

Short Communication

Inhibitory Effect of Azelastine on Allergic Itch-Associated Response in Mice Sensitized With Mosquito Salivary Glands Extract

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Received December 24, 2002; Accepted January 16, 2003

Abstract. We examined whether azelastine would inhibit itch-associated responses of mice to mosquito allergy. Repeated injections of mosquito salivary gland extract increased scratching and sensory nerve activity. Azelastine inhibited the increased scratching and nerve activity, while terfenadine was without effects. Dexamethasone did not affect the increased scratching. Azelastine suppressed high K⁺-induced increase in intracellular free Ca²⁺ in primary cultures of mouse sensory neurons. Direct inhibition by azelastine of sensory neurons may be at least involved in the anti-pruritic effect of azelastine. Histamine, substance P, and leukotriene B₄ may not play a key role in the itching of mosquito allergy.

Keywords: itch-associated response, mosquito allergy, azelastine

Mosquito bite-induced cutaneous reactions, such as wheal, flare, and itch, are allergic responses. In the immediate allergy reaction, wheal, erythema, and itch develop within a few minutes after the bite and usually subside in a short time. We have developed a mouse model of itch in mosquito allergy (1). Although there are no apparent responses to the first mosquito bite, mice show marked scratching when they have been repeatedly given mosquito bites or injections of the extract of mosquito salivary glands (SGE). The H₁ histamine receptor antagonist diminishes plasma extravasation after mosquito bites without effect on mosquito bite-induced scratching (1), suggesting the involvement of mediators other than histamine in itching. However, it remains unknown which mediators are involved in the itch of mosquito allergy.

Azelastine, an orally effective anti-allergic and anti-asthmatic drug, blocks H₁ histamine receptors and inhibits the production of chemical mediators including arachidonic acid metabolites (2). It directly affects eosinophils, neutrophils, macrophages, bronchial epithelium, and T cells (2). With regard to pruritic diseases, azelastine is used for the management of urticaria, atopic

dermatitis, and pruritus in hemodialysis patients (3, 4). It inhibits itch-associated responses of mice to intradermal injections of substance P and leukotriene B₄ (5). In this study, we investigated the effects of azelastine on itch-associated responses of mice with mosquito allergy.

We used male ICR mice (Japan SLC, Inc., Shizuoka), which were 5-week-old at the start of experiments, except acute experiments in which they were used at 5–7 weeks of age. They were housed in a room under controlled temperature (22 ± 2°C), humidity (55 ± 10%), and light (light on 07:00–19:00 h). Food and water were freely available. This study was approved by the Committee for Animal Experiments at Toyama Medical and Pharmaceutical University.

Azelastine hydrochloride (Eizai Co., Ltd., Tokyo), terfenadine (Sigma, St. Louis, MO, USA), and dexamethasone (Sigma) were suspended in 0.5% carboxymethyl cellulose. They were administered perorally 30 min (azelastine and terfenadine) or 60 min (dexamethasone) before antigen injection.

SGE was prepared from female adult mosquitoes *Aedes albopictus* (1). SGE (10 µg/50 µl) was injected intradermally into the caudal back twice a week for four weeks for sensitization and then into the rostral back for itch induction.

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Behavior observation was done as described (1). In brief, after at least 1-h acclimation to the experimental environment, mouse behaviors were videotaped with no experimenters present in the observation room. Playback served for counting the scratching.

The activity of cutaneous nerve branch was recorded as described (6). Briefly, under urethane (1.5 g/kg) anesthesia, the skin of the rostral back was turned inside out. The cutaneous nerve branch was then exposed, dissected free from surrounding tissues, and maintained in a mineral oil pool. Nerve activity was recorded extracellularly using bipolar electrodes of silver wire and an AC bioelectric amplifier (AB651; Nihon Kohden, Tokyo) with a band-pass filter of 1 kHz. SGE was injected intradermally into the receptive field.

For *in vitro* experiments, immediately after decapitation, the mouse vertebral column was cut out, placed in ice-chilled phosphate-buffered saline (pH 7.4), and dorsal root ganglia (DRGs) were dissected out. After 45-min incubation with collagenase (2 mg/ml; Wako Pure Chem., Osaka) at 37°C, DRGs were mechanically dispersed by passage through micropipettes and cell suspensions were centrifuged. After they were washed with phosphate-buffered saline, the pellets were suspended in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). The isolated DRG cells were plated onto poly-D-lysine-coated glass bottom dishes and maintained in the medium containing 8 µM Ara-C for 1 day. Neurons adhering to the glass coverslip were incubated in standard OPTI-MEM®I containing 10 µM Fluo-3 acetoxymethyl ester and 0.1% F-127 for 45 min. After 1-h washing in HEPES buffer (115 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂ · 2H₂O, 0.4 mM KH₂PO₄, 0.4 mM Na₂HPO₄ · 12H₂O, 20 mM HEPES, and 10 mM glucose), the concentration of intracellular free Ca²⁺ ([Ca²⁺]_i) in neurons (<25 µm) was determined at 488-nm excitation and 530-nm emission with a laser confocal microscope (Insight Plus-IQ; Meridian Instruments Far East, Tokyo). The neurons were excited with 30 mM KCl. Azelastine was added to the medium 30 min before KCl stimulation.

Values given are means and S.E.M. Statistical significance was determined with two-way repeated measures analysis of variance followed by post hoc Dunnett's test or Kruskal-Wallis one way analysis of variance on ranks followed by post hoc Dunn's test; differences were considered significant at *P* < 0.05.

Although an intradermal injection of SGE did not elicit scratching in naive mice, its repetition increased scratching and the eighth SGE injection markedly induced scratching (Fig. 1: A – C). The responses peaked during the initial 20-min period and almost subsided

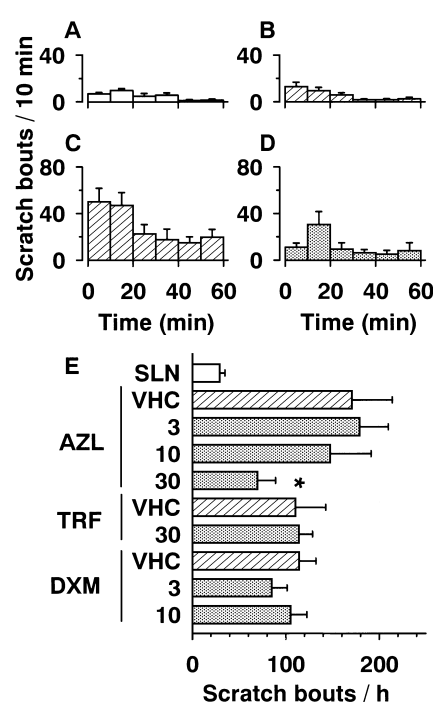


Fig. 1. Inhibition by azelastine of scratching following an injection of the extract of mosquito salivary gland. A) Scratching following saline (SLN) injection in naive mice. B) Scratching following the extract injection in naive mice. C) Scratching following the extract injection in sensitized mice. D) Effect of azelastine (AZL, 30 mg/kg) on scratching following the extract injection in sensitized mice. E) Effects of AZL, terfenadine (TRF), and dexamethasone (DXM) on scratching following the extract injection in sensitized mice. See text for sensitizing procedures. A series of several rapid scratches for about 1 s was counted as one bout of scratching. AZL, TRF, and DXM were administered perorally 30, 30, and 60 min before extract injection, respectively. Values represent the means and S.E.M. of 8 animals. **P* < 0.05, when compared with vehicle (VHC) (Dunnett's test).

by 60 min (Fig. 1C). This scratching was distinctly inhibited by peroral pretreatment with azelastine (30 mg/kg) (Fig. 1D). Azelastine (3 – 30 mg/kg) produced a dose-dependent inhibition of scratching, but terfenadine (30 mg/kg) and dexamethasone (3 and 10 mg/kg) were without effects (Fig. 1E).

SGE injection markedly increased the activity of the cutaneous nerve branch in a sensitized mouse, but it was without effects in the naive one (Fig. 2A). The activity was high during the initial 12-min period and subsided by 60 min after injection (Fig. 2B). It was markedly suppressed by azelastine (30 mg/kg) but not by terfenadine (30 mg/kg) (Fig. 2B).

High concentration of KCl (30 mM) increased [Ca²⁺]_i in the DGR neurons *in vitro*. Azelastine (30 and 100 µM) exerted a concentration-dependent inhibition of this increase (Fig. 3).

An intradermal injection of SGE markedly increased

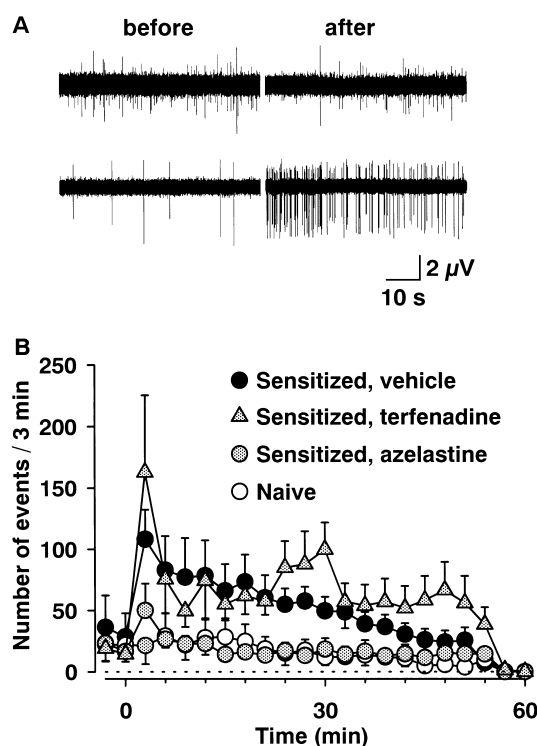


Fig. 2. Increased firing of cutaneous nerve branch following an injection of salivary gland extract and its inhibition by azelastine. A) Typical traces of cutaneous nerve firing. The traces show the impulse activity for 60 s before and 1 min after the injection of salivary gland extract in naive (upper traces) and sensitized mice (lower traces). B) Effects of azelastine and terfenadine on the increased firing following an injection of salivary gland extract in sensitized mice. See text for sensitizing procedures. Azelastine (30 mg/kg) and terfenadine (30 mg/kg) were administered perorally 30 min before the extract injection. Values represent the means and S.E.M. of 5–8 animals. Two-way repeated measures analysis of variance (sensitized groups): main effects of drug treatment, $F(2,18) = 12.81$, $P < 0.001$. Dunnett's test: $P < 0.05$ between azelastine and vehicle.

scratching in SGE-pretreated mice with no distinct effects in naive mice, the results being similar to our previous study (1). Repetitive pretreatment with SGE increases mosquito bite-induced scratching and plasma extravasation (1). SGE injection produced marked plasma extravasation in SGE-pretreated mice (E. Ohtsuka et al., unpublished data). These findings taken together suggest that SGE-induced scratching is due to the cutaneous reaction of immediate allergy. SGE injection increased the activity of cutaneous nerve in SGE-pretreated mice with a time-course similar to that of scratching. Intradermal injections of serotonin and histamine increase scratching and cutaneous nerve activity with similar time-courses (6). The spontaneous nerve activity is increased in chronically scratching mice (7). Thus, the nerve activity may be at least partly due to pruritic stimulation of primary afferent terminals in the

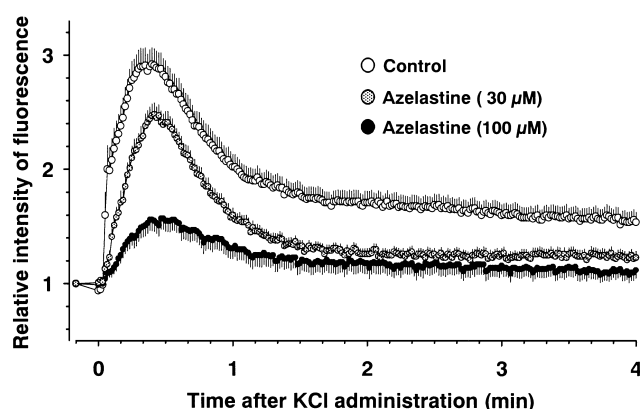


Fig. 3. Inhibition by azelastine of high K^+ -induced increase in intracellular Ca^{2+} in mouse dorsal root ganglion neurons. Primary cultures of mouse dorsal root ganglion neurons pre-loaded with Fluo-3 were stimulated by the increase of extracellular concentration of K^+ up to 30 mM. Azelastine (30 and 100 μ M) was added to the medium from 30 min before K^+ stimulation. $n = 24-38$. Kruskal-Wallis one way analysis of variance (peak effect at 54 s): $P < 0.05$. Dunn's test: $P < 0.05$ between 100 μ M azelastine and control.

periphery.

Azelastine inhibited SGE-induced scratching in sensitized mice. It blocks H_1 histamine receptors and reduces the locomotor activity at high doses, raising the possibility that the decrease of scratching was due to the central suppression. However, this agent inhibited SGE-induced firing of cutaneous nerve and scratching at the same dose, suggesting that its peripheral action is involved in the inhibition of SGE-induced scratching.

Azelastine (30 mg/kg) markedly inhibits histamine-induced scratching in mice (5). However, the involvement of the blockade of intracutaneous H_1 histamine receptors in this inhibition is not supported by the following findings. First, terfenadine (30 mg/kg) distinctly decrease histamine-induced scratching (1), but this H_1 antagonist at the same dose did not affect the SGE-induced scratching. Second, although terfenadine (30 mg/kg) almost abolishes mosquito bite-induced plasma extravasation, it does not affect mosquito bite-induced scratching (1). In addition, repeated injections of SGE increase scratching in mast cell-deficient mice as well as normal mice (1). Histamine plays an important role in itch-associated responses to passive cutaneous anaphylaxis in mice (8). However, the mast cell-histamine system may not play an important role in mosquito allergy-induced scratching in mice. It is interesting to investigate whether or not this phenomenon is generally observed in the itch of immediate type skin allergy.

Intradermal injections of substance P and leukotriene B_4 elicit scratching in mice (9, 10). Substance P produces leukotriene B_4 in the skin and the leukotriene B_4

antagonist inhibits substance P-induced scratching (11). Azelastine (30 mg/kg) inhibits substance P-induced production of leukotriene B₄ in the skin and suppresses scratching induced by either leukotriene B₄ or substance P (5). In the present experiments, dexamethasone (3 and 10 mg/kg, 1 h before SGE) did not affect SGE-induced scratching. Considering the findings that 1-h pretreatment with this steroid at a dose of 3 mg/kg markedly inhibits the substance P-induced production of leukotriene B₄ in the skin and scratching in mice (11), the results suggest that leukotriene B₄ and substance P do not play a key role in the itching of mosquito allergy. Emedastine (0.03–0.3 mg/kg) markedly suppresses scratching induced by either leukotriene B₄ or substance P in mice (12). In our preliminary experiments, however, the SGE-induced scratching was not affected by emedastine (0.3 mg/kg), supporting the above-mentioned idea.

Azelastine inhibited the depolarization-induced increase of $[Ca^{2+}]_i$ in the cultured sensory neurons, suggesting the direct action on neurons. There have been several reports on the inhibitory action of this agent on the intracellular Ca^{2+} mobilization. For example, azelastine diminishes the endothelin-induced increase of $[Ca^{2+}]_i$ in airway smooth muscles, without effects on vascular smooth muscles and cardiocytes (13). It also inhibits the platelet-activating factor-induced increase of $[Ca^{2+}]_i$ in peritoneal macrophages (14). Thus, azelastine acts directly on sensory neurons to inhibit intracellular Ca^{2+} mobilization, which may be at least partly involved in the inhibitory action on scratching. We do not rule out the possibility that local anesthetic action also participates in the inhibitory effect of azelastine, because this agent has been suggested to inhibit Na^+ channels as well as Ca^{2+} channels in cardiac muscle (15).

The mechanisms of itch of mosquito allergy remain unclear. However, leukotriene B₄ and substance P as well as histamine may not play important roles in this itching.

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