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Pharmacological characterization of a chronic pruritus model induced by multiple application of 2,4,6-trinitrochlorobenzene in NC mice

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Abstract

Female NC/Jic mice were sensitized and challenged repeatedly at 48 h intervals for 10 and 30 days by painting 1% 2,4,6-trinitrochlorobenzene (TNCB) on both ears. Mice challenged with TNCB for 30 days developed an inflammatory dermatitis with high immunoglobulin E (IgE) titer. Histological analysis with acidic Toluidine Blue staining revealed that dermal mast cells markedly differentiated and intensely degranulated, consistent with a dramatic increase in scratching behavior. A significant increase in total scratching events could be observed in mice treated with TNCB for a short period of 10 days. Extending the term of TNCB application to 30 days, the IgE titer and number of mast cells elevated significantly, and thus various drugs were evaluated pharmacologically by using the mice treated with TNCB for 30 days. Terfenadine and cyproheptadine attenuated the chronic scratching behavior. Tacrolimus and dexamethasone were less effective and cromolyn showed no effect. In addition, terfenadine and tacrolimus suppressed the degranulation of mast cells. The present chronic scratching model could be suitable to evaluate drugs effective for suppression of mast cell differentiation and degranulation by irritation, and may represent a promising tool to develop new drugs for inflammatory pruritus associated with, for example, atopic dermatitis.

Keywords: 2,4,6-Trinitrochlorobenzene; Atopic dermatitis; Chronic itching model; Degranulation; Mast cells

1. Introduction

Atopic dermatitis is one of the most refractory diseases today. The itching sensation causes patients to scratch, and the scratching behavior aggravates the skin condition in the patients (Wahlgren, 1999). This cycle has been called an "itch–scratch" vicious circle, and is an important target for treatment of atopic dermatitis.

Although it has been known that histamine induces an acute itching sensation in humans (Magerl et al., 1990; Handwerker et al., 1991; Baron et al., 2001), the mechanism underlying chronic itching is still unclear. It has often been reported that antihistamines are ineffective in relieving pruritus in atopic dermatitis (Klein and Clark, 1999), while immunoregulatory drugs such as tacrolimus ointment and cyclosporin A relieve the chronic itchy sensation (Harper et al., 2000; Reitamo et al., 2000; Alomar et al., 2004), suggesting that multiple factors in addition to histamine may be involved in chronic pruritus. For example, neurotrophins may be considered as important factors in the chronic itching sensation. Plasma levels of the nerve growth factor in atopic dermatitis patients were increased in comparison with non-atopic controls (Toyoda et al., 2002). Consequently, upregulated nervous factor leads to extension of the cutaneous nerve fibers in atopic lesion skin to epidermis (Tobin et al., 1992; Sugiura et al., 1997; Urashima and Mihara, 1998), neurotrophins may be considered as important factors evoked by innocuous stimulation (Simone et al., 1991) and mechanical or chemical stimuli inducing pain (Ikoma et al., 2004).

However, mediators inducing chronic scratching behavior have not been fully characterized and remedies for persistent itch have not been developed. Establishing an appropriate animal model is a prerequisite to clarify the mechanism leading to chronic pruritus and to develop drugs for controlling itching in chronic inflammatory diseases.

Previously, we established a dermatitis model by repeatedly applying 2,4,6-trinitrochlorobenzene (TNCB) to BALB/c mice

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for up to 90 days (Yamashita et al., 2005). Histological observation revealed that eosinophilic infiltration, acanthosis, hyperkeratosis, and dermal papilla were observed in the whole ear. In addition, continuous scratching behavior was shown in the TNCB-treated mice. We also suggested that differentiation and degranulation of mast cells play an important role in inducing chronic scratching behavior in the model by using T cell-deficient BALB/c-*nu*/*nu* mice and mast cell-deficient WBB6F1-W/W^{v} and Sl/Sl^{d} mice.

In the present study, we attempted to shorten the period necessary for inducing steady scratching behavior by using NC/Jic mice instead of BALB/c mice, because NC mice have been considered a good animal model for atopic dermatitis (Vestergaard et al., 1999). Furthermore, we tried to characterize the present model pharmacologically by drugs used clinically for treatment of atopic dermatitis: terfenadine, cyproheptadine, tacrolimus, dexamethasone, and cromolyn, and confirmed that one of the most important factors inducing chronic pruritus was degranulation of mast cells.

2. Materials and methods

2.1. Animals

NC/Jic mice obtained from Clea Japan, Inc. (Tokyo, Japan) were home-reared and housed in standard plastic cages. Female NC/Jic mice of 6- to 9- weeks old were used for experiments. The mice were kept under controlled temperature (23±2 °C), humidity (50±10%), and light (lights on from 06:00 to 18:00), but not in a specific pathogen-free environment, with food (CE-2, Clea Japan, Inc.) and water available *ad libitum*. Experimental procedures were approved by the Animal Care Committee of the Graduate School of Pharmaceutical Sciences, Nagoya City University, in accordance with the guidelines of the Japanese Council on Animal Care.

2.2. Reagents

2,4,6-Trinitrochlorobenzene and dexamethasone were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Terfenadine, cyproheptadine hydrochloride, and cromolyn sodium salt were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Tacrolimus hydrate was kindly provided by Fujisawa Pharmaceutical (Osaka, Japan). TNCB was dissolved in acetone/olive oil (4:1) as 1% (w/v) solution and used for the sensitization and elicitation. Terfenadine, cyproheptadine hydrochloride, tacrolimus hydrate, and dexamethasone were suspended in phosphate-buffered saline (PBS, pH 7.4) containing 5% polyoxyethylene sorbitan monooleate (Nacalai Tesque), and cromolyn sodium salt was dissolved in PBS.

2.3. Application of TNCB

Female NC/Jic mice were initially sensitized by painting 1% TNCB (20 μl) on the face of both ears. Six days later, they started to be challenged by painting 20 μl of 1% TNCB solution repeatedly, every 48 h, for 10 and 30 days. In the vehicle-treated group, acetone/olive oil (4:1) was applied instead of TNCB. The thickness of the right ear was measured with a dial thickness gauge (G-1A, Ozaki MFG. Co., Ltd, Tokyo, Japan) prior to each topical application of TNCB. We applied TNCB on both ears because it facilitated the measurement of scratching behavior although histological evaluation was conducted on only the right ear.

2.4. Evaluation of scratching behavior

Scratching behavior was measured using the MicroAct system (Neuroscience, Tokyo, Japan) where this behavior could be detected automatically, and analyzed objectively (Inagaki et al., 2002, 2003). Briefly, a ring-type magnet (1.0 mm inner diameter, 2.5 mm outer diameter, 2.0 mm height, 130–145 mT/cm^2) was attached to both ankles with steel wire (No. 34, TOHO, Co. Ltd, Hiroshima, Japan). Ten to 15 h after magnet placement, each mouse was placed in a plastic chamber (11 cm in diameter and 18 cm in height) surrounded by a round coil, and the current induced in the coil by movement of the magnets was amplified and recorded. Characteristic signals were identified as scratching behavior using the parameters: threshold, 0.12 V; event gap, 0.02 s; minimum duration, 0.40 s; maximum frequency, 18.0 Hz; minimum frequency, 5.0 Hz. The measurement of scratching behavior was started 48 h after the final application of TNCB, since non-specific stimuli induce unfavorable scratching episodes immediately after applying the solvent and the number of scratching events within 48 h post-application in the vehicle-treated group was equal to that in the TNCB-treated group (Yamashita et al., 2005). The mice were put in the system device and kept for 2 h for acclimation. Then the scratching behavior was recorded continuously for 12 h.

2.5. Drug administration

The drugs were injected intraperitoneally 4 h after the start of recording with the MicroAct system, and measurement of the scratching behavior was continued for a further 8 h. The doses of the drugs were as follows: terfenadine (30 and 100 mg/kg), cyproheptadine hydrochloride (10 and 30 mg/kg), tacrolimus hydrate (1 and 10 mg/kg), dexamethasone (1 and 3 mg/kg), and cromolyn sodium salt (100 and 300 mg/kg). For the control groups, PBS or PBS containing 5% polyoxyethylene sorbitan monooleate was injected instead of the drugs.

2.6. Histological observation

After recording the scratching behavior, we sacrificed the mice by lethal inhalation of ether vapor. Right ears of the mice were excised and fixed with 10% phosphate-buffered formalin (pH 7.2), and embedded in paraffin. Sections (3 μm) were stained with acidic Toluidine Blue (pH 4.1), and the number of mast cells per square millimeter of dermis in four sights chosen at random was counted. The phenotype of the mast cells was classified into degranulated (>10% of the cytoplasmic granules exhibiting fusion or discharge) or normal, and the ratio of degranulated cells was calculated (Wershil et al., 1988; Yano et al., 1989).

2.7. Determination of total immunoglobulin E (IgE) titer in serum

Blood was collected from the scarified mice and serum was stored at −20 °C until used. The concentration of total IgE in the serum was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (Yamashita et al., 2005).

2.8. Statistics

All results are given as means±S.E.M. Statistical analyses were conducted by two-tailed multiple *t*-test with Bonferroni correction following ANOVA, or two-tailed Student's or Aspin–Welch's *t*-test following *F*-test. These statistics were analyzed using the software Java Applets and Servlets for Biostatistics (programmed by H. Ono, http://chiryo.phar.nagoya-cu.ac.jp/javastat/JavaStat-e.htm), and synergistic interactions between factors Treatment×Time were calculated by two-factor factorial ANOVA using Microsoft Excel.

3. Results

3.1. Shortening the term necessary to induce chronic scratching behavior

TNCB was applied to NC/Jic mice epicutaneously every 2 days for 10 and 30 days. Average ear thickness of NC/Jic mice before application of TNCB or mice was 229.9±1.7 μm. Ear thickness increased dramatically up to 10 days after the first application (1406±56 μm in the TNCB-treated mice compared with 226.2±2.7 μm in the vehicle-treated ones) and reached a plateau. On day 30 it was 1359±33 μm in the TNCB-treated mice compared with 235.6±2.0 μm in the vehicle-treated ones. Repeated application of TNCB for 10 days induced a significant increase in scratching events when measured in the 12-h period following application. The scratching incidences were further increased slightly when the TNCB application was repeated for 30 days (Fig. 1A). Total serum IgE titer was significantly increased in mice treated with TNCB for 30 days (Fig. 1B). As shown in Fig. 2A, the number of mast cells in the ear tissues was significantly increased depending on the repeated application of TNCB. The ratio of degranulated mast cells to total mast cells in dermis was increased by applying TNCB repeatedly for 10 days and 30 days (Fig. 2B).

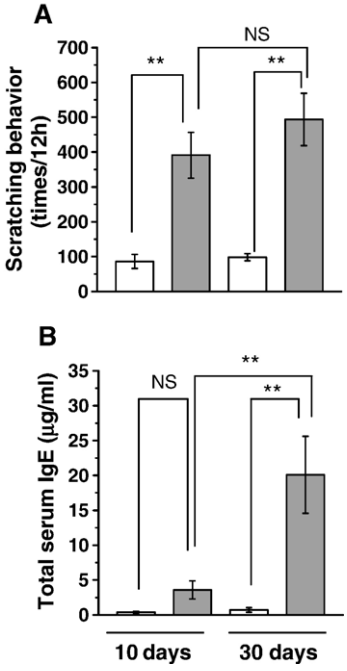

Fig. 1. Scratching behavior (A) and total IgE (B) in NC/Jic mice treated with TNCB (filled column) and vehicle (open column) repeatedly for 10 days and 30 days at 48 h intervals. Scratching behavior was recorded and analyzed with the MicroAct system for 12 h starting 48 h after the final challenge. Total serum IgE titer was assayed by ELISA. Each column represents the mean±S.E.M of six mice. The significance of differences was determined by the two-tailed multiple *t*-test with Bonferroni correction following ANOVA (three comparisons in four groups: $^{**}P<0.01$; NS, not significant).

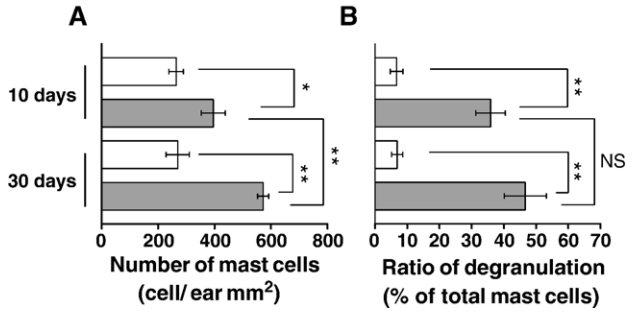

Fig. 2. Number of mast cells per square meter of dermis (A) and ratio of degranulated mast cells (B) in the ear of NC/Jic mice treated with TNCB (filled column) and vehicle (open column) repeatedly for 10 days and 30 days at 48 h intervals. Mast cells were histologically evaluated by acidic Toluidine Blue staining. They were classified as degranulated when more than 10% of the cytoplasmic granules exhibited fusion or discharge. Each column represents the mean±S.E.M. of six mice. Significant differences were determined by the two-tailed multiple *t*-test with Bonferroni correction following ANOVA (three comparisons in four groups: $^{*}P<0.05$, $^{**}P<0.01$; NS, not significant).

Total serum IgE titers and the number of mast cells were significantly increased in the mice treated with TNCB for 30 days compared with those treated for 10 days. Furthermore, a significant interaction between treatment and time was detected by two-factor factorial ANOVA regarding the total serum IgE level (Fig. 1B, $P<0.05$) and the number of the mast cells (Fig. 2B, $P<0.05$). Therefore, we decided to evaluate the effects of drugs on the scratching behavior in the model established by applying TNCB for 30 days. The increase in the scratching incidences continued until at least 2 weeks after final application of TNCB.

3.2. Pharmacological analysis of chronic scratching behavior

The effects of various drugs on chronic scratching behavior induced by TNCB were examined in NC/Jic mice. There was no difference of stationary scratching behavior before injection of the drugs among all of the experimental groups. Terfenadine, a non-sedative antihistamine, inhibited scratching behavior when injected at a dose of 100 mg/kg. Terfenadine (100 mg/kg) led to intense suppression of scratching behavior between 2 and 4 h after injection and the suppression was gradually decreased

thereafter (Fig. 3A). Cyproheptadine, an antagonist for both histamine- and serotonin-receptors, also inhibited the scratching behavior dose-dependently (Fig. 3B). In the mice injected with cyproheptadine, not only scratching behavior but also voluntary exercise was suppressed. Tacrolimus (10 mg/kg), an immunoregulation drug, reduced the number of scratching events between 2 and 4 h after intraperitoneal administration. Dexamethasone, a steroid drug, also inhibited the scratching behavior between 4 and 6 h after injection at a dose of 1 mg/kg (Fig. 3C and D). In contrast, cromolyn, a stabilizer of mast cells, had no effect on scratching behavior (Fig. 3E).

The effects of the drugs on the ratios of degranulated mast cells to total mast cells in the ear dermis were examined by Toluidine Blue staining (Fig. 4). Because the suppressive effects of the drugs on scratching behavior are most striking between 2 and 4 h post-drug administration, the ratio of mast cell degranulation was estimated at a time point 2 h after drug treatment. The assay was also carried out 8 h after drug injection. The ratio was significantly reduced 2 h after the injection of terfenadine (100 mg/kg) and tacrolimus (10 mg/kg), comparable to the decrease in scratching behavior between 2 and 4 h, and was restored to the control level thereafter. The time-course change in mast cell degranulation was quite consistent with that in the scratching behavior as seen in Fig. 3A and C. Cyproheptadine (30 mg/kg) slightly but insignificantly suppressed the ratio of degranulated mast cells while neither dexamethasone (1 mg/kg) nor cromolyn (300 mg/kg) inhibited degranulation of mast cells (data not shown).

4. Discussion

Here, we have shown that repeated application of TNCB to ears of NC/Jic mouse for a short period of 10 days induced

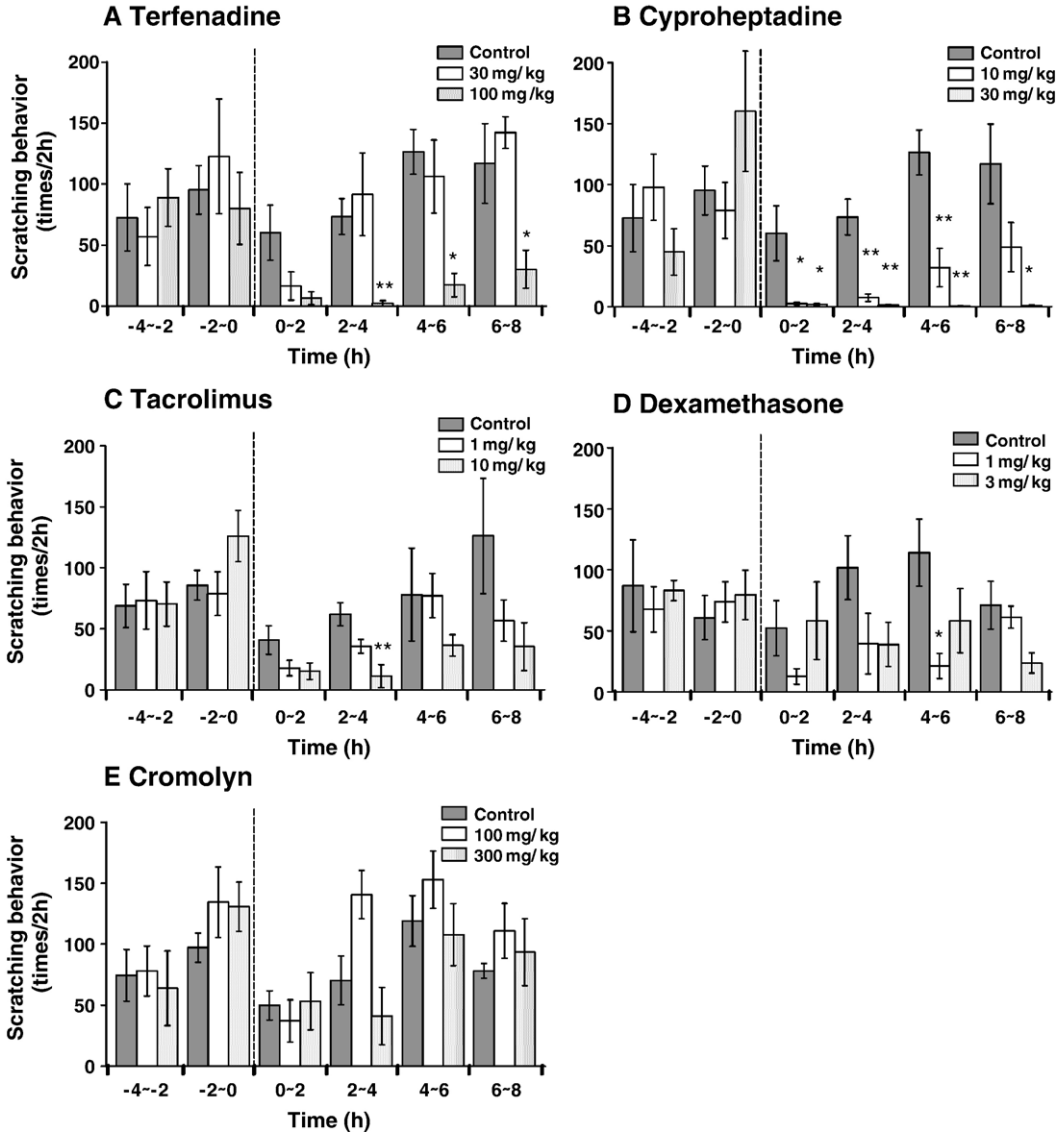

Fig. 3. Effects of various drugs on the chronic scratching behavior in NC/Jic mice induced by multiple application of TNCB for 30 days. Measurement of the scratching behavior commenced 4 h prior to drug injection and ended 8 h post-drug administration and the number of scratching events was counted in a fraction of a 2-h period. Each column represents the mean ± S.E.M. of 5 to 7 mice. Statistical analysis was carried out by the two-tailed multiple *t*-test with Bonferroni correction following ANOVA (two comparisons in three groups: $*P<0.05$, $**P<0.01$ *vs*. the control group at the corresponding time point).

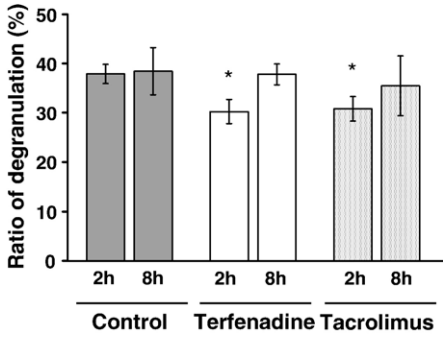

Fig. 4. Effects of terfenadine and tacrolimus on the ratio of degranulated mast cells in NC/Jic mice. The mice were treated with TNCB for 30 days. The right ear was collected from the mice 2 h and 8 h after drug injection and histologically evaluated by acidic Toluidine Blue staining. The number of mast cells per square millimeter of dermis was counted under a light microscope and the mast cells in which more than 10% of the cytoplasmic granules exhibited fusion or discharge were classified as degranulated. Each column represents the mean±S.E.M. of six mice. *$P<0.05$ *vs.* control group by Student's *t*-test.

marked ear eczema and upregulation of serum IgE concentration in parallel with an increase in scratching episodes. The chronic itchy pruritus with the elevated serum IgE level resembled human atopic dermatitis.

Although the serum IgE level was only slightly but insignificantly increased in the mice treated with TNCB for 10 days compared with that in vehicle treated mice ($P=0.056$, TNCB treated group for 10 days *vs.* vehicle treated one by Welch' s *t*-test), the number of scratching episodes in the TNCB-treated mice was significantly higher than that in the control mice. This may indicate that slight increase in IgE level is enough to induce degranulation of mast cells and scratching behavior.

In an earlier study, we established a chronic itching model with persistent scratching by applying TNCB repeatedly for 90 days to BALB/c mice to understand the molecular basis of pruritus in inflamed skin (Yamashita et al., 2005). Furthermore, we indicated that proliferation and degranulation of dermal mast cells play an important role in the scratching behavior using T cell- and mast cell-deficient mice and suggested that the model may be useful in understanding the molecular process controlling the development of itching in chronic inflammatory skin disease including atopic dermatitis. However, a major disadvantage of this model is that it takes 3 months to establish a persistent and stable scratching behavior accompanied by skin inflammation, which especially discourages the use of the model to screen drugs for treatment of inflammatory pruritus.

In the present investigation, we succeeded in detecting a significant increase in scratching episodes together with marked ear swelling and elevated serum IgE titers in 30 days by using NC mice instead of BALB/c mice. NC mice were first established from Japanese fancy mice as an inbred line. Atopic dermatitis-like skin lesions were reported to appear spontaneously in NC/Nga mice, derived from the original NC mice, when they were raised in non-sterile environments (Matsuda et al., 1997), and NC/Nga and NC/Jic mice, other strains of NC mice, have been used as animal models for elucidating the pathogenesis and developing new remedies for atopic dermatitis. It has currently been recognized that the symptoms do not always spontaneously develop even in non-sterile circumstances and that application of haptens or mite antigens is necessary to evoke the eczema stably (Sasakawa et al., 2001; Matsuoka et al., 2003; Gao et al., 2004). Consistent with these reports, TNCB treatment facilitated the incidence of severe inflammation and scratching behavior even after 10 days while such symptoms did not appear in vehicle-treated mice.

Terfenadine, a histamine H_1 receptor antagonist significantly inhibited scratching behavior. Since terfenadine suppressed the degranulation of mast cells, it may inhibit the scratching events by stabilizing the membrane of mast cells (Okayama et al., 1994; García et al., 1997) rather than blocking histamine H_1 receptors.

The inhibition of scratching behavior by cyproheptadine may indicate that serotonin induced the itching sensation in the present model. However, the number of scratching episodes was reduced in the cyproheptadine-treated mice more than that in the vehicle-treated group and lethargic appearance was observed in the mice administered with cyproheptadine at a dose of 30 mg/kg. Thus, it is likely that suppression of scratching behavior by cyproheptadine is due to its sedative effect.

In addition, tacrolimus inhibited the scratching behavior as well as the degranulation of mast cells. It was shown to inhibit mast cell degranulation *in vitro* (De Paulis et al., 1991; Sengoku et al., 2000) and early phase reaction in the mast cell-dependent biphasic cutaneous response *in vivo* (Katayama et al., 1996; Geba et al., 2001). The present results confirmed pharmacologically that tacrolimus inhibits the scratching behavior by suppressing mast cell degranulation *in vivo*. In fact, Alomar et al. (2004) reported that tacrolimus ointment was effective for chronic itch in atopic dermatitis patients.

Dexamethasone suppressed the scratching behavior from 4 to 6 h after injection at a dose of 1 mg/kg but not at 0.1 mg/kg and 10 mg/kg. Because mast cell degranulation was not suppressed by dexamethasone, and the dose-response relationship for scratch-inducing effects of serotonin or opioid peptides in mice was reported to be bell-shaped (Tohda et al., 1997; Yamaguchi et al., 1998, 1999), dexamethasone may inhibit mediators leading to chronic pruritus, which needs further investigation.

Although cromolyn was a stabilizer of mast cells, it could not suppress degranulation and scratching behavior. This result is consistent with a report describing that cromolyn failed to prevent acute scratching action induced by compound 48/80 (Sugimoto et al., 1998). It may be that cromolyn is too weak to suppress mast cell degranulation and the itching sensation induced by intense stimulation.

Histamine was reported to be a major mediator released from human mast cells to provoke the itching sensation (Handwerker et al., 1991) while serotonin induced the sensation slightly (Hägermark, 1992), although the itching sensation could not be suppressed by antihistamine in atopic dermatitis patients (Rukwied et al., 2000). In contrast, in most murine strains, scratching behavior was not induced by histamine (Inagaki et al., 2001) but was evoked by mast cell-derived serotonin (Yamaguchi et al., 1999; Maekawa et al., 2000). Furthermore, tryptase has been recognized to be the most abundant mediator present in mast cell granules, activating the proteinase-activated receptor-2 (PAR-2). PAR-2 is present in various tissues including the

primary sensory neurons (Steinhoff et al., 2000) and is involved in nociception (Kawabata et al., 2001). Steinhoff et al. (2003) reported that in atopic dermatitis patients, an intradermal injection of a tethered ligand for PAR-2 provoked enhanced itch in eczema. A PAR-2 antagonist inhibited acute scratching behavior induced by compound 48/80 in mice (Ui et al., 2006). Thus, tryptase is a factor derived from mast cells, inducing the itching sensation in both human and mouse and may play an important role as a mediator in our chronic model. Although mast cell-derived mediators leading to the itching sensation have to be identified in the future, the present results clearly indicate that degranulation of mast cells play a pivotal role in the incidence of scratching behavior and it will be an important target of drug development for curing chronic itch.

The result that not only degranulation but also number of mast cells were markedly increased by repeated application of TNCB suggests that differentiation of mast cells is another target for remedy of chronic itch. Stem cell factor (SCF), interleukin-3 (IL-3) and interleukin-4 (IL-4) are cytokines playing an essential role in differentiation of murine mast cells (Tsuji et al., 1990, 1995; Takagi et al., 1992). Preliminary measurement revealed no significant difference in SCF level either in serum or in ear tissues between TNCB-treated and vehicle-treated mice (data not shown). It is likely that neither IL-3 nor IL-4 are involved in mast cell differentiation in the present model because mast cells were differentiated in the T cell-deficient BALB/c-*nu*/*nu* mice as previously reported (Yamashita et al., 2005). Identification of the factors involved in the differentiation of mast cells in the present model is an interesting problem to be solved in the future.

In conclusion, we established the atopic dermatitis-like chronic pruritus model by epicutaneously applying TNCB to NC/Jic mice. The chronic scratching behavior was inhibited by suppressing the degranulation of mast cells using terfenadine and tacrolimus. The present atopic dermatitis-like model is a promising tool for development of new drugs specific for inflammatory pruritus.

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References

Alomar, A., Berth-Jones, J., Bos, J.D., Giannetti, A., Reitamo, S., Ruzicka, T., Stalder, J.F., Thestrup-Pedersen, K., 2004. The role of topical calcineurin inhibitors in atopic dermatitis. Br. J. Dermatol. 151 (Suppl 70), 3–27 (Dec).

Baron, R., Schwarz, K., Kleinert, A., Schattschneider, J., Wasner, G., 2001. Histamine-induced itch converts into pain in neuropathic hyperalgesia. NeuroReport 12, 3475–3478.

De Paulis, A., Cirillo, R., Ciccarelli, A., Condorelli, M., Marone, G., 1991. FK-506, a potent novel inhibitor of the release of proinflammatory mediators from human Fc epsilon RI+ cells. J. Immunol. 146, 2374–2381.

Gao, X.K., Nakamura, N., Fuseda, K., Tanaka, H., Inagaki, N., Nagai, H., 2004. Establishment of allergic dermatitis in NC/Nga mice as a model for severe atopic dermatitis. Biol. Pharm. Bull. 27, 1376–1381.

García, G., DeMora, F., Ferrer, L., Puigdemont, A., 1997. Effect of H1-antihistamines on histamine release from dispersed canine cutaneous mast cells. Am. J. Vet. Res. 58, 293–297.

Geba, G.P., Ptak, W., Askenase, P.W., 2001. Topical tacrolimus and cyclosporin A differentially inhibit early and late effector phases of cutaneous delayed-type and immunoglobulin E hypersensitivity. Immunology 104, 235–242.

Hägermark, O., 1992. Peripheral and central mediators of itch. Skin Pharmacol. 5, 1–8.

Handwerker, H.O., Forster, C., Kirchhoff, C., 1991. Discharge patterns of human C-fibers induced by itching and burning stimuli. J. Neurophysiol. 66, 307–315.

Harper, J.I., Ahmed, I., Barclay, G., Lacour, M., Hoeger, P., Cork, M.J., Finlay, A.Y., Wilson, N.J., Graham-Brown, R.A., Sowden, J.M., Beard, A.L., Sumner, M.J., Berth-Jones, J., 2000. Cyclosporin for severe childhood atopic dermatitis: short course versus continuous therapy. Br. J. Dermatol. 142, 52–58.

Ikoma, A., Fartasch, M., Heyer, G., Miyachi, Y., Handwerker, H., Schmelz, M., 2004. Painful stimuli evoke itch in patients with chronic pruritus: central sensitization for itch. Neurology 62, 212–217.

Inagaki, N., Nagao, M., Igeta, K., Kawasaki, H., Kim, J.F., Nagai, H., 2001. Scratching behavior in various strains of mice. Skin Pharmacol. Appl. Skin Physiol. 14, 87–96.

Inagaki, N., Igeta, K., Kim, J.F., Nagao, M., Shiraishi, N., Nakamura, N., Nagai, H., 2002. Involvement of unique mechanisms in the induction of scratching behavior in BALB/c mice by compound 48/80. Eur. J. Pharmacol. 448, 175–183.

Inagaki, N., Igeta, K., Shiraishi, N., Kim, J.F., Nagao, M., Nakamura, N., Nagai, H., 2003. Evaluation and characterization of mouse scratching behavior by a new apparatus, MicroAct. Skin Pharmacol. Appl. Skin Physiol. 16, 165–175.

Katayama, I., Otoyama, K., Yokozeki, H., Nishioka, K., 1996. Effect of mast cell modulators on IgE-mediated murine biphasic cutaneous reactions. Int. Arch. Allergy Immunol. 109, 390–397.

Kawabata, A., Kawao, N., Kuroda, R., Tanaka, A., Itoh, H., Nishikawa, H., 2001. Peripheral PAR-2 triggers thermal hyperalgesia and nociceptive responses in rats. NeuroReport 12, 715–719.

Klein, P.A., Clark, R.A., 1999. An evidence-based review of the efficacy of antihistamines in relieving pruritus in atopic dermatitis. Arch. Dermatol. 135, 1522–1525.

Maekawa, T., Nojima, H., Kuraishi, Y., 2000. Itch-associated responses of afferent nerve innervating the murine skin: different effects of histamine and serotonin in ICR and ddY mice. Jpn. J. Pharmacol. 84, 462–466.

Magerl, W., Westerman, R.A., Möhner, B., Handwerker, H.O., 1990. Properties of transdermal histamine iontophoresis: differential effects of season, gender, and body region. J. Invest. Dermatol. 94, 347–352.

Matsuda, H., Watanabe, N., Geba, G.P., Sperl, J., Tsudzuki, M., Hiroi, J., Matsumoto, M., Ushio, H., Saito, S., Askenase, P.W., Ra, C., 1997. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int. Immunol. 9, 461–466.

Matsuoka, H., Maki, N., Yoshida, S., Arai, M., Wang, J., Oikawa, Y., Ikeda, T., Hirota, N., Nakagawa, H., Ishii, A., 2003. A mouse model of the atopic eczema/dermatitis syndrome by repeated application of a crude extract of house-dust mite *Dermatophagoides farinae*. Allergy 58, 139–145.

Okayama, Y., Benyon, R.C., Lowman, M.A., Church, M.K., 1994. In vitro effects of H1-antihistamines on histamine and PGD2 release from mast cells of human lung, tonsil, and skin. Allergy 49, 246–253.

Reitamo, S., Wollenberg, A., Schöpf, E., Perrot, J.L., Marks, R., Ruzicka, T., Christophers, E., Kapp, A., Lahfa, M., Rubins, A., Jablonska, S., Rustin, M., 2000. Safety and efficacy of 1 year of tacrolimus ointment monotherapy in adults with atopic dermatitis. The European Tacrolimus Ointment Study Group. Arch. Dermatol. 136, 999–1006.

Rukwied, R., Lischetzki, G., McGlone, F., Heyer, G., Schmelz, M., 2000. Mast cell mediators other than histamine induce pruritus in atopic dermatitis patients: a dermal microdialysis study. Br. J. Dermatol. 142, 1114–1120.

Sasakawa, T., Higashi, Y., Sakuma, S., Hirayama, Y., Sasakawa, Y., Ohkubo, Y., Goto, T., Matsumoto, M., Matsuda, H., 2001. Atopic dermatitis-like skin lesions induced by topical application of mite antigens in NC/Nga mice. Int. Arch. Allergy Immunol. 126, 239–247.

Sengoku, T., Kishi, S., Sakuma, S., Ohkubo, Y., Goto, T., 2000. FK506 inhibition of histamine release and cytokine production by mast cells and basophils. Int. J. Immunopharmacol. 22, 189–201.

Simone, D.A., Alreja, M., LaMotte, R.H., 1991. Psychophysical studies of the itch sensation and itchy skin ("alloknesis") produced by intracutaneous injection of histamine. Somatosens. Motor Res. 8, 271–279.

Steinhoff, M., Vergnolle, N., Young, S.H., Tognetto, M., Amadesi, S., Ennes, H.S., Trevisani, M., Hollenberg, M.D., Wallace, J.L., Caughey, G.H., Mitchell, S.E., Williams, L.M., Geppetti, P., Mayer, E.A., Bunnett, N.W., 2000. Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. Nat. Med. 6, 151–158.

Steinhoff, M., Neisius, U., Ikoma, A., Fartasch, M., Heyer, G., Skov, P.S., Luger, T.A., Schmelz, M., 2003. Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. J. Neurosci. 23, 6176–6180.

Sugimoto, Y., Umakoshi, K., Nojiri, N., Kamei, C., 1998. Effects of histamine H1 receptor antagonists on compound 48/80-induced scratching behavior in mice. Eur. J. Pharmacol. 351, 1–5.

Sugiura, H., Omoto, M., Hirota, Y., Danno, K., Uehara, M., 1997. Density and fine structure of peripheral nerves in various skin lesions of atopic dermatitis. Arch. Dermatol. Res. 289, 125–131.

Takagi, M., Nakahata, T., Kubo, T., Shiohara, M., Koike, K., Miyajima, A., Arai, K., Nishikawa, S., Zsebo, K.M., Komiyama, A., 1992. Stimulation of mouse connective tissue-type mast cells by hemopoietic stem cell factor, a ligand for the c-kit receptor. J. Immunol. 148, 3446–3453.

Tobin, D., Nabarro, G., Baart de la Faille, H., van Vloten, W.A., van der Putte, S.C., Schuurman, H.J., 1992. Increased number of immunoreactive nerve fibers in atopic dermatitis. J. Allergy Clin. Immunol. 90, 613–622.

Tohda, C., Yamaguchi, T., Kuraishi, Y., 1997. Intracisternal injection of opioids induces itch-associated response through mu-opioid receptors in mice. Jpn. J. Pharmacol. 74, 77–82.

Toyoda, M., Nakamura, M., Makino, T., Hino, T., Kagoura, M., Morohashi, M., 2002. Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. Br. J. Dermatol. 147, 71–79.

Tsuji, K., Nakahata, T., Takagi, M., Kobayashi, T., Ishiguro, A., Kikuchi, T., Naganuma, K., Koike, K., Miyajima, A., Arai, K., 1990. Effects of interleukin-3 and interleukin-4 on the development of "connective tissue-type" mast cells: interleukin-3 supports their survival and interleukin-4 triggers and supports their proliferation synergistically with interleukin-3. Blood 75, 421–427.

Tsuji, K., Koike, K., Komiyama, A., Miyajima, A., Arai, K., Nakahata, T., 1995. Synergistic action of interleukin-10 (IL-10) with IL-3, IL-4 and stem cell factor on colony formation from murine mast cells in culture. Int. J. Hematol. 61, 51–60.

Ui, H., Andoh, T., Lee, J.B., Nojima, H., Kuraishi, Y., 2006. Potent pruritogenic action of tryptase mediated by PAR-2 receptor and its involvement in anti-pruritic effect of nafamostat mesilate in mice. Eur. J. Pharmacol. 530, 172–178.

Urashima, R., Mihara, M., 1998. Cutaneous nerves in atopic dermatitis. A histological, immunohistochemical and electron microscopic study. Virchows Arch. 432, 363–370.

Vestergaard, C., Yoneyama, H., Murai, M., Nakamura, K., Tamaki, K., Terashima, Y., Imai, T., Yoshie, O., Irimura, T., Mizutani, H., Matsushima, K., 1999. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. J. Clin. Invest. 104, 1097–1105.

Wahlgren, C.F., 1999. Itch and atopic dermatitis: an overview. J. Dermatol. 26, 770–779.

Wershil, B.K., Murakami, T., Galli, S.J., 1988. Mast cell-dependent amplification of an immunologically nonspecific inflammatory response. Mast cells are required for the full expression of cutaneous acute inflammation induced by phorbol 12-myristate 13-acetate. J. Immunol. 140, 2356–2360.

Yamaguchi, T., Kitagawa, K., Kuraishi, Y., 1998. Itch-associated response and antinociception induced by intracisternal endomorphins in mice. Jpn. J. Pharmacol. 78, 337–343.

Yamaguchi, T., Nagasawa, T., Satoh, M., Kuraishi, Y., 1999. Itch-associated response induced by intradermal serotonin through 5-HT2 receptors in mice. Neurosci. Res. 35, 77–83.

Yamashita, H., Michibata, Y., Mizukami, H., Ogihara, Y., Morita, A., Nose, M., 2005. Dermal mast cells play a central role in the incidence of scratching behavior in mice induced by multiple application of the hapten, 2,4,6-trinitrochlorobenzene. Exp. Dermatol. 14, 438–444.

Yano, H., Wershil, B.K., Arizono, N., Galli, S.J., 1989. Substance P-induced augmentation of cutaneous vascular permeability and granulocyte infiltration in mice is mast cell dependent. J. Clin. Invest. 84, 1276–1286.