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Original Article

Scratching behavior in NC/Nga mice with dermatitis: Involvement of histamine-induced itching

Yuki Hashimoto,[1] Norikazu Takano,[1] Atsushi Nakamura,[1] Shiro Nakaike,[1] Zhigian Yu,[2] Yasuo Endo[2] and Iwao Arai[1]

[1]Pharmacology Laboratory, Medicinal Research Laboratories, Taisho Pharmaceutical Co. Ltd, Saitama and [2]Department of Molecular Regulation, Graduate School of Dentistry, Tohoku University, Sendai, Japan

Abstract

Background: Itching, a typical symptom in dermatitis, including atopic dermatitis (AD), leads to scratching. In the present study, we investigated whether histamine-induced itching is involved in scratching behavior seen in NC/Nga (NC) mice, in which AD-like dermatitis is induced spontaneously.

Methods: The severity score of dermatitis, the number of scratches, histamine content, activity of the histamine-forming enzyme histidine decarboxylase (HDC), the number of mast cells and the effect of histamine receptor antagonists were examined.

Results: We found two types of scratching behavior in NC mice, one of a short duration (0.3–1.0 s) and the other of a longer duration (over 1.0 s). The number of short scratchings and HDC activity increased with age (4–13 weeks) in parallel with the severity of dermatitis in NC mice raised in conventional surroundings. The number of longer-duration scratchings had increased before any apparent development of dermatitis and this type of scratching behavior increased at 13 weeks, as did histamine content and mast cell number in the skin. There were no changes in these parameters in NC mice raised in specific pathogen-free surroundings during the experimental period. Chlorpheniramine (a histamine H_1 receptor antagonist), but not histamine H_2 and $H_{3/4}$ receptor antagonists, decreased the number of short scratchings. None of the histamine receptor antagonists decreased the number of long scratchings, whereas tacrolimus reduced both short and long scratching behavior.

Conclusions: The findings of the present suggest that histamine produced by enhanced HDC activity is involved in the induction of short scratchings in NC mice.

Key words: atopic dermatitis, histamine, itching, NC/Nga mice, scratching.

Introduction

Itching that leads to scratching is a common symptom among various forms of dermatitis, including atopic dermatitis (AD), and, consequently, constitutes a major diagnostic criterion.[1] Because itching is a psychological response to the manifestation of dermatitis, it is thought that patients with AD may benefit from scratching behavior. However, AD patients often complain of intense itching leading to excessive scratching. Despite the considerable increase in the number of AD patients, the mechanism underlying the development of itching in AD is unclear.

Histamine is a well-known endogenous amine that exhibits a variety of effects on central and peripheral tissues. These effects are mediated through at least four histamine receptors: H_1–H_4. Stimulation of histamine H_1 receptors induces vasodilatation, increased capillary permeability, contractions of various smooth muscles and itching and/or pain; thus H_1 receptors are generally thought to play an important role in allergy.[2] Histamine H_2 receptors regulate gastric acid secretion and H_3 receptors control the release of histamine mainly in the central nervous system.[3] The molecular identity of the

Correspondence: Dr Yuki Hashimoto, Pharmacology Laboratory, Medicinal Research Laboratories, Taisho Pharmaceutical Co. Ltd, 1-403, Yoshino-cho, kita-ku, Saitama-city, Saitama 331-9530, Japan. Email: yuki.hashimoto@po.rd.taisho.co.jp

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H_4 receptor was recently reported[4] and expression of this type of receptor on eosinophils and mast cells has also been reported.[5] Although involvement of H_4 receptors in the immune system was suggested, biological knowledge of the roles of these receptors is limited. Histamine, which is synthesized from histidine by histidine decarboxylase (HDC), is stored in mast cells. Generally, it has been thought that histamine is formed in mast cells and preformed (or pooled) histamine is released from mast cells. However, histamine is also formed in cells other than mast cells (possibly vascular endothelial cells, granulocytic precursor cells and immune competent cells) via the induction of HDC and the histamine thus formed is released rapidly (without being stored) from the cells in which it is produced.[6–9]

NC/Nga (NC) mice, which originated from Japanese fancy mice, have been established as an inbred strain by Kondo *et al*.[10] and these mice develop eczema when kept in conventional, but not in specific pathogen-free (SPF) environments.[11] The eczema manifests itself as lesions characterized by edema, hemorrhage, erosion, dryness and alopecia (on the ears, back, neck and facial regions).[11] Therefore, the development of dermatitis in NC mice has been considered to be a model of human AD.[11] A method for evaluating scratching behavior in mice was reported[12] and our recent analyses on NC mice demonstrated that there are two types of scratching behavior, one of a short duration (0.3–1.0 s) and the other of a longer duration (over 1.0 s).[13]

In the present study, we examined the number of scratchings, the severity of skin lesions, histamine content and HDC activity in dorsal skin, as well as the number of mast cells, during the development of dermatitis in NC mice. In addition, we examined the effects of histamine H_1, H_2 and $H_{3/4}$ receptor antagonists on the scratching behavior in NC mice and compared them with the effects of tacrolimus, an immunosuppressant.

METHODS

Animals

Conventional NC mice (CNV-NC) and SPF NC mice (SPF-NC; 4-, 7-, 10-, 13- and 15-week-old, males) were purchased from SLC (Shizuoka, Japan) and kept in our laboratory (lights on 07.00 h; lights off 19.00 h; controlled temperature (23 3 C) and humidity 55 15%). Food and tap water were provided *ad libitum*. All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Taisho Pharmaceutical Co. Ltd and Tohoku University.

Agents

Tacrolimus (Prograf®; Fujisawa, Osaka, Japan), chlorpheniramine maleate (Wako, Osaka, Japan), famotidine and thioperamide (Sigma-Aldrich, St Louis, MO, USA) were used in the present study. Drugs were suspended in 0.5% (w/v) sodium carboxymethyl cellulose.

Evaluation of dermatitis

The severity of the dermatitis was graded on a four-point scale as follows: 0, no symptoms; 1, light; 2, mild; 3, severe. This scoring was allocated for the severity of erythema (hemorrhage), edema, excoriation (erosion) and scaling (dryness). A score of 3 was defined as the severity seen in a given tissue in most NC mice at 15 weeks of age. The total score of the above four symptoms for each mouse was taken as the score for the mouse (i.e. minimum 0 and maximum 12).

Measurement of scratching behavior

Scratching was detected automatically and evaluated objectively using Micro Act (Neuroscience, Tokyo, Japan).[13] Briefly, with mice anesthetized, a small magnet (1 mm in diameter, 3 mm long) was implanted subcutaneously into both hind paws at least 6 h before the measurement of scratching behavior. The mouse was placed in an observation chamber (11 cm in diameter, 18 cm high) that was surrounded by a round coil. The electric current induced in the coil by the movement of the magnets implanted in the hind paws was amplified and recorded. Under the present experimental conditions, the apparatus detected both short-duration (0.3–1.0 s) and long-duration (> 1.0 s) scratchings consecutively.[13] Analysis parameters of Micro Act for detecting waves were as follows: threshold 0.1 V; event gap 0.2 s; duration 0.3–1.0 or over 1.0 s; maximum frequency 20 Hz; minimum frequency 2 Hz.

Assay of HDC activity in skin

The activity of HDC was assayed using a method reported previously,[14] but with slight modification.[15] Briefly, mice were decapitated and the dorsal skin of each mouse (approximately 200 mg) was removed and stored in a jar with dry ice. Approximately two-thirds of

the skin was put into a cooled tube with phosphorylated cellulose (50 mg) and 2.5 mL ice-cold 0.02 mol/L phosphate buffer (pH 6.2) containing pyridoxal 5 -phosphate (20 μmol/L) and dithiothreitol (200 μmol/L), then homogenized using an Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany). The supernatant obtained after centrifugation of the homogenate (10 000 *g* for 15 min at 4 C) was used as the enzyme solution. The histamine in the tissues was bound to the phosphorylated cellulose and was removed almost completely from the enzyme solution by centrifugation. Reaction mixture (1 mL) containing the enzyme solution was incubated at 37 C for 14 h with histidine. After the enzyme reaction had been terminated by the addition of 2 mL of 0.5 mol/L $HClO_4$, the histamine formed during the incubation was separated by chromatography on a small phosphorylated cellulose column and quantified fluorometrically. The activity of HDC was expressed as pmol histamine formed during a 1 h period of incubation by the enzyme and contained in 1 g (wet weight) of skin (pmol/h per g).

Determination of histamine

The remaining part of the skin used in the HDC assay was used for measuring histamine. Histamine extracted with 0.4 mol/L $HClO_4$ was separated by chromatography and determined fluorometrically, as described previously.[14]

Histological study

Toluidine blue staining of the dorsal skin was examined. Hair on the skin was shaved off using an electric razor, then the skin was removed and fixed in 10% buffered formalin solution. A block of forehead skin was removed and embedded in paraffin (by conventional methods), cut into 3–4 μm sections, stained with Toluidine blue and the number of mast cells in the dermis counted.

Evaluation of histamine receptor antagonists on spontaneous scratching behavior

Before the administration of drugs, the scratching behavior of each mouse over 24 h (15.00–15.00 h) was measured. Just after this measurement, each test drug or vehicle was given orally to the mouse, then the scratching behavior of the mouse was again measured for an additional 24 h. Scratching behavior before and after drug administration was tentatively called scratching 'Pre' and scratching 'Post', respectively. The sum of the number of scratchings by each mouse for every 1 h was recorded during the experiment. The effects of drugs did not last for a long period of time and Pre scratching numbers differed among the mice. Thus, in the present study, the total number of Post scratchings for each mouse for the initial 9 h (15.00–23.00 h) was expressed as a percentage of that of the number of Pre scratchings.

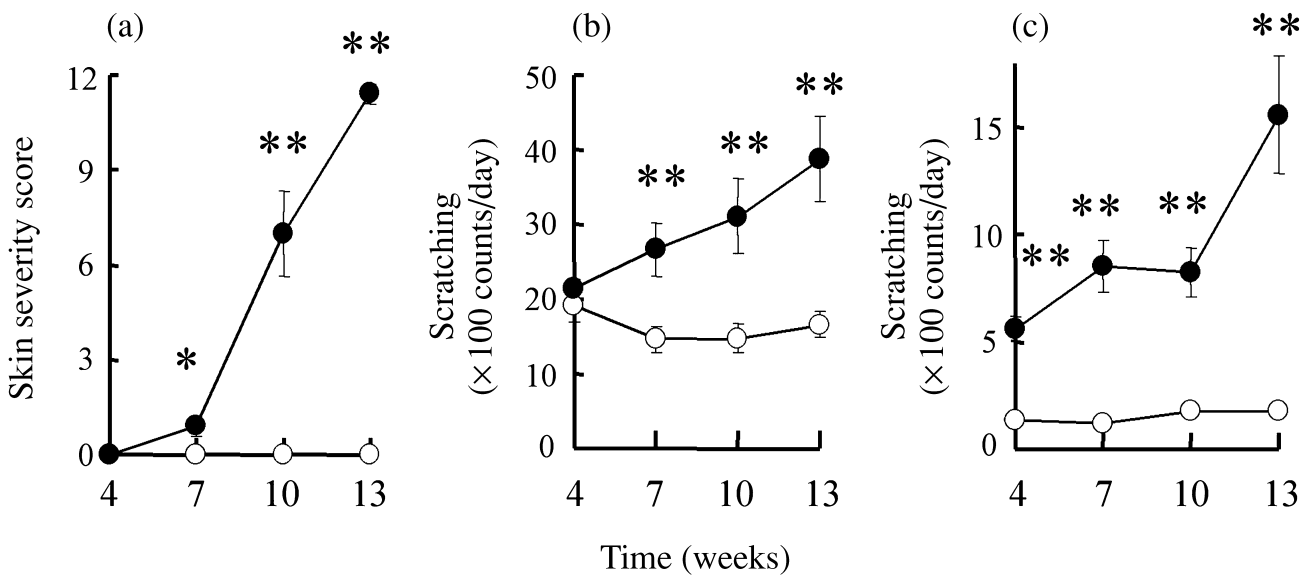

Fig. 1 Development of dermatitis and increases in the scratching behavior in NC/Nga (NC) mice. (a) The severity of the dermatitis was assessed as scores, as described in the Methods, and (b) short- and (c) long-duration scratching behaviors were measured at 4, 7, 10 and 13 weeks of age in NC mice raised in either specific pathgoen-free (○) or conventional (●) conditions. Data are the mean SEM from 10 mice. *$P < 0.05$, **$P < 0.01$ compared with specific pathogen-free NC/Nga mice.

Statistical analysis

Experimental values are given as the mean SEM. Differences in severity scores of dermatitis were analyzed using Mann–Whitney *U*-tests. Differences in scratching behavior, HDC activity, histamine levels and the number of mast cells were analyzed using Student's *t*-test or Welch's test after *F*-test. The evaluation of histamine receptor antagonists was analyzed using Student's *t*-test or Welch's test after *F*-test, with Bonferroni correction. $P < 0.05$ was considered to be statistically significant.

RESULTS

Development of dermatitis in NC mice

In CNV-NC at age 4 weeks, no dermatitis was detected, but dermatitis became apparent at 7 weeks and thereafter gradually increased up to 13 weeks (Fig. 1a). The hair on CNV-NC mice became progressively rougher, especially on the head and neck. Erythema, hemorrhage, excoriation, erosion, scaling and dryness were observed on the ears, face, nose and neck. However, no such eczematous lesions developed in SPF-NC.

Scratching behaviors of NC mice

The number of short-duration (0.3–1.0 s) and long-duration (>1.0 s) scratchings did not increase in SPF-NC during the experimental period. In contrast, in CNV-NC, the number of short scratchings increased with the age of the mouse. There was a statistical difference between SPF-NC and CNV-NC at 7, 10 and 13 weeks of age (Fig. 1b). The number of long scratchings was apparent and statistically higher in CNV-NC than in SPF-NC throughout the experimental period (Fig. 1c). Notably, in CNV-NC, even at 4 weeks, at which time the development of dermatitis was not detected, the number of long scratchings was significantly higher than that in SPF-NC. It should also be noted that the number of long scratchings in CNV-NC increased markedly at 13 weeks.

Histidine decarboxylase activity and histamine content in the skin of NC mice

In SPF-NC, HDC activity, histamine levels and mast cell numbers did not increase during the experimental period (Fig. 2). In CNV-NC, HDC activity in the skin increased with age and there was a statistical difference between SPF-NC and CNV-NC at 7, 10 and 13 weeks (Fig. 2a).

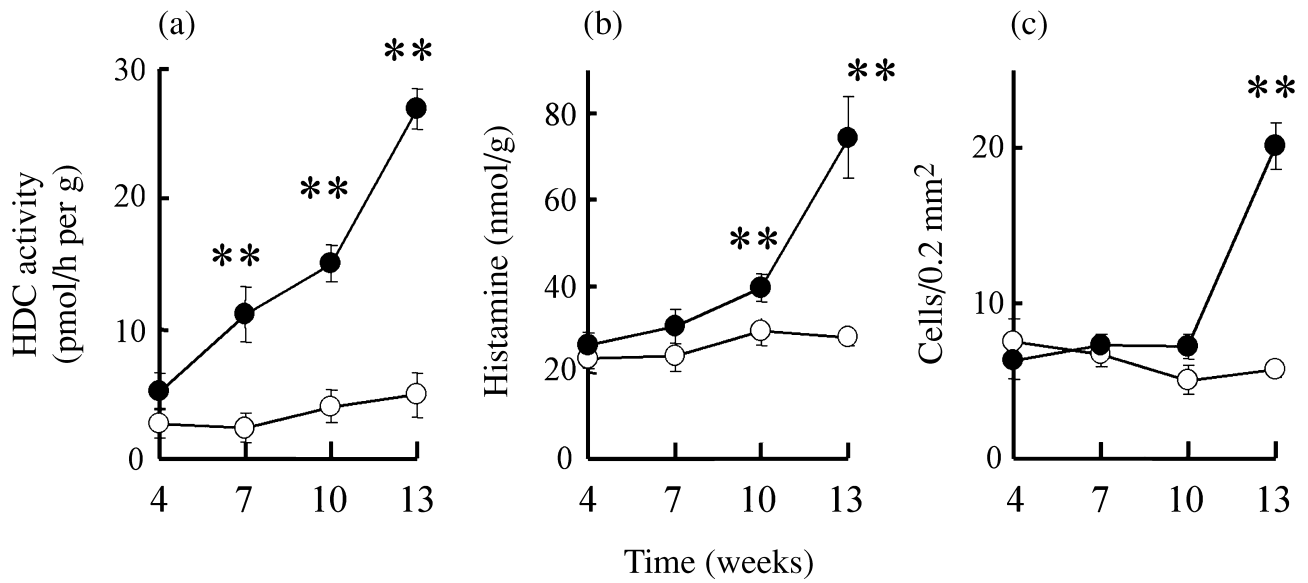

Fig. 2 (a) Histidine decarboxylase (HDC) activity, (b) histamine levels and (c) mast cell numbers in the skin of NC/Nga (NC) mice raised in either specific pathgoen-free (○) or conventional (●) conditions. Mice were decapitated at 4, 7, 10 and 13 weeks and a part of the back skin was subjected to assay of HDC activity (a) and histamine content (b). Another part of the skin was subjected to Toluidine blue staining and the number of mast cells (c) was counted. Data are the mean SEM from 10 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with specific pathogen-free NC/Nga mice.

The level of histamine in CNV-NC was statistically higher than that in SPF-NC at 10 and 13 weeks (Fig. 2b). The level of histamine in CNV-NC increased markedly at 13 weeks compared with its increase during the period 4–10 weeks (Fig. 2b).

Histological comparison of the skin of NC mice

The number of mast cells in the skin of CNV-NC was significantly higher than that of SPF-NC only at 13 weeks (Fig. 2c) and these changes were confirmed histologically (Fig. 3). In addition, in agreement with the results of investigations of histamine content shown in Fig. 2b, there was no significant difference in the number of mast cells between SPF-NC and CNV-NC at ages of 4–10 weeks (Fig. 3).

Effects of histamine H_1, H_2 and $H_{3/4}$ receptor antagonists on scratching behavior in NC mice with skin lesions

For evaluation of the antiscratch properties of drugs, CNV-NC was used at 15 weeks of age, a time when dermatitis had been established. Changes in the number (total number for every 1 h period during 24 h) of Pre and Post scratchings are shown in Figs 4,5, respectively. Administration of vehicle to the mice had no detectable effect on either short-duration (Fig. 4a) or long-duration (Fig. 5a) scratching. Chlorpheniramine (an H_1 receptor antagonist) tended to decrease the number of short scratchings for the initial several hours after its administration (Fig. 4b), but its effect on the long-duration scratching was only marginal (Fig. 5b). The effects of famotidine (an H_2 receptor antagonist) and thioperamide (an $H_{3/4}$ receptor antagonist) on the short-duration (Fig. 4c,d) and long-duration (Fig. 5c,d) scratchings were also only marginal. Tacrolimus (an immunosuppressant) tended to decrease the number of both short- and long-duration scratchings (Figs 4e,5e). However, it should be noted that in this type of comparison, the differences did not reach statistical significance.

However, when the data for Post scratchings are compared in terms of percentage of Pre scratchings, as described in the Methods, the effects of chlorpheniramine and tacrolimus on the short-duration scratchings were statistically significant. Tacrolimus was also effective at reducing the number of long-duration scratchings (Fig. 6b). Famotidine and thioperamide showed no significant effects on either short- or long-duration scratchings, even in this type of comparison (Fig. 6a,b).

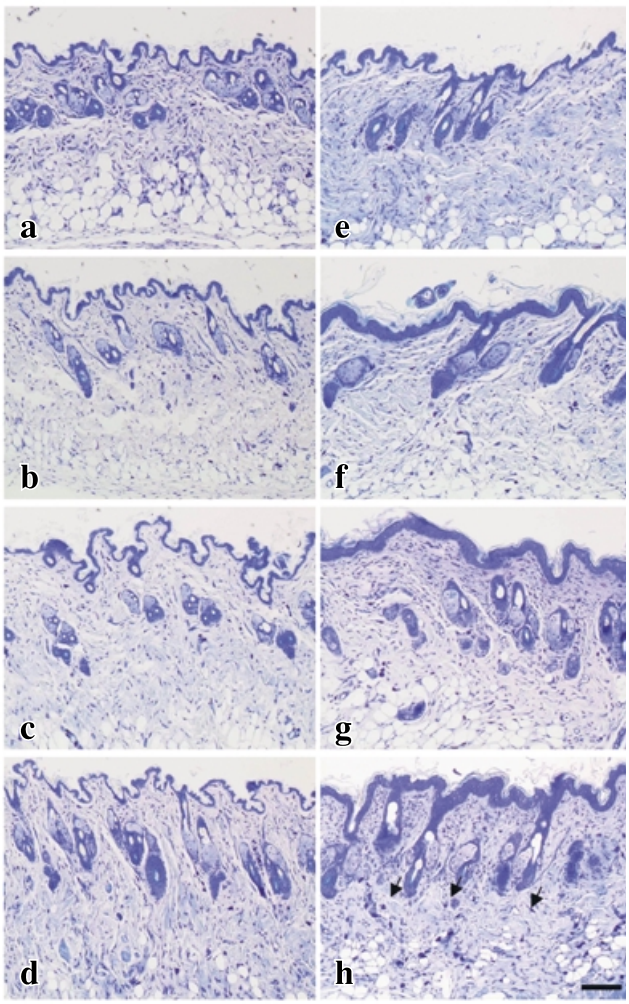

Fig. 3 Toluidine blue staining of the skin. Hair on the skin of the back was shaved off using an electric razor and the skin was removed and fixed in 10% buffered formalin solution. A block of skin was removed, embedded in paraffin, cut into 3–4 μm sections and stained with Toluidine blue. (a–d) Specific pathogen-free NC/Nga mice and (e–h) conventional NC/Nga mice at 4 (a,e), 7 (b,f), 10 (c,g) and 13 weeks of age (d,h). Arrowheads indicate mast cells. Bar, 100 μm.

Discussion

The findings of the present study may be summarized as follows:

1. Threre are two types of scratching behaviors in NC mice, one of a short duration (0.3–1.0 s) and the other of a long duration (over 1.0 s).

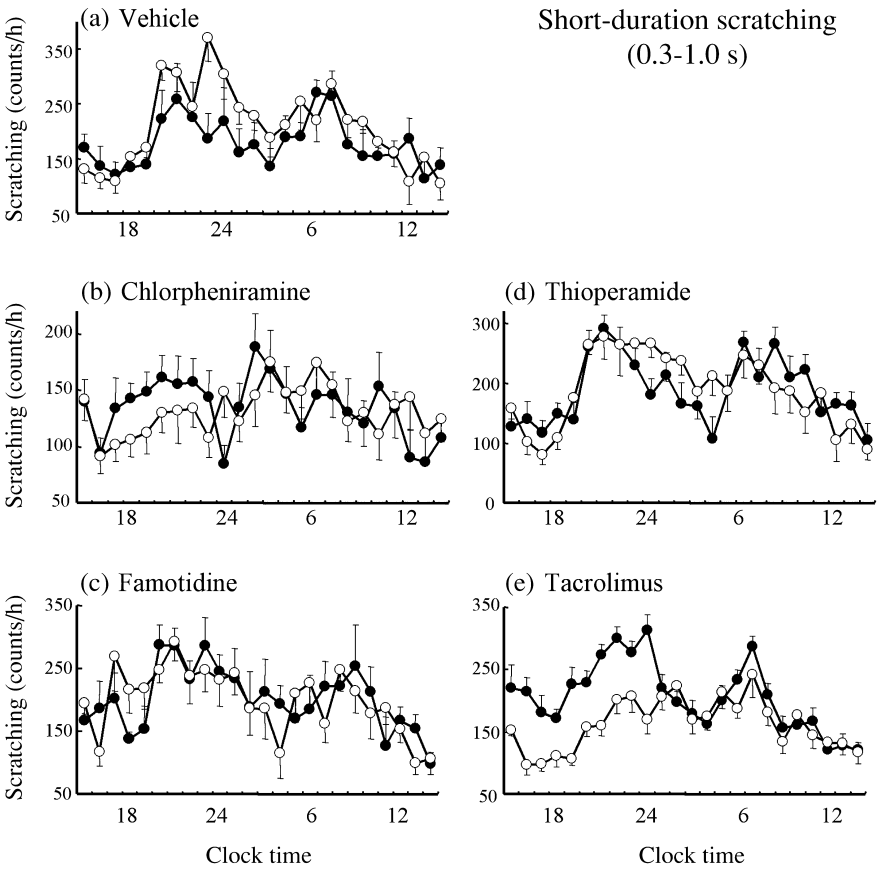

Fig. 4 Effects of histamine H_1, H_2 and $H_{3/4}$ receptor antagonists on the number of short-duration scratchings. For evaluation of the antiscratching properties of drugs, conventional NC/Nga mice at 15 weeks of age were used. Scratching behaviors were measured for 24 h before (●) and after (○) the administration of each drug, as described in the Methods. The number of short-duration scratchings (0.3–1.0 s) of mice in each group for every 1 h are averaged. Data are the mean SEM for six to 12 mice.

2. The number of short-duration scratchings and HDC activity increased with age (4–13 weeks) in parallel with the severity of dermatitis in NC mice raised in conventional surroundings.
3. The number of long-duration scratchings had increased before any apparent development of dermatitis and this type of scratching increased only between 10 and 13 weeks.
4. Histamine content and mast cell numbers in the skin also increased significantly only between 10 and 13 weeks and at 13 weeks, respectively.
5. There were no changes in these parameters in NC mice raised in SPF surroundings during the experimental period.
6. A histamine H_1 receptor antagonist (but not H_2 and $H_{3/4}$ receptor antagonists) decreased the number of short-duration scratchings. None of these histamine antagonists decreased the number of long-duration scratchings.
7. Tacrolimus (an immunosuppressant) reduced both short- and long-duration scratchings.

We discuss these findings in the following paragraphs.

Involvement of histamine in short-duration scratching

Scratching aggravates skin lesions and causes more itching.[16] Histamine is a potent inducer of itching in humans.[17] The efficacy of antihistamine agents for the treatment of itches was noted in AD patients,[18] as well as in mice,[19] and various antihistamines are prescribed for AD patients. However, there is controversy regarding the role of histamine in AD. Patients often complain that itching does not cease when such drugs are taken and histamine may not be a major pruritogen in AD.[20] Although some investigators did not find any, or only marginal, effects of histamine H_1 receptor antagonists,[21] others have reported a significant reduction in pruritus following administration of histamine H_1 receptor antagonists.[22] Intradermal injection of histamine into NC mice was reported not to induce scratching, suggesting that histamine has no important role in the spontaneous itch–scratch response in NC mice.[23] However, Inagaki *et al*. indicated that high doses of histamine would induce systemic effects, which prevent the observation of

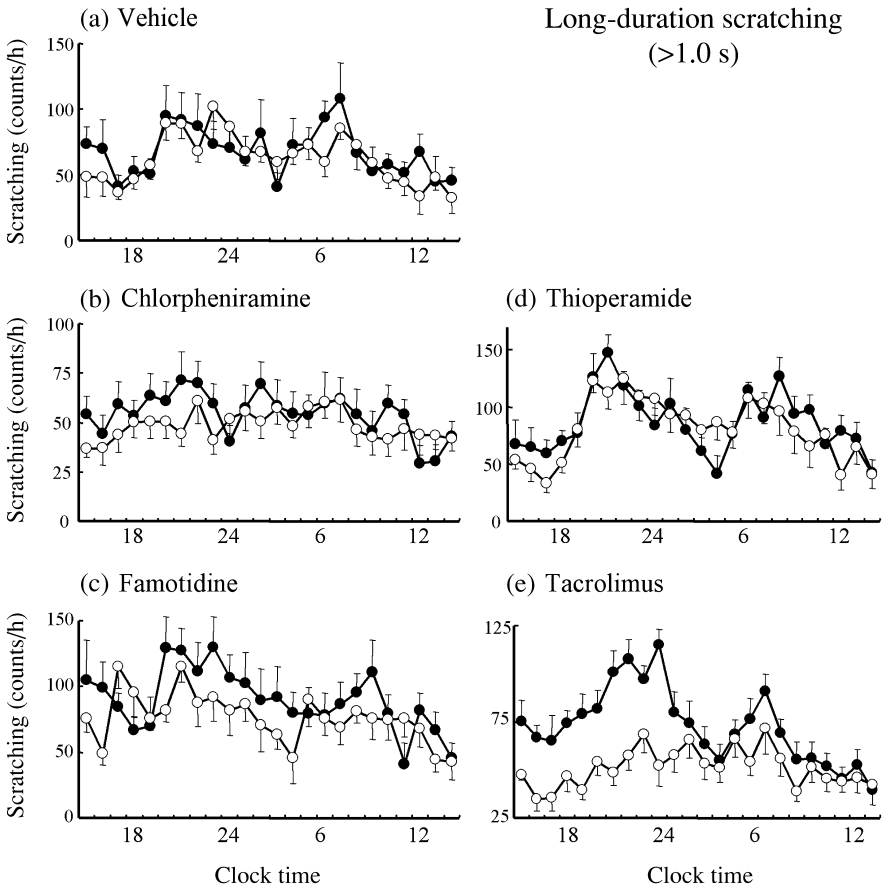

Fig. 5 Effects of histamine H_1, H_2 and $H_{3/4}$ receptor antagonists on the number of long-duration scratchings. Data on long-duration scratching (>1.0 s) observed in the experiments shown in Fig. 4 are shown for 24 h before (●) and after (○) the administration of each drug. The number of long-duration scratchings of mice in each group for every 1 h are averaged. Data are the mean SEM for six to 12 mice.

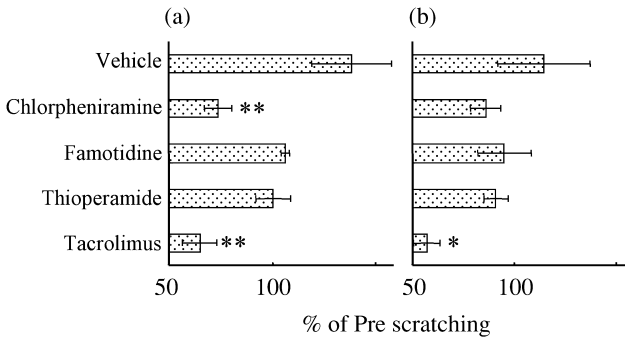

Fig. 6 Comparison of the effects of drugs on (a) short- and (b) long-duration scratchings as a percentage of Pre scratching. The number of short-duration (0.3–1.0 s) and long-duration (>1.0 s) scratchings for each mouse for the initial 9 h (15.00–23.00 h) shown in Figs 4,5 were summed and the values of Post scratching were expressed as a percentage of Pre scratching. Mean values were compared among groups. Data are the mean SEM for six to 12 mice. *$P < 0.05$, **$P < 0.01$ compared with vehicle.

scratching.[24] In the present study, we observed parallel increases in skin HDC activity (but not histamine content and the number of mast cells), dermatitis scores and the number of short-duration scratchings. In addition, short-duration scratchings were significantly suppressed by the histamine H_1 receptor antagonist chlorpheniramine. These results suggest that the itches inducing short-duration scratching are mediated, at least in part, by the release of histamine that was newly formed via elevation of HDC activity, although involvement of preformed histamine released from mast cells was not excluded. However, tacrolimus inhibited short-duration scratchings. Therefore, other mechanisms inhibited by tacrolimus are involved in the induction of short-duration scratchings.

Elevation of HDC activity in the skin of NC mice during the development of dermatitis

As mentioned in the Introduction, histamine is also formed in and released from cells other than mast cells

via induction of HDC activity in response to inflammatory cytokines or inflammatory materials derived from bacteria (such as Lipopolysaccharide and peptidoglycan).[6–9] Because NC mice develop dermatitis in conventional, but not SPF, environments we speculate that microbial components may have a role in the development of dermatitis. We assume that the enhanced activity of HDC and the production and release of histamine from non-mast cells in the skin, in which microbial infection may be involved, may contribute to the induction of short-duration scratching via histamine H_1 receptors in CNV-NC. Interestingly, however, the application of histamine has been reported to reduce itch ratings[25] and axon reflex flares in AD patients,[26] suggesting that histamine is not only an inflammatory mediator, but also an anti-inflammatory mediator in AD.

Effects of drugs

Tacrolimus ointment is an effective drug for the treatment of AD patients.[27] In the present study, tacrolimus decreased the number of both short- and long-duration scratchings, whereas chlorpheniramine decreased only the number of short-duration scratchings. Although these results suggest that chlorpheniramine reduced short-duration scratching behavior by blocking H_1 receptors, antihistamines have a soporific effect and they are anecdotally useful to relieve pruritus at night.[28] In the present study, chlorpheniramine 10 mg/kg did not show sedative properties (data not shown). Because chlorpheniramine 30–100 mg/kg was most effective at reducing both short- and long-duration scratchings (data not shown), these findings seem to be due to its sedative effects.

It was reported that keratinocytes have histamine H_2 receptors and affect immune responses[29] and H_4 receptors have been shown to be expressed on eosinophils and mast cells.[5] However, in the present study, famotidine and thioperamide had only marginal effects on the short- and long-duration scratchings.

Contribution of mast cells to dermatitis

In agreement with histamine contents in the skin, our histological study showed that there was a marked increase in the number of mast cells at 13 weeks in CNV-NC mice and no notable difference during 4–10 weeks in either SPF-NC or CNV-NC mice. These results suggest that although the notable increase in histamine content in the skin at 13 weeks is due to an increase in mast cells, the mast cell is not a source of a pruritogen for short-duration scratching. However, it has also been reported that the inhibition of mast cell activation resulted in suppression of the development of dermatitis in NC mice with severe dermatitis.[30,31] Because symptoms of dermatitis initially appeared as itching (and, thus, scratching) in NC mice, it is likely that mast cells infiltrate into the skin as a result of scratching. Although the role of mast cells in AD patients is unclear, mast cells may play divergent roles in both the exacerbation of inflammation and in the healing of wounds.[32]

Contribution of long-duration scratching to dermatitis in NC mice

Our findings that the time-course of the development of short- and long-duration scratchings differed (the former had already appeared even at 4 weeks of age and the former (but not the latter) was suppressed by chlorpheniramine) suggest that the mechanisms of short- and long-duration scratchings also differ. Mast cells in the skin increase in number at a later period (13 weeks) and, hence, may be involved in part of the long-duration scratching behavior. We have no data concerning the substance(s) responsible for long-duration scratching. Because steroids and immunosuppressants (such as tacrolimus) inhibit cytokine production by T cells[33] and interleukin-2 has been shown to play a role in causing pruritus and inflammation in the skin of AD patients,[34] more studies are needed to elucidate the mechanism involved in long-duration scratching behavior. In addition, one possible reason why antihistamines exhibit no discernible benefit in some patients may be because they are ineffective at reducing the long-duration scratching observed in NC mice.

In conclusion, the findings of the present study suggest that histamine H_1 receptors are involved in the induction of short- but not long-duration scratching, although the latter may be a more important target for reducing the severity of dermatitis in NC mice or, possibly, in AD patients.

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