INTRODUCTION

Atopic dermatitis (AD) is one of the most common chronic inflammatory and pruritic skin disorders, marked by alternating periods of relapse and remission (Rudikoff and Lebwohl, 1998; Barnetson and Rogers, 2002). In the field of dermatology, glucocorticoids are the most widely used therapy for AD. Topical, as well as systemic, glucocorticoid therapy can induce numerous cutaneous side effects. The potency, and in particular the duration of the therapy determine the occurrence and severity of these effects. The most important cutaneous side effects of glucocorticoid treatment are skin atrophy and disturbance of wound healing (Schäcke et al., 2002).

Itching can often occur as a rebound phenomenon after withdrawal of glucocorticoids, leading to their reinstatement and chronic use, as the symptoms are always exacerbated after withdrawal. Often, scratching can intensify the itching and even cause further damage to the skin, a phenomenon dubbed the "itch-scratch cycle" (Pfenninger and Zainea, 2001). For these reasons it is important to investigate the mechanism of itching after withdrawal of glucocorticoid treatment, and to control it, although the underlying factors responsible still remain unclear. For studies in this field, it is important to select an appropriate animal model, and some such models have been reported to mimic the exacerbation of dermal inflammation, known as the rebound phenomenon, following withdrawal of long-term glucocorticoid therapy in AD patients (Inoue et al., 2003; Tamura et al., 2005).

A recent study has suggested that glucocorticoids may exacerbate irritant chemical-triggered scratching...
through an increase in nerve growth factor (NGF) levels and nerve fiber density at the application site (Fujii et al., 2010). On the other hand, no previous study has investigated the effect of glucocorticoids on the augmentation of pruritus in AD models. AD, exacerbated by chronic exposure to antigens, is a common and distinctive form of allergic skin disease associated with eczematous skin lesions, characterized by a predominance of T-helper cell type (Th) 2 in lesional skin and elevated serum levels of IgE in response to allergens (Cooper, 1994; Ohmen et al., 1995). AD is a chronic dermatosis bearing clinical, histological and immunological similarities to chronic allergic contact dermatitis, and multiple hapten challenges to normal murine skin produce a chronic Th2-like hypersensitivity reaction with multiple features of human AD (Wang et al., 2007; Man et al., 2008).

Our aim in the present study was to create a novel animal model of glucocorticoid-induced pruritus in mice with chronic allergic contact dermatitis, with the expectation that the data would help to elucidate the mechanism of itching caused by long-term glucocorticoid treatment and contribute to safer and more effective usage of topical glucocorticoids.

MATERIALS AND METHODS

Animals
All experiments and procedures were approved by the Chiba University Institutional Animal Care and Use Committee. Female BALB/c mice, 6 weeks of age, were obtained from Japan SLC Inc. (Shizuoka, Japan), and housed under a controlled light (07:00-19:00 hr) and temperature (24°C) regime with food and water available ad libitum. Hair on the abdominal area was clipped at least 1 day before the experiment.

Reagents
Dexamethasone was obtained from Sigma Chemical (St. Louis, MO, USA) and 2, 4, 6-trinitro-1-chlorobenzene (TNCB) was obtained from Tokyo Chemical (Tokyo, Japan). Dexamethasone and TNCB were dissolved in acetone at 0.03% and 1.0% (w/v), respectively.

TNCB-induced chronic allergic contact dermatitis
The experimental protocols are illustrated in Fig. 1. The hair on the abdominal region of the mice was shaved with a hair clipper 1 day before sensitization. On day -7, these mice were sensitized by a single epicutaneous application of 100 μl of TNCB solution to the shaved abdomen, and on day 0 challenged with 10 μl/ear of TNCB solution. TNCB was then applied repeatedly to each ear three times a week until day 46. For assessment of the influence of glucocorticoids, dexamethasone was applied topically every day from day 14 to 46 at a volume of 10 μl/ear to both sides 30 min before TNCB solution. For withdrawal of dexamethasone (WD), application of dexamethasone was terminated at day 34 and acetone was applied instead from day 35. For non-application of...
TNCB (Nil) and non-application of dexamethasone (Nil and vehicle), acetone alone was applied to both ears.

**Measurement of ear thickness**

Right and left ear thickness was measured with a micrometer (Mitsutoyo, Kanagawa, Japan) under light ether anesthesia, 24 hr after each challenge. Ear swelling was calculated by averaging the thickness values for both ears.

**Pruritus measurement**

The number of bouts of scratching behavior was counted for 2 hr immediately after TNCB challenge on days 11, 32, 37 and 46. Pruritus was evaluated by automatic counting of the scratching bouts using MicroAct (Neuroscience Inc., Tokyo, Japan), as reported previously (Inagaki et al., 2003).

**Expression of cytokine mRNA in skin**

After evaluation of pruritus on day 46, the ears of the mice were sampled. Each specimen was homogenized, and the total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was prepared from the RNA by reverse transcription using a PrimeScript RT reagent Kit (TAKARA Bio INC., Shiga, Japan). Real-time quantitative PCR was performed on a Step One TM Real Time PCR System (Applied Biosystems Inc., Carlsbad, CA, USA) using SYBR Premix Ex Taq for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mouse interleukin (IL)-4, mouse interferon (IFN)-γ and mouse nerve growth factor (NGF) in accordance with the manufacturer’s instructions (TAKARA Bio INC.). Results are expressed as the relative mRNA level corrected with reference to GAPDH mRNA as an internal control.

**Measurement of serum IgE**

Blood was collected by retro-orbital bleeding on day 46. The serum was then obtained by centrifugation at 1,000 x g for 20 min at 4°C and stored at -30°C until use. Serum IgE levels were determined using a commercial sandwich ELISA assay (Bethyl Laboratories Inc., Montgomery, TX, USA).

**Statistical analysis**

All data are presented as mean ± S.E.M. of 7 mice. Statistical significance was analyzed using Dunnett’s method for multiple comparisons or Student’s t test. Differences at p < 0.05 were considered statistically significant. All statistical analyses were conducted using StatLight software (Yukms Co., Ltd., Tokyo, Japan).

### RESULTS

#### Effects of topical application of dexamethasone on ear thickness

Long-term repeated exposure of mouse ears to TNCB induced progressive and chronic allergic contact dermatitis characterized by skin swelling, which reached a plateau on day 10. Topical dexamethasone, started on day 14, significantly inhibited the increase in ear thickness from day 15 and throughout the experimental period (Fig. 2). The inhibitory effect of dexamethasone changed gradually towards exacerbation after withdrawal of dexamethasone on day 34 (Fig. 2).

#### Effects of topical application of dexamethasone on scratching behavior

Repeated challenge with TNCB evoked an increase in the number of scratching bouts. On day 11, the number of scratching bouts was increased to 50.7 in TNCB-treated mice, compared with 22.3 in acetone-treated mice (Nil). Repeated topical application of dexamethasone, starting from day 14, resulted in an increase in the number of scratching bouts throughout the experimental period, and the count reached 220 on day 46 (Fig. 3). On the other hand, the increased in the number of scratching bouts induced by topical dexamethasone was reduced to the level observed...
in vehicle-treated mice after discontinuation of the treatment on day 34.

**Effects on the expression of mRNA for Th1 and Th2 cytokines in mouse ears**

To evaluate the relative dominance of Th1 or Th2 cytokines in the lesional ear after repeated application of TNCB, and to elucidate the effect of dexamethasone, we analyzed the expression of mRNA for IL-4 and IFN-γ using a quantitative PCR method. The expression of IL-4 mRNA detected in ears taken 3 hr after the last challenge was significantly (55 times) higher than that in acetone-treated ears. On the other hand, the expression of IL-4 mRNA, which had been decreased by 66% in dexamethasone-treated ears, was further increased after discontinuation of topical dexamethasone. The levels of IFN-γ mRNA were increased by repeated challenge with TNCB, but the extent of the increase was only about half that seen for IL-4 mRNA. In addition, neither topical dexamethasone nor its withdrawal affected the increase in the levels of IFN-γ mRNA (Fig. 4).

**Effects on serum IgE levels**

The total serum IgE level was significantly increased by repeated challenge with TNCB. Dexamethasone significantly ameliorated this increase. On the other hand, the decrease in the IgE level induced by dexamethasone was reversed after its discontinuation (Fig. 5).

**Effects on the expression of mRNA for neural factor in mouse ears**

To clarify the mechanism by which dexamethasone exacerbates scratching behavior, we investigated its effect on the levels of NGF mRNA in lesional skin. The level of NGF in lesioned ears taken at day 46 was slightly decreased in comparison with that in acetone-treated ears. Topical dexamethasone enhanced the level of NGF mRNA 2.3-fold relative to the vehicle group. Furthermore, the level of NGF mRNA was significantly increased in the dexamethasone withdrawal group (Fig. 6).

**DISCUSSION**

Single application of a contact sensitizing agent such as TNCB is able to induce a contact hypersen-
sitivity response, as is seen typically in delayed-type hypersensitivity, in animals that have been previously sensitized with the same agent (Gray, 1993). Repeated elicitation with such agents not only increases the serum level of IgE but also induces a shift in the cutaneous cytokine milieu from a Th1 to a Th2 profile (Kitagaki et al., 1997), followed by the development of chronic allergic contact dermatitis that is similar to AD clinically, histologically and immunologically. A recent in vivo study has shown that 1 week of glucocorticoid challenge increased the scratching triggered by an irritant chemical (Fujii et al., 2010). However, the influence of glucocorticoid has not been investigated using models of chronic allergic contact dermatitis. We therefore investigated the effects of long-term topical application of dexamethasone in a mouse model of chronic allergic contact dermatitis induced by repeated challenge (Kitagaki et al., 1995).

In the present study, to examine the role of glucocorticoids in the pruritic response of AD patients, we created a novel animal model of pruritus by repeated topical application of dexamethasone to mice with TNCB-induced dermatitis.

Repeated application of TNCB induced a chronic allergic contact dermatitis characterized by significant skin swelling throughout the experimental period. Topical application of 0.03% dexamethasone (0.003 mg/ear/day) significantly inhibited the increase in ear thickness during the treatment period. It has been reported that long-term topical, as well as systemic, glucocorticoids can induce numerous side effects. In our study, the well known side effects of glucocorticoids, such as reduction of body weight and atrophy of the spleen and thymus, were also observed (data not shown). The concentration of dexamethasone used in this study was moderate to low relative to that used clinically. The serum concentration of corticosterone was markedly decreased after the dexamethasone treatment, but the concentration recovered after withdrawal of the dexamethasone (data not shown).

Repeated topical application of TNCB caused an increase in the number of scratching bouts, accompanied by skin lesions, being twice as frequent as that in non-treated mice. Topical dexamethasone increased the number of scratching bouts in time-dependent manner, but after its withdrawal, a significant reduction of scratching was observed on day 46 (Fig. 3). It was therefore considered that the enhancement of scratching behavior in this animal model was caused by the topical dexamethasone. Withdrawal of the dexamethasone caused relapse of the dermatitis, but relieved the pruritus. This observation suggests...
that some kind of response accompanying the dermatitis, for example the production of prostaglandin D_{2} from activated mast cells, might have contributed to the reduction of pruritus. The number of scratching bouts after dexamethasone withdrawal was the same as that in mice that continued to receive dexamethasone on day 37, which was 2 days after dexamethasone withdrawal. This might have been due to the fact that the half-life of topical dexamethasone is 3 days.

An obvious increase of IL-4 mRNA, suggesting a response of the Th2 type, was observed upon application of TNCB to mouse ears, being consistent with other reports (Kitagaki et al., 1997). Topical dexamethasone suppressed the increase of IL-4 mRNA, and this suppression was well correlated with the effect on ear thickness. On the other hand, withdrawal of dexamethasone significantly enhanced the level of IL-4 mRNA. Upon withdrawal of topical dexamethasone, the mice might have been at a stage of exacerbation of ear dermatitis toward the level seen in the TNCB control, and this might have been associated with a marked shift in the cytokine milieu towards a Th2 profile. In addition, the ear thickness exceeded that in the TNCB control after continuous observation for a long period (data not shown), in agreement with previous reports (Inoue et al., 2003; Tamura et al., 2005).

On the other hand, neither topical dexamethasone nor its withdrawal affected the level of mRNA for IFN-γ, which is a Th1 cytokine. These results suggest that the Th1 type response is unlikely to be involved in the pruritic response induced by topical dexamethasone.

Topical dexamethasone attenuated the increase in the total serum IgE level caused by repeated application of TNCB, contrary to exacerbation of the scratching count, suggesting that the level of IgE is unlikely to be associated with the pruritic response induced by topical dexamethasone. On the other hand, the high correlation between the serum IgE level and ear swelling indicates that IgE might be involved in the inflammatory response (Matsuda et al., 1997; Nagai et al., 1997).

The level of mRNA for NGF was increased by topical application of dexamethasone to the ear, and remained high even after its discontinuation. It has been reported that an enhancement of the NGF level is associated with an increase in the scratching count in animals receiving topical application of glucocorticoids (Fuji et al., 2010). In view of the fact that the pruritic response was reduced by withdrawal of topical dexamethasone without any change in the NGF level, some form of system for inhibition of the pruritic response may exist in the skin, and this system may be suppressed by topical application of dexamethasone. Further study will be needed to investigate this possibility.

In the present study, we created a novel animal model of glucocorticoid-induced pruritus by long-term topical application of dexamethasone to mice with contact dermatitis. This animal model will be useful for investigating the mechanism of pruritus induced by long-term application of topical glucocorticoids in clinical use, and for the screening of novel compounds that could be more effective than histamine H_{1} receptor antagonist for preventing the rebound phenomena following withdrawal of topical glucocorticoids.

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REFERENCES


Novel pruritus model induced by topical glucocorticoid

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