cutaneous Reaction in Passively Sensitized Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mg/kg)</th>
<th>Inhibition rate (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IPR (1 h)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>10</td>
<td>58.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50.9</td>
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<tr>
<td></td>
<td>30</td>
<td>63.8</td>
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</table>

Fraction 2 of *M. sinensis* and prednisolone were administered i.p. to the passively sensitized mice 2 h before DNFB challenge. Ear swelling was assessed 1 h (IPR) and 24 h (LPR) after the challenge. Each value represents the mean of 3 mice. Ear swelling in IPR and LPR of control mice was 48.3 ± 4.5 and 44.7 ± 4.2 μm, respectively.

**Effect of Fraction 2 on Vascular Permeability Induced by Compound 48/80, Histamine and Serotonin** Compound 48/80, which induces the degranulation of mast cells, and mediators derived from mast cell granules such as histamine and serotonin, are known to cause extravasation and edema.11,12 The extent of vascular permeation increased within 1 h corresponding to IPR after the DNFB challenge in passively sensitized mice.61 We next investigated the effect of fraction 2 on vascular permeability induced by compound 48/80, histamine or serotonin associated with IPR. As shown in Fig. 2, the dye-leakage induced by compound 48/80, histamine or serotonin was inhibited by amilorax (membrane stabilizer), diphenhydramine (H1 receptor antagonist) or methysergide (5-HT receptor antagonist), respectively. The pretreatment with fraction 2 significantly inhibited the vascular permeability induced by these stimuli.

**Effect of Fraction 2 on Scratching Behavior** We investigated the effect of fraction 2 on scratching behavior elicited by the DNFB-challenge in passively sensitized and non-sensitized mice. The time course and total number of scratching behaviors after the challenge are shown in Fig. 3. The time...

**Fig. 2. Effect of Fraction 2 of *M. sinensis* on Vascular Permeability Induced by Compound 48/80, Histamine and Serotonin**

Fraction 2 (10 mg/kg, i.p.), amilorax (30 mg/kg, p.o.), methysergide (10 mg/kg, i.p.), or diphenhydramine (30 mg/kg, i.p.) were administered 2 h, 1 h, 2 h, and 30 min before the injection of compound 48/80 (100 μg/site), histamine (5 μmole/site) or serotonin (5 μmole/site) into the mouse ear, respectively. Mice were injected i.v. with 0.5% Evans blue 20 min before the permeation inducers, and sacrificed 20 min after the injection. Exuded blue dye was extracted from the ears and spectrophotometrically measured at 620 nm. Each value represents the mean (O.D.) ± S.D. of 5–8 mice. *p < 0.05; **p < 0.01 by Mann-Whitney U-test.

**Fig. 3. Time Course Study of Scratching Behavior after DNFB Challenge in Mice**

Mice were sensitized with anti-DNP IgE mAb 24 h before the challenge of 0.1% DNFB in 100% ethanol (anti-DNP IgE mAb/0.1% DNFB). Non-sensitized mice were challenged with vehicle (ethanol) or 0.1% DNFB (=vehicle or −0.1% DNFB, respectively). a) Scratching behavior of the challenged sites with the hind paws was enumerated in 15 min intervals. b) Total number of scratching incidences was estimated over 2 h. Each value represents the mean ± S.D. of 4 mice. *p < 0.05 by two-way repeated-measures ANOVA.
course of scratching behavior was evaluated by counting the scratching behavior per 15-min period for 2h in IPR and LPR. Scratching behavior in IPR was observed in both sensitized and non-sensitized mice after DNFB challenge, but not in non-sensitized mice after the challenge of vehicle (ethanol) alone. The total number of scratching behaviors in IPR of sensitized mice after the challenge obviously increased as compared with non-sensitized mice (Fig. 3b). On the other hand, scratching behavior in LPR was not shown in any tested groups.

Since the above results demonstrated that the administration of prednisolone and fraction 2 markedly inhibited the IgE-mediated ear swelling in both IPR and LPR after DNFB challenge, we next investigated their effect on the scratching behavior as well as ear swelling for 2h after DNFB challenge in sensitized and non-sensitized mice (Figs. 4 and 5). Fraction 2 (1—30 mg/kg) as well as prednisolone (10 mg/kg) significantly inhibited the ear swelling and accompanied scratching behavior in both sensitized and non-sensitized mice. Since prednisolone and fraction 2 inhibited both ear swelling and scratching behavior in non-sensitized mice after DNFB challenge (Fig. 5), the effect of fraction 2 may be due to the inhibition of both IgE-mediated and non-IgE mediated inflammatory and itching reactions.

**Locomotion of Mice Treated with Fraction 2**

We demonstrated that fraction 2 inhibited scratching behavior as well as ear swelling induced by a DNFB challenge, and this suggests that the inhibitory effect is concerned with the inhibition of vascular permeability induced by histamine and serotonin derived from mast cells. Histamine antagonists, including diphenhydramine, are known to have alternative mechanisms in addition to the ability to antagonize the peripheral histamine receptor. Sedation through the suppression
of the central nervous system is considered to be one such inhibitory mechanism of this kind of antagonist.\textsuperscript{13,14} Therefore, we examined the sedative activities of fraction 2 and other anti-allergy agents by a locomotion assay of mice (Fig. 6). Fraction 2 as well as diphenhydramine significantly inhibited the locomotor activity. In contrast, prednisolone did not show any activity. Amlexanox tended to inhibit the locomotor activity, but its effect was not significant. These findings indicate that the inhibition of the scratching behavior by fraction 2 may be partly associated with its sedative property.

DISCUSSION

Most anti-allergic agents are effective at inhibiting the IPR of biphatic skin reactions in patients with atopic dermatitis but not the LPR.\textsuperscript{15} We have recently reported that the glycoprotein fraction (fraction 2) of \textit{M. sinensis} was effective in the inhibition of the IgE-mediated biphatic cutaneous reaction (both IPR and LPR) in passively and actively sensitized mice.\textsuperscript{3} To further extend our previous study, we investigated the anti-allergic mechanism of fraction 2 for IPR after DNFB challenge in passively sensitized mice. In allergic responses, allergen exposure to IgE-sensitized mast cells caused an increase in vascular permeability accompanied by the release of chemical mediators such as histamine and serotonin,\textsuperscript{16} which are considered to be pruritogenic factors.\textsuperscript{17–19} As shown in Fig. 2, amlexanox (membrane stabilizer), diphenhydramine (H1 receptor antagonist) and methysergide (5-HT receptor antagonist) significantly inhibited the vascular permeability induced by compound 48/80, histamine and serotonin, respectively. Fraction 2 markedly inhibited the dye-leakage elicited by all these stimuli. These results suggest that the inhibitory effect of fraction 2 on IPR of IgE-mediated skin reaction in response to DNFB is partly due to the inhibition of the increase in vascular permeability.

Classical histamine receptor antagonists have also been shown to possess sedative activity.\textsuperscript{20,21} In the locomotor behavioral determination, fraction 2 and diphenhydramine markedly inhibited the locomotion of mice, while prednisolone failed to inhibit (Fig. 6). These results suggest that the anti-inflammatory action by fraction 2 is similar to that of classical histamine receptor antagonists. Also, fraction 2 as well as prednisolone inhibited both IPR and LPR, but diphenhydramine inhibited only IPR (data not shown). Therefore, the inhibition of LPR by fraction 2 may be an alternative mechanism.

Pruritus is a common symptom of cutaneous inflammatory diseases including atopic dermatitis, and is associated with a desire to scratch.\textsuperscript{22–24} Scratching worsens the cutaneous condition. Scratching behavior was observed for 2 h after DNFB challenge (corresponding to IPR) in both IgE-sensitized and non-sensitized mice (Fig. 3). The frequency of scratching in sensitized mice was obviously more than that in non-sensitized mice. Fraction 2 inhibited the scratching behavior of IgE-sensitized mice after the challenge in a dose-dependent manner (Fig. 4). Moreover, fraction 2 resulted in a significant inhibition of scratching behavior as compared with prednisolone in non-sensitized mice (Fig. 5). Thus, the H1 receptor antagonist-like property of fraction 2 may be partly responsible for the inhibitory effect on IPR and scratching behavior, although the properties of fraction 2 have not yet been clarified.

In conclusion, we demonstrated that fraction 2 markedly inhibited the ear swelling and scratching behavior in IPR after the DNFB challenge in both IgE-sensitized and non-sensitized mice. The inhibitory effect on the inflammatory reaction is partly due to the protection of vascular permeability induced by chemical mediators. Since fraction 2 inhibited locomotor activity, the inhibition of scratching responses may be associated with its sedative action.

Acknowledgements This work was supported in part by a Grant-in-Aid for Cancer Research from the Japanese Ministry of Education, Science, Sports and Culture of Japan (No. 09254101).

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