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Responsiveness of C Neurons in Rat Dorsal Root Ganglion to 5-Hydroxytryptamine-Induced Pruritic Stimuli In Vivo

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Hachisuka J, Furue H, Furue M, Yoshimura M. Responsiveness of C neurons in rat dorsal root ganglion to 5-hydroxytryptamine-induced pruritic stimuli in vivo. *J Neurophysiol* 104: 271–279, 2010. First published May 19, 2010; doi:10.1152/jn.00938.2009. Itching is a common symptom in dermatologic diseases and causes restless scratching of the skin, which aggravates the condition. The mechanism of the itch sensation, however, is enigmatic. The present study included behavioral tests and electrophysiological recordings from rat dorsal root ganglion (DRG) neurons in vivo to analyze the response to pruritic stimuli induced by topical application of 5-hydroxytryptamine (5-HT) to the skin. Topically applied 5-HT to the rostral back evoked scratching, whereas application of the vehicle did not. Following subcutaneous injection of the opioid receptor antagonist naloxone, the number of scratches decreased, suggesting that the scratching was preferentially mediated by itch but not pain sensation. To elucidate the firing properties of DRG neurons in response to topically applied 5-HT, intracellular recordings were made from DRG neurons in vivo. None of the $A\beta$ and $A\delta$ neurons responded to 5-HT; in contrast, 25 of 91 C neurons (27%) exhibited repetitive firing in response to 5-HT, which could be classified into two firing patterns: one was a transient type, characterized by low firing frequency that decreased within 5 min; the other was a long-lasting type, having high firing frequency that continued increasing after 5 min. The time course of the firing pattern of long-lasting C neurons was comparable to the scratching behavior. Intriguingly, the long-lasting-type neurons had a significantly smaller fast afterhyperpolarization than that of the 5-HT-insensitive neurons. These observations suggest that the long-lasting-firing C neurons in rat DRG sensitive to 5-HT are responsible for conveying pruritic information to the spinal cord.

INTRODUCTION

Itching is an unpleasant sensation that can occasionally degrade the quality of life. There are some similarities between itching and pain; both are unpleasant sensations and most pruritogens, such as histamine, can also produce pain (Dray 1995; Schmelz et al. 2003). The behavioral response in rats or mice to these two types of stimuli, however, has some differences. Itch stimuli evoke scratching or biting, whereas noxious stimuli evoke a withdrawal reflex, flinching, or licking (Kuraishi et al. 1995, 2008; Nojima et al. 2004). Opioids, which are commonly used for alleviation of pain, elicit itching (Hales 1980; Jeon et al. 2005; Maxwell et al. 2005; Slappendel et al. 2000); conversely, the opioid receptor antagonist naloxone enhances pain but inhibits the itch sensation (Metze et al. 1999; Robertson et al. 2008).

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To elucidate the characteristics of itch-sensing primary afferents, electrophysiological recordings of these afferents have been made. Microneurography reveals that histamine applied to human skin elicits low-frequency activity in polymodal C fibers (Handwerker et al. 1991; Torebjörk 1974; Van Hees and Gybels 1972). Tuckett and Wei recorded the activity of A and C fibers in cats and found that only polymodal C fibers were activated by the application of cowhage, which is pruritic in humans (Tuckett and Wei 1987a,b). Polymodal C fiber that responds to cowhage is also reported in monkeys (Johanek et al. 2008) and humans (Namer et al. 2008). Histamine-sensitive polymodal C fibers are also responsive to mustard oil, which is known to activate TRPA1, and there is no significant difference in the discharge patterns caused by itching or burning stimuli (Handwerker et al. 1991). A new subtype of C fibers that is insensitive to mechanical stimuli was reported to respond to itch stimuli (Schmelz et al. 1997). However, these fibers also respond to algogens such as capsaicin or bradykinin (Schmelz et al. 2003).

Several chemicals have been used experimentally to elicit the itch sensation. However, the itch-inducing potency of these chemicals differs between species. Histamine, for instance, is a well-known pruritogen in humans. It evokes scratching in ICR mice, hairless guinea pig, and monkeys (Inagaki et al. 2001; Johanek et al. 2008; Woodward et al. 1995), but it is generally less effective in ddY mice and Sprague–Dawley rats (Jinks and Carstens 2002; Kuraishi et al. 1995). Instead of histamine, 5-hydroxytryptamine (5-HT) is often used in mice and rat itch models (Inagaki et al. 2001; Nojima and Carstens 2003a,b; Nojima et al. 2003; Thomsen et al. 2001; Yamaguchi et al. 1999). Interestingly, 5-HT is only weakly pruritic for humans (Schmelz et al. 2003). 5-HT is released from aggregated platelets and mast cells in rats (Weisshaar et al. 1997). 5-HT produces itching when applied to the human skin (Weisshaar et al. 1997) and is considered to be a cause of pruritus accompanied by polycythemia vera (Fitzsimons et al. 1981). In rats, intradermal injection of 5-HT into the rostral back evokes a scratch response and this behavior is depressed by subcutaneous injection of the opioid receptor antagonist naltrexone (Nojima and Carstens 2003a; Nojima et al. 2003). These observations indicate that 5-HT is a potent pruritogen in rats and suitable for clarifying the properties of afferent fibers conveying the itch sensation to the spinal cord.

In the present study, we intended to answer the following questions. *1*) What types of primary afferents are responsible for the reaction to topically applied 5-HT on a receptive field? and *2*) What is the characteristic feature of itch-sensing neurons? To address these questions, we made intracellular recordings from rat dorsal root ganglion (DRG) neurons in vivo and

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analyzed the response to itch stimulation induced by topical application of 5-HT to the skin.

METHODS

All experimental procedures involving the use of animals were approved by the Committee on the Ethics of Animal Experiments, Kyushu University, and were in accordance with the Guidelines of the Japanese Physiological Society. All efforts were made to minimize animal suffering and the number of animals used for the studies. At the end of the experiments, the rats were given an overdose of urethane and then exsanguinated.

Behavioral experiments

In all, 43 male Sprague–Dawley rats (aged 6–9 wk) were used. They were housed under controlled temperature and light. Food and water were freely available. The hair of the rostral back, right hindlimb, thigh, and hip was clipped one day before the behavioral experiments. Before the experiments, the animals were placed in an acrylic cage for about 1 h for acclimation. Behavioral changes were recorded by digital video camera for 1 h. On the clipped rostral back, 5-HT (1% [47 mM] in 99% ethanol, 50 μl) and the vehicle (99% ethanol, 50 μl) were applied on the target skin by using a micropipette instead of being injected intradermally, to avoid eliciting pain by inserting a needle and expanding the skin. The number of scratches to the application site by the hindpaw was counted. We used ethanol as the vehicle. Ethanol can activate TRPV1 and is painful on a skin wound. However, we did not believe it would be painful in this study for two reasons. One is that topical application of ethanol is often used in studies on humans and rarely evokes pain (Hatem et al. 2006; Namer et al. 2008; Wasner et al. 2004) and the other is that the amount of penetration of topically applied ethanol to the skin is small (Pendlington et al. 2001). We also applied 1% 5-HT (50 μl) to the right hindpaw and observed biting behavior since the electrophysiological experiments were performed from L4 to L6, mainly L5 DRG neurons, which innervate the hindpaw, thigh, and hip. In mouse itch models, biting behavior was elicited by 5-HT injection or under the condition of chronic dermatitis or dry skin (Kuraishi et al. 2008; Maekawa et al. 2002; Nojima et al. 2004). In contrast, a formalin application, which is known to initiate pain behavior, causes licking in mice (Abbott et al. 1995; Hunskaar et al. 1985; Tjølsen et al. 1992). Thus the biting behavior is considered as itch-related behavior. In some experiments, rats were given a subcutaneous injection of naloxone (1 $mg \cdot kg^{-1}$) or saline at a volume of 1 $ml \cdot kg^{-1}$ into the back skin 15 min before the topical application of 5-HT.

Electrophysiological recordings

In all, 88 rats were used for the electrophysiological experiments. After anesthesia of the rats with urethane (1.2 $g \cdot kg^{-1}$, administered intraperitoneally), laminectomy was performed at the lumbar level and the right DRG (L4–L6, mainly L5) was carefully exposed with a rongeur. Animals were fixed rigidly in a stereotaxic apparatus and then the skin flaps were stretched by nylon fibers to make a pool for perfusion of DRG. Connective tissues covering the surface of the DRG were removed using fine forceps and the exposed DRG was perfused with Krebs solution (in mM: NaCl, 117; KCl, 3.6; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; glucose, 11; and $NaHCO_3$, 25) equilibrated with 95% O_2-5% CO_2 at 37 ± 1°C. In vivo intracellular recordings were made using 1.5-mm thick-walled, borosilicate glass pipettes filled with a solution containing 4 M potassium acetate or 0.1 M potassium chloride. Tip resistance was 30–60 MΩ with 4 M potassium acetate and 80–150 MΩ with 0.1 M potassium chloride. Microelectrodes with shank and tip that were relatively shorter and larger, respectively, than those for conventional intracellular recordings were used. Although the input resistance of these electrodes was much higher than that of the electrodes containing 3 M potassium chloride, 0.1 M potassium chloride was more suitable for obtaining stable recordings from small C neurons. Signals were amplified using an Axoclamp 2B (Axon Instruments/Molecular Devices, Sunnyvale, CA) in bridge mode. Analogue data were digitized by a Digidata 1440A (Axon Instruments/Molecular Devices) and analyzed by pCLAMP 10 software (Axon Instruments/Molecular Devices). We determined the receptive fields by applying nonnoxious mechanical stimuli with a paint brush or noxious stimuli with toothed forceps. If the neurons were insensitive to either nonnoxious or noxious stimuli, then electrical stimulation with a monopolar electrode was applied for further confirmation of the receptive fields of recorded neurons. If a receptive field was not found, the neurons were discarded. When we recorded more than two neurons from the same animal, we made sure that each neuron had different receptive fields. DRG neurons were classified into three types according to the conduction velocity of the corresponding fiber: C fibers <2.0 ms^{-1}, Aδ fibers 2.0–10 ms^{-1}, and Aβ fibers >10 ms^{-1}, referring to published reports (Lawson and Waddell 1991; McCarthy and Lawson 1989; Pinto et al. 2008; Villière and McLachlan 1996). Conduction velocity was estimated by the length from the stimulating point to the DRG and the latency between onset of electrical stimulation and orthodromic action potential. In some neurons, the mechanical threshold was determined by von Frey filaments. Subsequently, we gently applied the vehicle (ethanol 20 μl) and then 5-HT (serotonin hydrochloride 20 μl; Sigma) on the receptive field by using a micropipette. We observed the activity of the neurons ≥2 min after applying the drugs to evaluate whether they were responsive to the drugs. Capsaicin (0.05% in ethanol 20 μl; Wako) was applied to the receptive fields of eight 5-HT-insensitive neurons. We did not test the effect of capsaicin on 5-HT-sensitive neurons, since we could not exclude the possibility that previously applied 5-HT would redissolve into the capsaicin solution and activate the 5-HT receptors. C neurons that had membrane potential more positive than −50 mV were excluded from further analysis; however, the firing response of those neurons to 5-HT application or mechanical stimuli was included in the present study due to the difficulty in obtaining recordings from C neurons. Neurons showing spontaneous firing without 5-HT or mechanical stimuli were discarded.

Statistical analysis

Total scratching and biting was evaluated by unpaired *t*-test. Electrophysiological data were evaluated by Mann–Whitney *U* test or Kruskal–Wallis *H* test. $P < 0.05$ was considered significant.

RESULTS

Scratching and biting behavior in response to topically applied 5-HT

Topically applied 5-HT to the rostral back evoked scratching behavior, whereas the vehicle did not (Fig. 1, *A* and *B*; $n = 8$ in each group). The scratching began within 5 min after application of 5-HT and reached a peak within 10 min; the scratching frequency reached 30 scratches per 5 min and then gradually decreased. The scratching behavior lasted for >40 min. There was a significant delay in the beginning of scratching after 5-HT application, probably due to the diffusion time for 5-HT to reach the nerve terminals. The mean total number of scratch bouts in 1 h induced by 5-HT was 197 ± 27 (mean ± SE, $n = 8$). Following subcutaneous injection of naloxone (1 $mg \cdot kg^{-1}$), the scratching was reduced to less than one third (Fig. 1*C*, vehicle 172 ± 38, $n = 8$; naloxone 51 ± 18, $n = 7$), which was statistically significant ($P < 0.01$), suggesting that the scratching is due to itch but not pain sensation.

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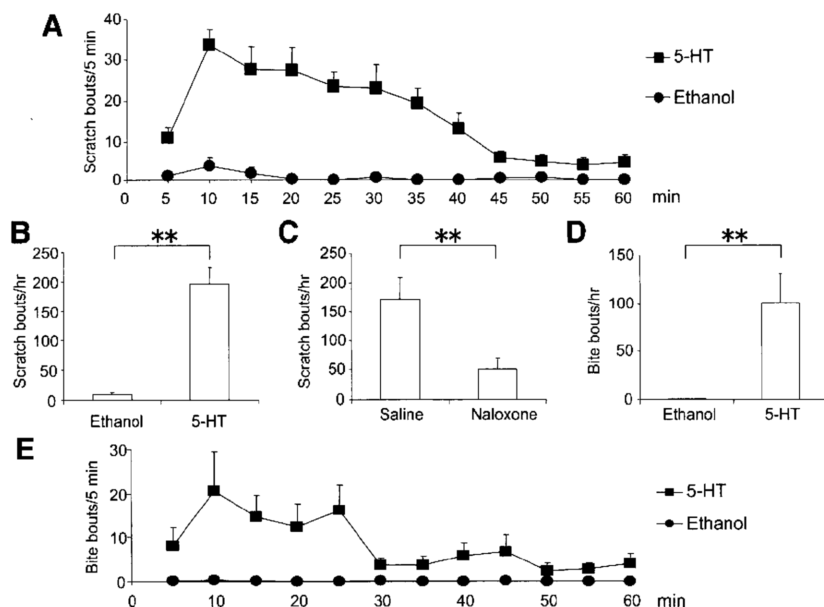

FIG. 1. Behavioral response to topically applied 5-hydroxytryptamine (5-HT, serotonin) and vehicle. *A*: time course of scratching induced by topically applied 1% 5-HT (square) or vehicle (ethanol: circle) to the skin of the rat rostral back. The number of scratching bouts was counted every 5 min (n = 8). *B*: total number of scratching bouts (n = 8 in each group). $^{**}P < 0.01$ (unpaired t-test). *C*: the effect of naloxone. Saline or naloxone (1 mg/kg) was injected subcutaneously 15 min before applying 1% 5-HT (n = 8 saline, n = 7 naloxone). $^{**}P < 0.01$ (unpaired t-test). *D*: total number of biting bouts induced by topically applied 1% 5-HT or vehicle (ethanol) to the rat hindpaw (n = 6 in each group). $^{**}P < 0.01$ (unpaired t-test). Data are presented as the mean and SE.

Before performing an intracellular recording analysis, behavioral changes induced by the topically applied 5-HT from the sole to the ankle of the right hindpaw were tested. Rats exhibited biting behavior to the 5-HT-applied region but not to the vehicle-applied region (Fig. 1*D*; n = 6 each group). The biting time course was similar to the scratching time course (Fig. 1*E*). These observations further indicate that 5-HT applied at the hindpaw, thigh, or hip also initiates itch sensation in rats.

Response of C neurons to topically applied 5-HT

Intracellular recordings were made from 29 neurons with Aβ, 25 neurons with Aδ, and 91 neurons with C-fiber in L5 DRG in vivo (Table 1). Receptive fields of the recorded neurons were identified by applying mechanical or electrical stimulation to the skin. Subsequently, ethanol (99%, 20 μl) or 5-HT (1%, 20 μl) was applied to the receptive field. Neither Aβ nor Aδ neurons responded to topical application of 5-HT (Table 1). Twenty-five of the 91 C neurons (27%) showed orthodromic firing in response to 5-HT application (Table 1). Vehicle application did not produce firings, except one Aδ neuron and one C neuron, which might be cool-sensitive neurons because they responded to the gentle application of ice.

TABLE 1 *Summary of the recorded neurons*

Factor	5-HT-Insensitive	5-HT-Sensitive: Transient	5-HT-Sensitive: Long-Lasting
A. A β neurons (n = 29)			
Touch	17 (59%)	0	0
Pinch	11 (38%)	0	0
Mechanically insensitive	1 (3%)	0	0
Total	29 (100%)	0	0
B. Aδ neurons (n = 25)			
Touch	3 (12%)	0	0
Pinch	19 (76%)	0	0
Mechanically insensitive	3 (12%)	0	0
Total	25 (100%)	0	0
C. C neurons (n = 91)			
Touch	0	1 (1%)	1 (1%)
Pinch	56 (62%)	12 (13%)	11 (12%)
Mechanically insensitive	10 (11%)	0	0
Total	66 (73%)	13 (14%)	12 (13%)

Figure 2 shows an example of a 5-HT-sensitive C neuron (Fig. 2, *A–D* were recorded from the same neuron). This neuron was also sensitive to touch (arrows) and pinch stimuli (Fig. 2, *A* and *B*). Continuous firing began 32 s after topical application of 5-HT (Fig. 2, *A* and *D*) and was recorded for ≤50 min (Fig. 2*D*). As noticed from the continuous recording (Fig. 2*A*), action potential was followed by just a small-amplitude afterhyperpolarization (AHP) (Fig. 2*C*). We classified these neurons as a long-lasting type. These neurons continued to fire for a long time and the peak firing frequency was observed >5 min after 5-HT application. Twelve of the 91 C neurons (13%) were considered to be the long-lasting type and were responsive to noxious stimuli, except one neuron that responded to nonnoxious stimuli (Table 1). We found another type of 5-HT-sensitive C neuron that responded to topically applied 5-HT; however, unlike the long-lasting type, the peak firings were found within 5 min and most of them stopped firing within a few minutes (Fig. 3*A*). These neurons were sensitive to pinch stimuli (Fig. 3*B*). We classified these neurons as a transient type and they were also responsive to mechanical stimuli, except for one neuron that responded to nonnoxious stimuli (Table 1). Thirteen C neurons (14%) were considered to be the transient type (Table 1). This type also had a small-amplitude AHP. Figure 4 shows an example trace of a 5-HT-insensitive C neuron. This type of neuron was sensitive to pinch stimuli (Fig. 4*B*). The 5-HT-insensitive C neurons (73%) were largely sensitive to noxious stimuli, but not to nonnoxious stimuli. A substantial number of 5-HT-insensitive C neurons (11%) were insensitive to mechanical stimuli. 5-HT-insensitive neurons exhibited a relatively large-amplitude AHP, as shown in Fig. 4*C*.

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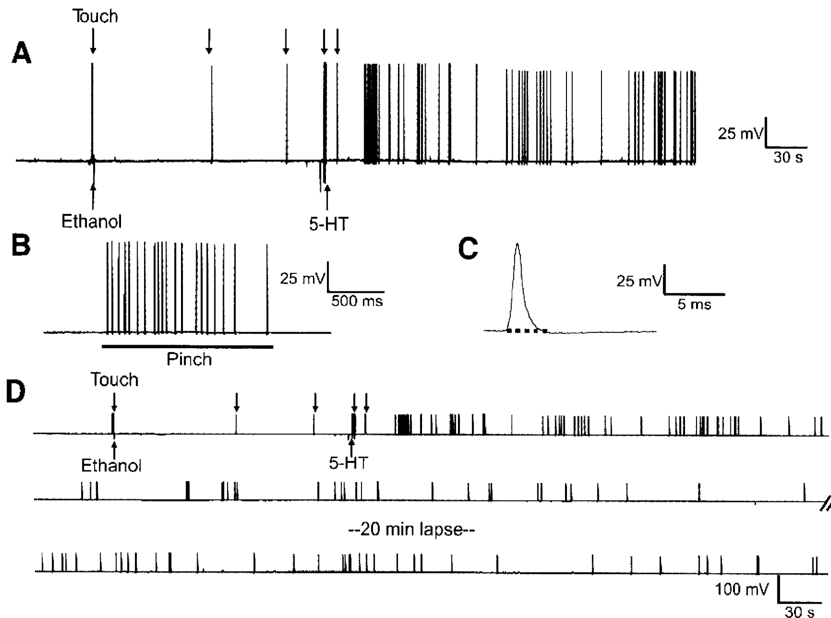

FIG. 2. Representative trace of 5-HT-sensitive C neuron (long-lasting type). *A*, *B*, *C*, and *D* were obtained from the same neuron. *A*: this neuron was sensitive to touch stimulation. Topical application of vehicle (ethanol) did not evoke action potential. After topical application of 1% 5-HT to the hindpaw, the neuron fired continuously. *B*: action potential to pinch stimuli. *C*: single action potential of long-lasting-type C neuron, which was followed by a small-amplitude afterhyperpolarization (AHP). *D*: continuous firing was recorded for ≤50 min. There was a 20-min time lapse between the 2nd and 3rd traces.

Firing properties of 5-HT-sensitive neurons in response to 5-HT application

Next, we compared the firing properties of long-lasting and transient-type 5-HT-sensitive C neurons. The long-lasting type showed a continuous increase in the number of firings 5 min after the application of 5-HT (Fig. 5*A*). On the other hand, the number of firings in the transient type reached a peak within 3 min (median 2.5 min, range 1–3 min, $n = 13$) and then decreased (Fig. 5*B*). The long-lasting type exhibited a significantly higher firing rate than that of the transient type in the first 5 min (long-lasting type: median 70 spikes/min, range 15–288 spikes/min, $n = 12$; transient type: median 18 spikes/min, range 3–70 spikes/min, $n = 13$; $P < 0.01$; Fig. 5*C*). Furthermore, the long-lasting-type neurons began to fire much earlier than the transient firing neurons (long-lasting type: median 26 s, range 12–67 s, $n = 12$; transient type: median 75 s, range 14–115 s, $n = 13$; $P < 0.05$; Fig. 5*D*).

Properties of action potential

The conduction velocity of 5-HT-insensitive and -sensitive C neurons was not significantly different. The median velocity was 0.6 ms^{-1} in 5-HT-insensitive neurons (range 0.3–1.9 ms^{-1}, $n = 66$), 0.6 ms^{-1} in transient-type neurons (range 0.5–1.5 ms^{-1}, $n = 13$), and 0.5 ms^{-1} in long-lasting-type neurons (range 0.5–0.7 ms^{-1}, $n = 12$). There were no significant differences in height or duration of action potential among 5-HT-insensitive, transient, and long-lasting types (height, 5-HT-insensitive: median 83.9 mV, range 55.7–104.5 mV, $n = 42$; transient: median 80.6 mV, range 67.8–93.9 mV, $n = 8$; long-lasting: median 75.0 mV, range 56.7–102.7 mV, $n = 11$; duration, 5-HT-insensitive: median 1.6 ms, range 0.9–3.5 ms, $n = 42$; transient: median 1.6 ms, range 1.3–2.8 ms, $n = 8$; long-lasting: median 2.4 ms, range 0.8–4.9 mV, $n = 11$). Interestingly, the long-lasting type had a significantly smaller fast AHP than that of the 5-HT-insensitive and transient types (Fig. 6, *A*, *B*, and *C*; 5-HT insensitive: median 7.8 mV, range 0–16.5 mV, $n = 42$; transient: median 3.0 mV, range 0–10.0 mV, $n = 8$; long-lasting: median 0 mV, range 0–5.3 mV, $n = 11$) and they were significantly different. The long-lasting-type neurons also showed no apparent slow AHP following the fast AHP, even though the action potential had a large Ca^{2+} component in the falling phase.

Application of algogenic agents to 5-HT-insensitive C neurons

To clarify whether 5-HT-insensitive C neurons were responsive to algogenic stimuli, we topically applied capsaicin

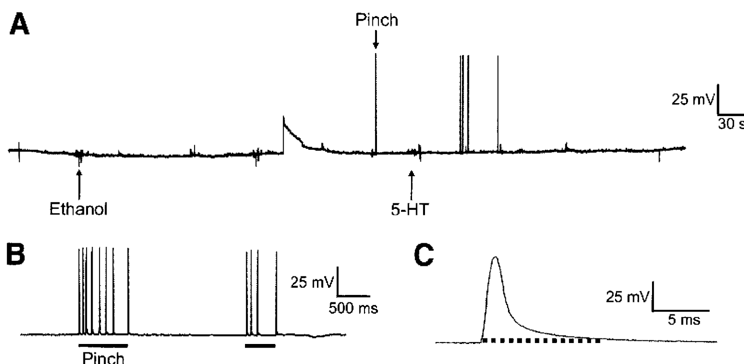

FIG. 3. Representative trace of 5-HT-sensitive C neuron (transient type). *A*, *B*, and *C* were obtained from the same neuron. *A*: topical application of vehicle (ethanol) did not evoke action potential. After topical application of 1% 5-HT, spikes were elicited, although the firing ceased within a few minutes. *B*: action potential initiated by pinch stimuli. *C*: single action potential of transient-type C neuron. This neuron showed afterdepolarization rather than AHP.

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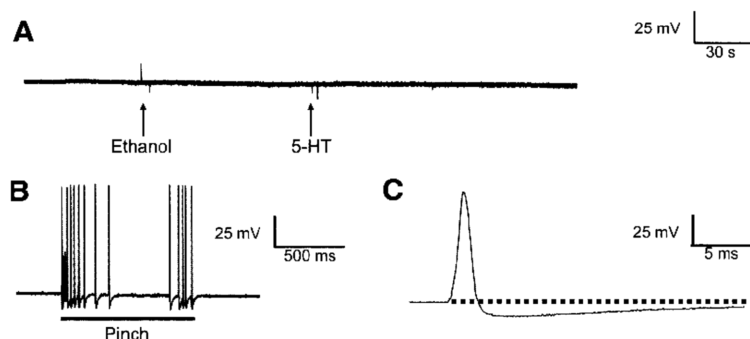

FIG. 4. Representative trace of 5-HT-insensitive C neuron. *A*, *B*, and *C* were obtained from the same neuron. *A*: neither vehicle (ethanol) nor 5-HT evoked action potential. *B*: action potential to pinch stimuli. *C*: single action potential of 5-HT-insensitive C neuron. The action potential was followed by a large-amplitude AHP.

(0.05% in ethanol) to the receptive fields (Fig. 7, *A* and *B*). Before capsaicin application, 5-HT was topically applied to test whether a neuron responded to 5-HT (Fig. 7*A*) and then noxious pinch stimulation was added. The neuron shown in Fig. 7*A* responded to pinch stimuli but not to 5-HT. In the same neuron, topical application of capsaicin elicited continuous firing lasting for >5 min with long latency (median 62 s, range 46–77 s, $n = 2$). Two of eight neurons were responsive to capsaicin.

Location of the receptive fields

Figure 8 shows the location of the receptive fields that we recorded. They were located mostly on the hindpaw and hip. The distribution of long-lasting C neurons, transient C neurons, and 5-HT-insensitive C neurons did not vary.

DISCUSSION

In the present study, intracellular recordings were made from C neurons in rat DRG in vivo to elucidate the firing properties of neurons in response to topically applied 5-HT to the skin, which elicited an itch-associated scratching response in rats. A small population of C neurons responded to the topical application of 5-HT and these 5-HT-responsive C neurons could be divided into two subtypes based on their firing duration, firing rate, and latency of the response. One type of C neurons showed long-lasting firing, higher firing frequency, and short latency. The firing duration was comparable to the behavioral changes induced by 5-HT topically applied to the hindlimb. The other type of C neurons exhibited transient firing that lasted for <5 min, low firing frequency, and long latency. These observations suggest that the long-lasting-type C neurons may express 5-HT receptors on the peripheral nerve endings and play an important role in carrying the itch sensation to the spinal cord in rats. The other possibility is that the shorter delay and longer duration of the 5-HT response could also be due to a higher density of 5-HT receptors.

Several chemicals have been reported to induce the itch sensation, including histamine, 5-HT (Nojima and Carstens 2003a,b; Thomsen et al. 2001; Yamaguchi et al. 1999), substance P (Andoh et al. 1998), trypsin (Ui et al. 2006), and prostaglandin E2 (Neisius et al. 2002). Histamine is the best-known itch mediator in humans, but not in rodents (Kuraishi et al. 1995), whereas 5-HT is a potent pruritogen in rats (Thomsen et al. 2001). In the majority of behavioral studies, 5-HT is administered by intradermal injection, whereas in the present study, topical application was used, which has the advantage of avoiding mechanical pain stimulation induced by intradermal injection. Similar to the results obtained in previous studies, the topical application of 5-HT evoked scratch behavior, which was reduced by prior application of naloxone, known to enhance the sensation of pain. In agreement with this finding, it is well known that intrathecal injection of an opioid initiates an itch sensation that can be blocked by naloxone (Hales 1980; Jeon et al. 2005; Maxwell et al. 2005; Slappendel et al. 2000). When 5-HT was injected into the hindpaw, rats bit the injected region instead of scratching, since it is difficult for them to scratch their hindpaw (Kuraishi et al. 2008). In the itch model that we used in the present study with topical application of 5-HT to the hindpaw, biting behavior was also observed. In contrast, an algogenic agent, such as formalin, applied to the

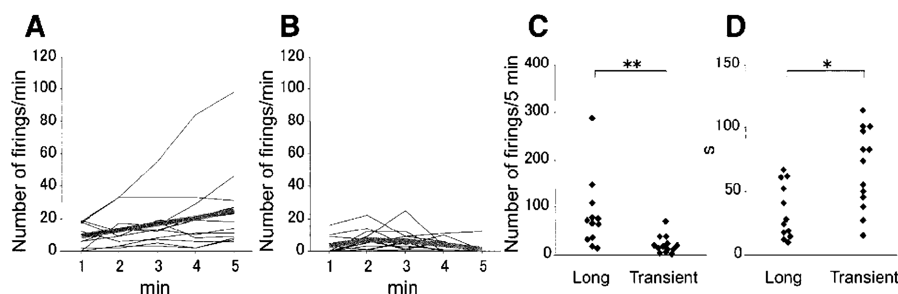

FIG. 5. Firing property induced by topically applied 5-HT. *A*: firing time course of long-lasting-type C neuron. The number of firings is plotted for every minute until 5 min ($n = 12$). The mean number of firings is shown as a gray line. *B*: firing time course of transient-type C neuron. The number of firings is plotted for every minute until 5 min ($n = 13$). The mean number of firings is shown as a gray line. *C*: total number of firings within 5 min of long-lasting-type neuron ($n = 12$) and transient-type neuron ($n = 13$). $**P < 0.01$ (Mann–Whitney U test). *D*: latency between application of 5-HT and the first evoked action potential (long-lasting: $n = 12$; transient: $n = 13$). $*P < 0.05$ (Mann–Whitney U test).

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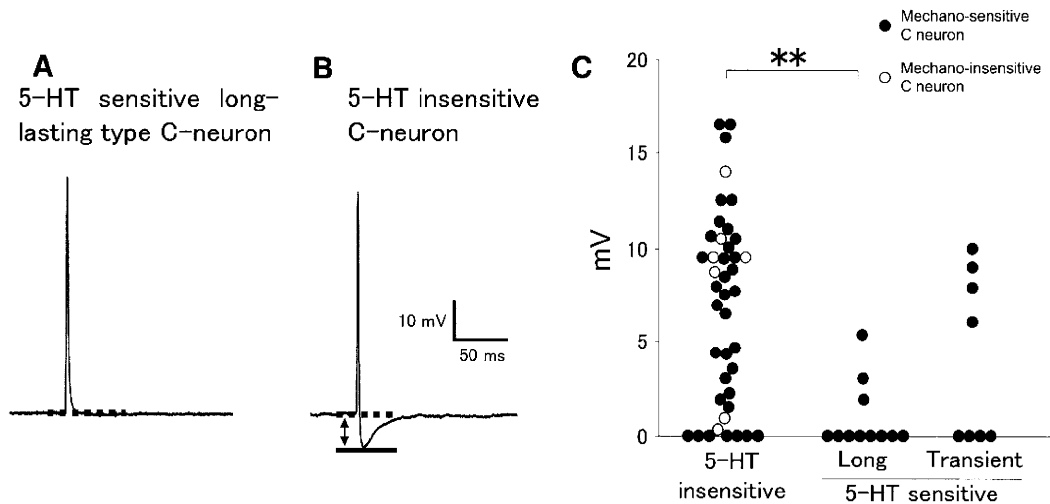

FIG. 6. Comparison of amplitude of AHP among C neurons. *A*: representative action potential of 5-HT-sensitive long-lasting-type neuron. *B*: representative action potential of 5-HT-insensitive C neuron. Note that obvious AHP was observed in 5-HT-insensitive C neuron. *C*: amplitudes of AHP of 5-HT-insensitive neurons (n = 42), 5-HT-sensitive long-lasting neurons (n = 11), and 5-HT-sensitive transient neurons (n = 8) are plotted. Filled circles represent mechanosensitive C neurons and open circles represent mechanoinsensitive C neurons. $^{**}P < 0.01$ (Kruskal–Wallis H test).

hindpaw initiated licking but not biting behavior, further confirming the effect of 5-HT in eliciting the itch sensation in rats (Abbott et al. 1995; Hunskaar et al. 1985; Tjølsen et al. 1992).

The 5-HT-sensitive long-lasting-firing C neurons started firing earlier than the C neurons of the transient type. This suggests that the long-lasting C-afferent terminals exist at a more superficial layer of the skin than those of the transient type. Numerous fine nerve endings are spread out at a level just below the epidermis (Shelly and Arthur 1957) and free nerve endings in the dermoepidermal junction are regarded as a receptor for itch sensation (Wahlgren 1992). It is reported that patients with atopic dermatitis have an increased number of nerve fibers in the skin (Tobin et al. 1992; Urashima and Mihara 1998) and nerve growth factor–mediated sprouting of nerve fiber is found in patients with contact dermatitis (Kinkelin et al. 2000). These reports support our findings. Interestingly, the onset of scratching response was later than the onset of action potentials of long-lasting neurons. There may be two reasons why scratching appears later: one is that an itchy sensation that produces a behavioral change needs the accumulation of activity of itch-related neurons; the other is that transient-type neurons may transmit painful sensation and they may suppress the itch sensation transiently in the first several minutes. However, we do not have enough evidence at present to make such hypotheses.

In the present study, topically applied 5-HT to the receptive field evoked repetitive firing in a small population of C neurons but not in Aβ and Aδ neurons. Previous electrophysiological observations support the contribution of C-afferent fibers to the itch sensation; for instance, cowhage selectively activates polymodal C fibers in rats and monkeys (Johanek et al. 2008; Tuckett and Wei 1987b) and histamine activates a subset of C fibers in humans (Handwerker et al. 1991; Schmelz et al. 1997, 2003). Recently, it was reported that the histamine-induced itch sensation is mediated by the activation of TRPV1 receptors expressed at C afferents via an arachidonic acid metabolite produced by histamine receptor activation (Shim et al. 2007). These observations are consistent with our findings. However, it is possible that multiple pathways might be responsible for the itch sensation and it is still not clear whether 5-HT-sensitive C neurons belong to the previously reported itch-responsible primary afferents.

From our behavioral study, naloxone inhibited itch-associated scratching behavior. The site of opioid action on the itch sensation pathway has not been identified. It is well known that the μ-receptor agonist DAMGO ([D-Ala, N-Me-Phe, Gly-ol]-

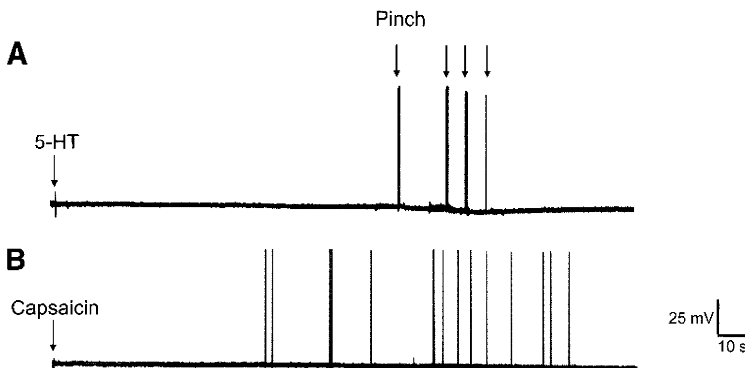

FIG. 7. Representative trace of 5-HT-insensitive C neuron that responded to pinch stimuli and topically applied capsaicin. *A*: 5-HT did not evoke firing. *B*: continuous firing was elicited following topical application of capsaicin to the same neuron.

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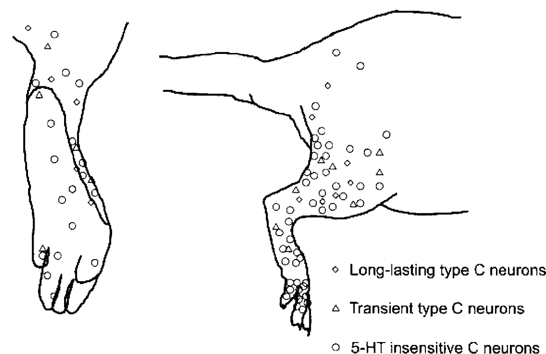

FIG. 8. Distribution of the receptive fields of long-lasting-type C neurons (diamond), transient-type C neurons (triangle), and 5-HT-insensitive C neurons (circle).

enkephalin) applied to the spinal cord inhibits the release of glutamate from the primary afferents, including C fibers (Ikoma et al. 2007; Kohno et al. 1999) and causes membrane hyperpolarization in substantia gelatinosa neurons (Yoshimura and North 1983), indicating that the analgesic effect of opioid is exerted both on the primary afferents and spinal interneurons. In contrast, subcutaneous injection of naltrexone does not affect the activity of cutaneous nerves innervating the chronic dermatitis area (Maekawa et al. 2002). Moreover, neither systemic morphine nor naltrexone affected the 5-HT-evoked c-Fos-like immunoreactivity in the superficial laminae of the spinal dorsal horn, whereas morphine significantly attenuated the intradermal capsaicin-induced immunoreactivity (Nojima et al. 2003). Therefore the μ-opioid receptor may modulate the itch sensation at the supraspinal level; however, further experiments are necessary to reveal how opioid receptors act on itch transmission.

Interestingly, the 5-HT-sensitive long-lasting-firing neurons had a significantly small, fast AHP. In general, nociceptive DRG neurons have a broader action potential than that of low-threshold mechanoreceptive neurons in the same conduction velocity group (Djouhri et al. 1998; Fang et al. 2005; Ritter and Mendell 1992). Nociceptive C neurons have a broader action potential and larger AHP than those of C neurons responsive to nonnoxious stimuli (Djouhri et al. 1998; Fang et al. 2005). The amplitude of AHP observed in this study was smaller than that previously reported (Fang et al. 2005), probably due to the more negative resting membrane potential of C neurons in our study. However, the resting membrane potential in three types of neurons was not significantly different in the present study; therefore the 5-HT-sensitive long-lasting C neurons may have smaller AHP compared with that of 5-HT-insensitive C neurons.

Regarding the issue of whether itch-sensing primary afferents respond to mechanical stimuli, Fang et al. (2005) reported that 54% of C fibers were nociceptors, 11% were nonnociceptors, and 34% were unresponsive to mechanical stimuli. The mechanical threshold of C fiber ranges from 16 to 608 mN (Pogatzki et al. 2002). In the present study, almost all 5-HT-sensitive C neurons responded to mechanical noxious stimuli and several were responsive to nonnoxious stimuli. Thus the 5-HT-sensitive neurons also responded to mechanical stimulation, indicating that the 5-HT-sensitive C neurons are polymodal. In this study, we did not apply thermal stimulation or capsaicin to the receptive fields of 5-HT-sensitive neurons. 5-HT receptor mRNAs were expressed in rat dorsal root ganglion neurons (Nicholson et al. 2003). 5-HT_{2A} induces thermal hyperalgesia in acute injury and inflammation in rats (Tokunaga et al. 1998). Activation of metabotropic 5-HT receptors enhances the TRPV1 function in primary afferent neurons (Ohta et al. 2006). This evidence suggests that 5-HT-sensitive C neurons might be part of the polymodal neurons. Furthermore, we applied capsaicin to the 5-HT-insensitive neurons, 85% of which were activated by noxious mechanical stimuli, and some of them responded to capsaicin (Fig. 7), indicating that the 5-HT-insensitive primary C neurons include polymodal neurons. It is not known from our study whether 5-HT-responsive C neurons are the only pruritogen-sensitive primary afferents. Endopeptidases elicit a pure itch sensation without any wheal or flare (Arthur and Shelley 1955). It was found that itching could be induced by electrical stimulation to the wrist without generating an axon reflex (Ikoma et al. 2005). It has been reported that histamine and another typical itch agent, cowhage, activate separate populations of spinothalamic tract neurons (Davidson et al. 2007), implying that the itch sensation is not mediated by a single system. Recently, it was reported that gastrin-releasing peptide receptors mediate itch sensation in the spinal cord (Sun and Chen 2007). Gastrin-releasing peptide is expressed in a subset of small and medium-sized DRG neurons that are peptidergic unmyelinated fibers (Sun and Chen 2007). Since it is still unknown whether long-lasting-type 5-HT-sensitive C neurons express gastrin-releasing peptide or peptidergic markers such as substance P and calcitonin gene-related peptide, further experiments are needed to answer the question.

The 5-HT receptor subtype responsible for activation of the primary afferent is still unknown. In DRG, the mRNA for 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{3B}, and 5-HT_4 receptors was detected in small-diameter neurons (Nicholson et al. 2003). It is known that 5-HT_{2A} and 5-HT_3 receptors have a role in pain sensation (Eschalier et al. 1989; Giordano and Rogers 1989; Okamoto et al. 2002; Tokunaga et al. 1998). In vitro electrophysiological experiments showed that 5-HT modulates the activity of primary afferents. Activation of axonal 5-HT_3 receptors enhances membrane excitability and modulates action potential trains in unmyelinated nerve fibers (Lang et al. 2006). Ohta et al. (2006) showed that 5-HT changed TRPV1 functions through the activation of 5-HT_{2A} and 5-HT_7 receptors in cultured DRG neurons. It is reported that 5-HT-evoked itching is mediated by 5-HT_2 receptors (Nojima and Carstens 2003b; Yamaguchi et al. 1999); however, this study does not indicate the principal receptor for 5-HT-induced itching. Because the latency of firing after topical application of 5-HT was within 1 min in the majority of 5-HT-sensitive long-lasting C neurons, these data may suggest that 5-HT directly activates 5-HT receptors; however, we cannot exclude the possibility that the activation of long-lasting C neurons was mediated by surrounding tissues such as keratinocytes or mast cells with the release of certain chemicals.

In conclusion, the itch-sensing primary afferents might be polymodal C fibers that responded to topically applied 5-HT, resulting in continuous firing for ≤50 min. This long-lasting high-frequency firing was, in part, due to the small amplitude of fast AHP and the absence of slow AHP, even though the neurons had large Ca^{2+} components in the action potential.

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The functional significance of the transient-firing neurons evoked by 5-HT has not been clarified in the present study. In view of the fact that 5-HT is also an algesic substance and considering the long duration of the scratching behavior demonstrated in the present study, we assumed that the transient firing observed in a subpopulation of C neurons may be responsible for carrying pain sensation to the spinal dorsal horn.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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CORRESPONDENCE

Symptom flares of atopic dermatitis during the Japanese cedar pollen season – a Website questionnaire study

Japanese cedar (JC) pollinosis is a common disease in Japan, and the prevalence is increasing dramatically [1]. The influence of pollen on atopic dermatitis (AD) has been much studied and it is reported that many AD patients can have symptom flares from February to May [2, 3], when the airborne JC pollen reaches a high level in Japan. In addition, a symptom flare is likely to be seen among AD patients who also have JC pollinosis [2]. However, to the best of our knowledge, there have only been a few reports discussing precisely this point. Thus, we examined the frequency of symptom flares of AD during the JC pollen season through a questionnaire on our Website.

We put out a questionnaire for AD patients with the title, "About changes in atopic dermatitis symptoms during the Japanese cedar pollen season", for 2 months from March 1st to April 30th 2007, which was maintained by our department (http://www.kyudai-derm.org/atopy/). The list of questions and details of the results are shown in *table 1*.

We obtained 188 responders. Of the 188 AD patients, 137 (72.9%) had also JC pollinosis. Based on our Website questionnaire, most of the patients with JC pollinosis (130 of 137, 94.9%) had experienced symptom flares of AD during the JC pollen season, and the symptom flares tended to occur on uncovered areas such as the face and neck, with frequencies as high as 77.9% (95 of 122) for erythema and 75.0% (93 of 124) for itch. These results suggest a close correlation between the exacerbation of AD and JC pollinosis and/or pollen.

Because the barrier function of the skin is damaged, AD patients can be sensitized more easily, and eczematous lesions can arise quickly due to attached allergens. This theory can explain not only the high complication rate associated with JC pollinosis but also the symptom flares, which occur mainly on uncovered areas during the pollen season.

In our study, 18.1% of AD patients also had asthma, and 26.5% of these had experienced symptom flares of asthma during the pollen season while 70.6% had symptoms that did not change. Although the questions were not limited to the patients with JC pollinosis, these results may suggest a closer correlation between AD and JC pollinosis than between AD and asthma or asthma and JC pollinosis.

There are limitations in our Website study. Responders were considered to be AD patients by self-reporting, and we cannot prove that all of them were doctor-diagnosed. The theme of the questionnaire may have made the responder population biased towards patients with JC pollinosis and against patients without JC pollinosis. We did not evaluate the presence of symptom flares in AD patients without JC pollinosis, and the presence of symptom flares in asthma patients was evaluated without being limited to the asthma patients with JC pollinosis.

In conclusion, JC pollen can be an exacerbating factor in AD, and it may make symptoms worse, especially on uncovered areas directly exposed to pollen. The relationship of JC pollinosis with AD may be closer than with asthma, which should be examined further through future studies. ■

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Conflict of interest. *None.*

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Table 1. A questionnaire shown on our Website and details of the answers

n=188	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71≤
	20	38	67	46	12	3	2	0
	10.6%	20.2%	35.6%	24.5%	6.4%	1.6%	1.1%	0%
Q2. What is your gender?								
n=188					Male		female	
					75		113	
					39.9%		60.1%	
Q3. Do you have Japanese cedar pollinosis?								
n=188					Yes		No	
					137		51	
					72.9%		27.1%	
Q4. Do your atopic dermatitis symptoms change during the Japanese cedar pollen season i.e.about from February to May? (Only for the patients who answered "Yes" on Q3)								
n=137					Yes		No	
					130		7	
					94.9%		5.1%	
Q5. How does your erythema change during that season? (Only for the patients who answered "Yes" on Q4)								
n=130			Better		No Change		Worse	
			0		8		122	
			0%		6.2%		93.8%	
Q6. Where do you have worse erythema? You can choose more than one answer. (Only for the patients who answered "Worse" on Q5)								
n=122			Face and Neck		Extremities		Trunk	
			95		61		54	
			77.9%		50.0%		44.3%	
Q7. How does your itch change during that season? (Only for the patients who answered "Yes" on Q4)								
n=130			More		No Change		Less	
			124		6		0	
			95.4%		4.6%		0%	
Q8. Where do you have more itch? You can choose more than one answer. (Only for the patients who answered "More" on Q7)								
n=124			Face and Neck		Extremities		Trunk	
			93		70		63	
			75.0%		56.5%		50.8%	
Q9. Do you have asthma?								
n=188			Yes		No			
			34		154			
			18.1%		81.9%			
Q10. Do your asthma symptoms change during the Japanese cedar pollen season i.e. about from February to May? (Only for the patients who answered "Yes" on Q9)								
n=34			Better		No Change		Worse	
			1		24		9	
			2.9%		70.6%		26.5%	

"Q" means an abbreviation of "question".

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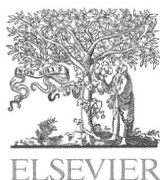

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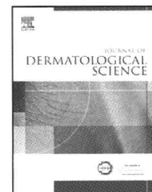

Collared mice: A model to assess the effects of scratching☆

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ABSTRACT

Background: There is no current method to precisely assess pruritus despite its importance as a major symptom in many skin diseases. Pruritus induces scratching that worsens various inflammatory skin diseases.
Objective: The purpose of this study was to determine the effects of scratching on allergic skin reactions using murine contact hypersensitivity (CH) as a model and to assess classical "anti-pruritic" agents using this model.
Methods: We utilized plastic collars which were placed around the necks of mice to prevent them from scratching their ears during the development of CH. This allowed us to assess ear swelling as an index of CH, obviating the effects of scratching that occurs during the development of CH.
Results: Following elicitation, the ear swelling of these "collared" mice was decreased by approximately 50%, compared to control mice in which collars were not used, suggesting that scratching contributes to the ear swelling that is measured as an index of CH. Using this model, we assessed the anti-pruritic effects of antihistamines, corticosteroids, non-steroidal antiinflammatory and sedative agents. All agents decreased CH when collars were not used. When collars were used, all agents, other than the sedatives, appeared to suppress CH, indicating their antiinflammatory effects. Sedative agents did not decrease CH in collared mice, indicating that their inhibitory effects in CH may be entirely due to their sedative effects.
Conclusions: This model enables the dissection of the various elements assessed when measuring CH in mice and may provide a simple tool to assess or screen potential anti-pruritic agents.
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1. Introduction

There is no current method to precisely assess pruritus, despite its being a prominent symptom in many skin diseases [1] such as atopic dermatitis, psoriasis, contact dermatitis, prurigo, or in systemic diseases such as diabetes, cirrhosis or chronic renal failure [1]. Pruritus is a symptom that affects quality of life both physically and psychologically and, as a consequence of scratching, aggravates preexisting skin eruptions. Considerable effort has been expended to assess pruritus in order to better understand mechanisms involved as well as to develop better anti-pruritic agents. Since pruritus is a perception, verbal itch score [2] or visual analogue scales that were originally developed for assessing pain sensation [3] have limited value because of their subjectivity. As well, since pruritus is, by definition, almost inexorably associated with scratching, various measures of scratching have been developed such as a sensitive limb movement meter [4], or videotaping the movements of patients during sleep [5]. Assessing scratching behavior is certainly an objective method, but analyzing hours of records along with the limited availability of such expensive automated recoding systems [6,7], remain a challenge.

Many antiinflammatory agents have been developed for clinical use (such as antihistamines, glucocorticosteroids, and others) and their anti-pruritic properties have been objectively evaluated using animal models [8,9]. In this study, we have developed a new, simple method to assess the effect of various agents on the scratching behavior in mice. In this assay, scratching is assessed as a physiological skin response using a murine model of contact hypersensitivity (CH). We utilized light-weight plastic collars which were placed around the necks of mice to prevent them from scratching or grooming their ears during the development of CH. The effects of scratching were assessed on the ear swelling assay of CH. Various agents with known sedative and/of anti-pruritic properties were also assessed using this assay.

2. Materials and methods

2.1. Reagents and monoclonal antibodies

Monoclonal antibodies (mAb) to CD16/CD32 (Fcγ III/II receptor), CD45 and MHC class II (MHC II) either unconjugated

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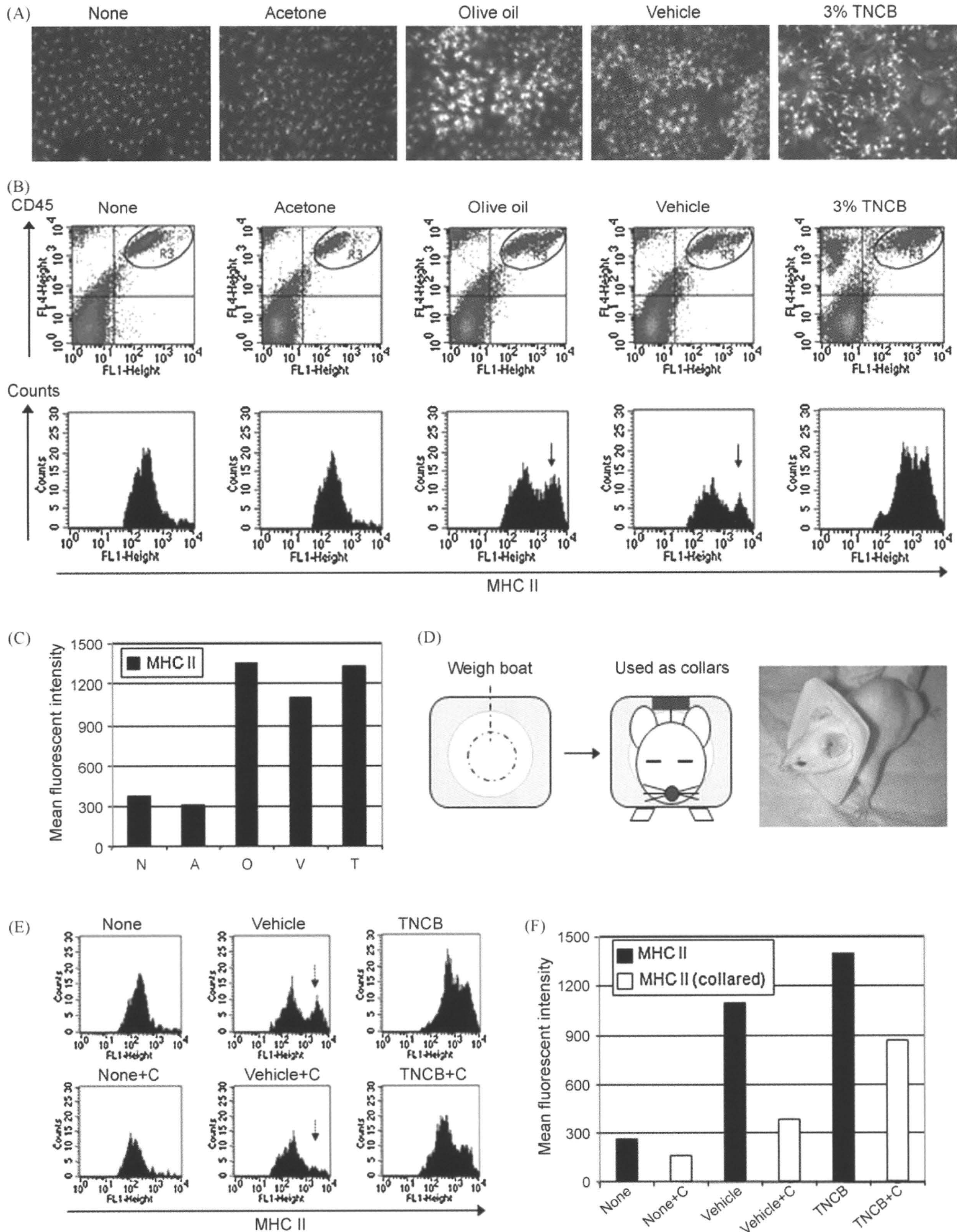

Fig. 1. Scratching behavior contributes to the activation of epidermal LC. Activated MHC II^{high} LC were observed at 24 h after painting in the epidermal sheets from mice painted with olive oil, vehicle (acetone + olive oil) and 3% TNCB, but not with acetone alone (A). Note that the appearance of the MHC II^{high} LC population (B, arrows) and (C) high MHC II-mean fluorescence intensity (MFI) of LC in mice painted with olive oil or vehicle or TNCB. A light-weight plastic collar was placed around the neck to prevent mice from scratching ears (D). LC exhibited high MHC II in mice painted with vehicle (E), but use of collars diminished the LC upregulation of MHC II in vehicle-painted mice (F). MHC II: MHC class II, MFI: mean fluorescent intensity, C: use of collars.

or conjugated with fluorescein isothiocyanate or with allophycocyanin were purchased from BD Bioscience Pharmingen (San Diego, CA). Propidium iodide, deoxyribonuclease I (DNase I), ammonium thiocyanate, olive oil, chlorpheniramine, prednisolone, aspirin, and phenobarbital were purchased from SIGMA-Aldrich (St. Louis, MO). Hexadecyl trimethylammonium bromide and o-dianisidine were purchased from Sigma Chemical Co. (St. Louis, MO).

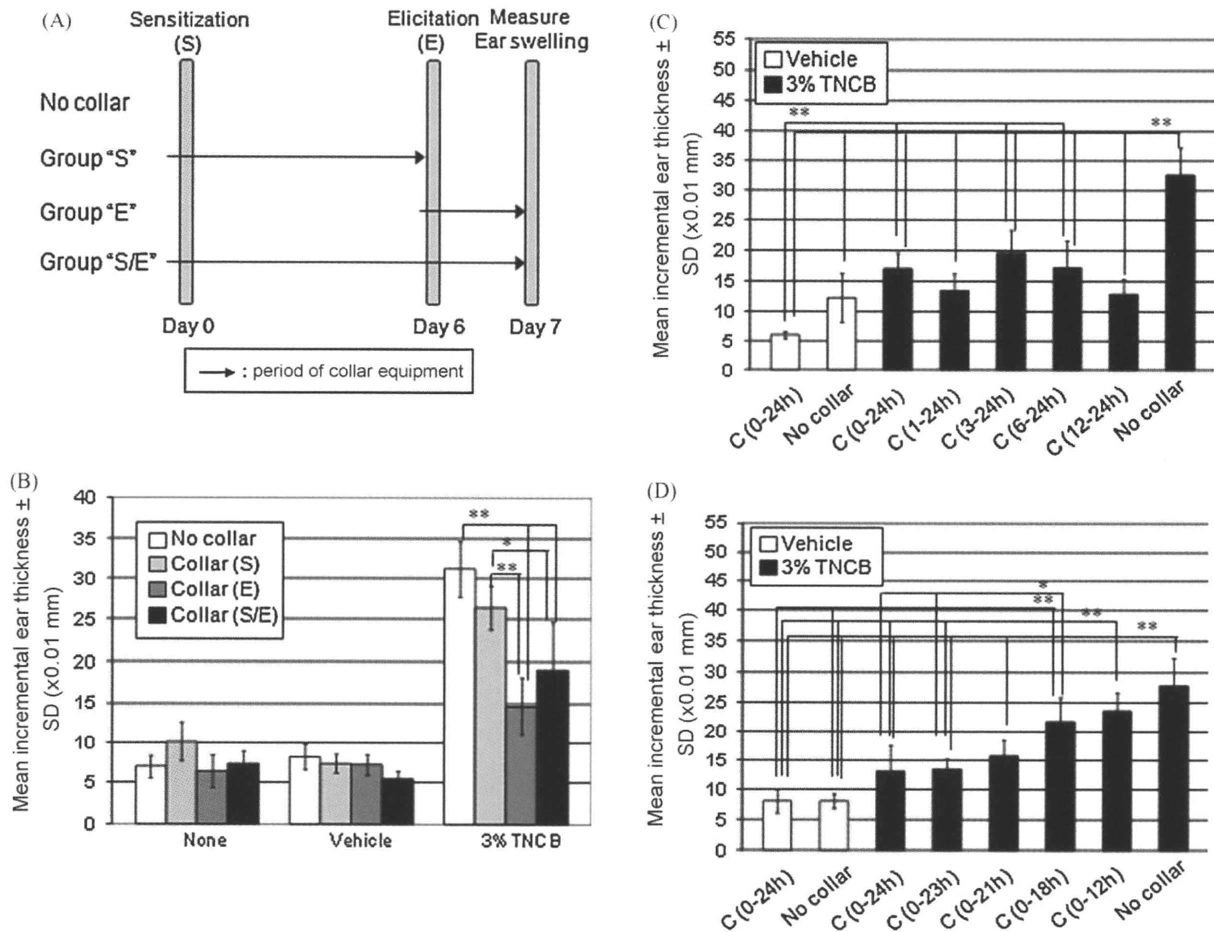

Fig. 2. Scratching behavior contributes to the ear swelling of CH. Collars were used during the sensitization and/or elicitation of CH to prevent mice from scratching the ears (A). Ear swelling of CH significantly decreased when collars were used during the elicitation phase (B). Applying the collars during the last 12 h, of the 24 h after elicitation significantly inhibited ear swelling (C). The longer the collars were used in the elicitation phase the greater the suppression of CH (D). S: sensitization, E: elicitation, S/E: both sensitization and elicitation, h: hour(s), C: use of collars, SD: standard deviation. Note that some description of statistical analysis, such as those between vehicle-treated group and sensitized group, were omitted in graph 2B for clarity. $^{**}p < 0.01$, $^{*}p < 0.05$.

Sodium phosphate and potassium phosphate were purchased from ICN Biomedicals Inc. (Aurora, OH). Acetone was purchased from Mallincrodt Baker Inc. (Paris, KY). Trinitrochlorobenzene (TNCB) was purchased from Polyscience Inc. (Warrington, PA). 1× phosphate buffered saline (PBS) and 1× Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Woodland, CA). Trypsin was purchased from USB Corporation (Cleveland, OH). 0.5 M EDTA was purchased from Quality Biological Inc. (Gaithersburg, MD).

2.2. Animals and protocols for contact hypersensitivity

BALB/c mice (7–12-week old) were purchased from the National Cancer Institute Frederick Cancer Research and Developmental Center (Frederick, MD). On day 0, the right ear or shaved abdomen of each mouse was sensitized with 3% TNCB solution in a vehicle (1:4 of acetone:olive oil) of 12 μl (ear) or of 100 μl (abdomen), respectively. On day 6, the left ear of the sensitized mouse was painted with a total of 20 μl of 1% TNCB solution (in 4:1 of acetone:olive oil) and ear swelling was measured using a dial thickness gauge at 24 h after elicitation. (Mice were sensitized on the right ears except for those used in experiments for Fig. 4B–E where intact unchallenged ears were needed for assays as negative controls.) Non-sensitized mice not painted or painted with vehicle alone served as negative controls. In these experiments, light plastic weigh boats were utilized as collars and were placed around the neck to prevent mice from scratching or grooming their ears at the time of sensitization or elicitation of CH. These types of collars had been used in prior studies that assessed early and late activation events in CH (unpublished data, S. Katz). In some experiments, chlorpheniramine (60 mg/kg), prednisolone (40 mg/kg), aspirin (200 mg/kg) and phenobarbital (90 mg/kg) was administered intraperitoneally at the time of or just after the elicitation phase of CH. The animal protocol has been approved by IRB of NIH.

2.3. Preparation of epidermal single cell suspensions and flow cytometric analysis

Split ears of mice were floated dermal side down on 0.5% trypsinized PBS and incubated for 45 min at 37 °C. The epidermis was then gently detached from the dermis, and put into HBSS with 5% FBS and 0.025% DNase I. Epidermal cell suspensions were obtained by pipetting and filtering the epidermis through 100-μm nylon mesh. Epidermal Langerhans cells (LC) were enriched using a lympholyte M-density gradient and were washed once with PBS containing 5% FBS and 2 mM EDTA before further procedures. Cells prepared as above were Fc-blocked with CD16/CD32 mAb for 10 min and incubated with various antibodies for 20 min at 4 °C. Cells were then washed twice and were analyzed using a Becton

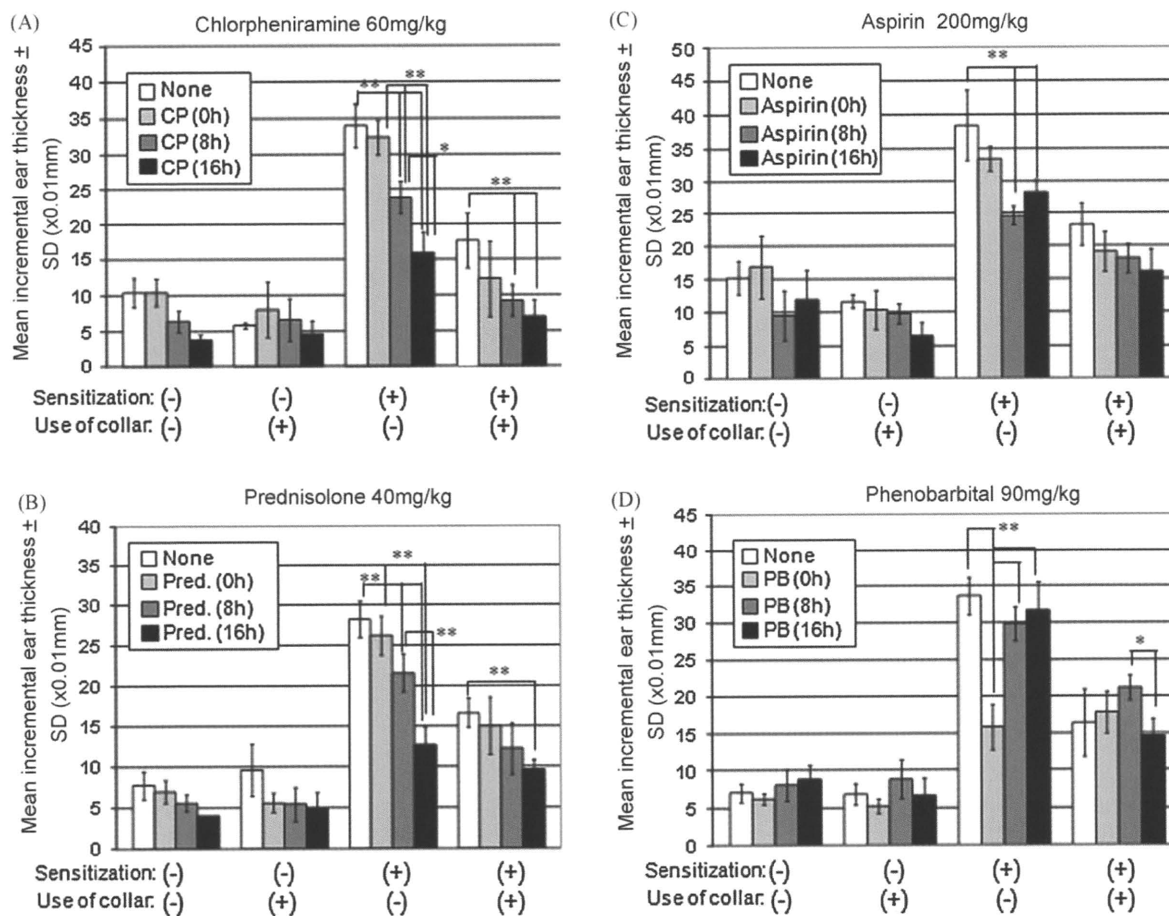

Fig. 3. Inhibition of CH by various agents. Various agents were administrated intraperitoneally immediately before (at 0 h), 8 and 16 h after elicitation, and ear swelling were measured at 24 h after elicitation. The administration of chlorpheniramine (A), prednisolone (B), and aspirin (C) significantly decreased CH both in collared- and control mice, and these drug appeared more effective when used in the later phases of elicitation (A–C). The administration of phenobarbital decreased CH when used at 0 h, but not when administered at 8 or 16 h after elicitation. Note that, in contrast to other agents, phenobarbital did not decrease CH in collared mice, as compared to that of non-treated collared mice (D). CP: chlorpheniramine, Pred.: prednisolone, PB: phenobarbital, SD: standard deviation. Note that statistical analysis is described only within each group to show that the effects of agents depends considerably on the timing of its application. $^{**}p < 0.01$, $^{*}p < 0.05$.

Dickinson FACS Calibur flow cytometer and CELLQuest software version 3.3 (San Jose, CA).

2.4. Preparation of epidermal sheets

Epidermis was gently detached from the dermis after incubation of split ears with 0.038% ammonium thiocyanate solution in 0.05 M sodium/potassium phosphate buffer for 20 min at 37 °C. After acetone fixation for 10 min at −20 °C, epidermal sheets were incubated with anti-MHC II mAb for 30 min at room temperature, washed thrice by PBS, then incubated with goat anti-mouse serum conjugated with FITC for 30 min at room temperature. The epidermal sheets were observed through a Nikon Eclipse TE300 fluorescent microscope (Tokyo, Japan), and images were captured by IPLab version 3.6.3 (Scanalytics Inc., Fairfax, VA).

2.5. Assessing myeloperoxidase (MPO) activity in situ

Dermal infiltration with polymorphonuclear cells (PMN) was assessed by tissue MPO activity in situ as previously described [10,11]. A six millimeters punch biopsy was used to punch a hole through both the challenged (elicited) and control ears of each mouse. These tissues were obtained at 6, 12 and 24 h after elicitation and were then weighed. Ear swelling was determined by subtracting the weight of the control ear from that of challenged ear in each mouse. These punched out ears were then homogenized in a 50 mM potassium phosphate, pH 6.0 buffer containing 0.5% hexadecyl trimethylammonium bromide using a FastPrep® homogenizer (GMI Inc., Ramsey, MN), and the collected supernatants were assessed for MPO activity.

2.6. Statistical analysis

Statistical analyses were performed using CA-Cricket Graph III ver. 1.5.3 (Computer Associates Intern. Inc., Islandia, NY) and Microsoft Excel 2001 (Microsoft corp., Seattle, WA), and p-values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Scratching contributes to the activation of epidermal LC

Epidermal sheets were examined 24 h after painting either 3% TNCB solution, vehicle [acetone plus olive oil (1:4)], acetone or olive oil (alone). Clusters of large sheets of MHC II^{high} dendritic cells were evident in mice painted with TNCB, olive oil, and vehicle whereas non painted and acetone (alone) painted ears did not show such MHC II upregulation (Fig. 1A). In flow cytometric analysis using epidermal cell suspensions, CD45^{+} MHC II^{high} (LC)

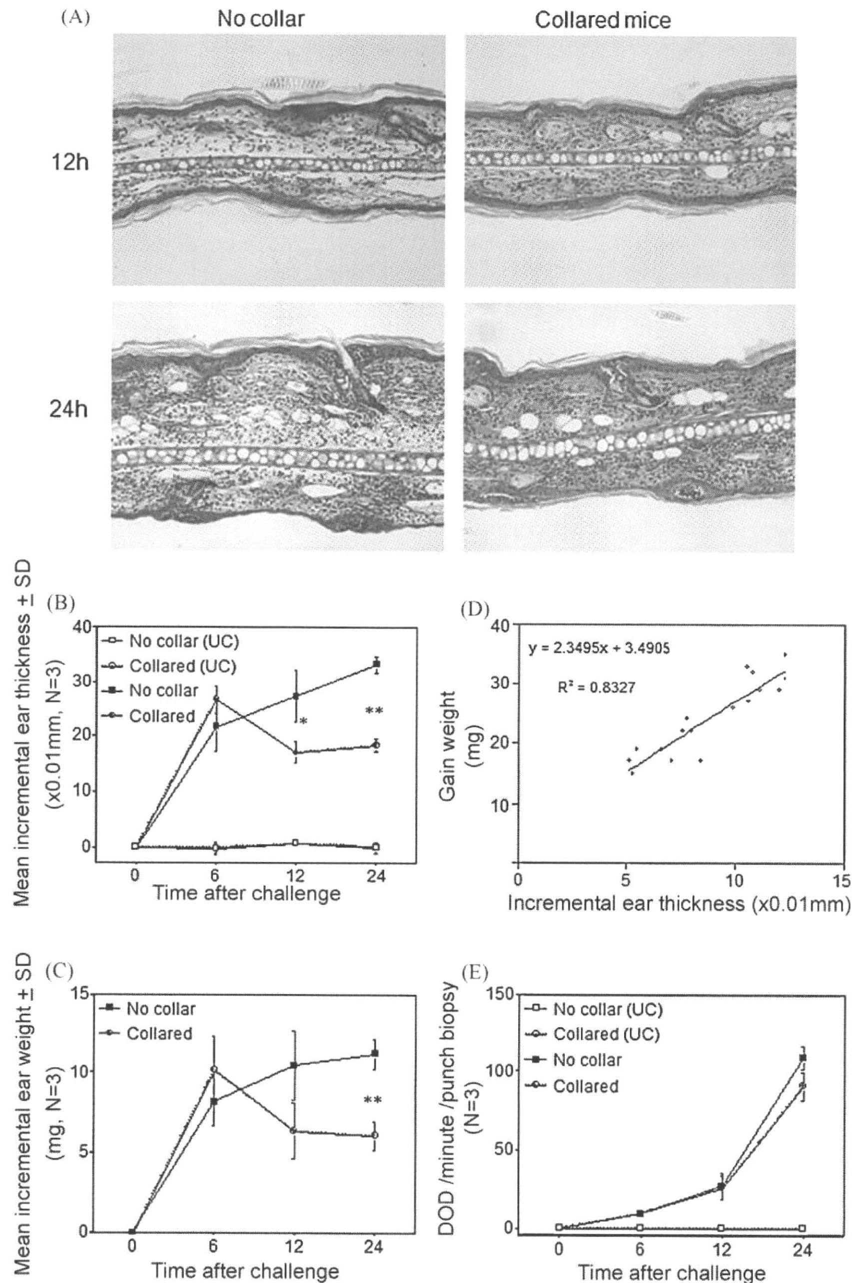

Fig. 4. Use of collars does not decrease inflammatory infiltration in the elicited dermis of CH. Histologically, the dermis of collared mice was less edematous at 12 and 24 h after elicitation compared to that of control mice, but the inflammatory infiltration appeared similar in the both groups (A). Ear swelling of CH decreased in collared mice at 12 and 24 h after elicitation as measured by conventional dial gauging (B) and by weighing punched out ears (C). The two measures for ear swelling showed a very high correlation (D). MPO activity in situ of challenged ears was no different between control- and collared mice (E). UC: unchallenged ears, SD: standard deviation. $^{**}p < 0.01$, $^{*}p < 0.05$.

were identified in mice painted with olive oil, vehicle and 3% TNCB, but not with acetone alone (Fig. 1B and C). Plastic collars were used around the neck to prevent mice from scratching their ears (Fig. 1D). The use of collars completely diminished LC upregulation of MHC II in ears of mice treated with vehicle and partially diminished MHC II expression in TNCB-painted ears (Fig. 1E and F).

3.2. Scratching contributes to the expression of CH in mice

The effects of collars were examined on sensitization and/or elicitation of CH as shown (Fig. 2A). Ear swelling of CH significantly decreased when collars were used during the elicitation phase of CH (Fig. 2B). Use of collars during the last 12 h, but not during the first 12 h, of the 24 h after elicitation appeared more critical to demonstrate the inhibitory effects on ear swelling of CH (Fig. 2C and D).

3.3. Phenobarbital decreases CH in control mice, but not that in collared mice

Various agents with possible anti-pruritic properties were assessed for their effects on the ear swelling response using control non-collared sensitized mice and collared mice. Use of each of these agents decreased CH when collars were not used (Fig. 3A–D). In contrast to the effects of other agents, phenobarbital was

effective only when used at the time of elicitation (0 h). In addition, when phenobarbital was administered, there was no decrease in ear swelling in the collared mice (Fig. 3A–C).

3.4. Use of collars decreases ear swelling, but does not affect inflammatory infiltration in skin exhibiting CH

Histological examination revealed that elicited ears of collared mice appeared to be less edematous but had a similar degree of inflammatory infiltration in the dermis as compared to the ears of control mice (Fig. 4A). Ear swelling was assessed by conventional thickness dial gauging (Fig. 4B) and by weighing punched out ears

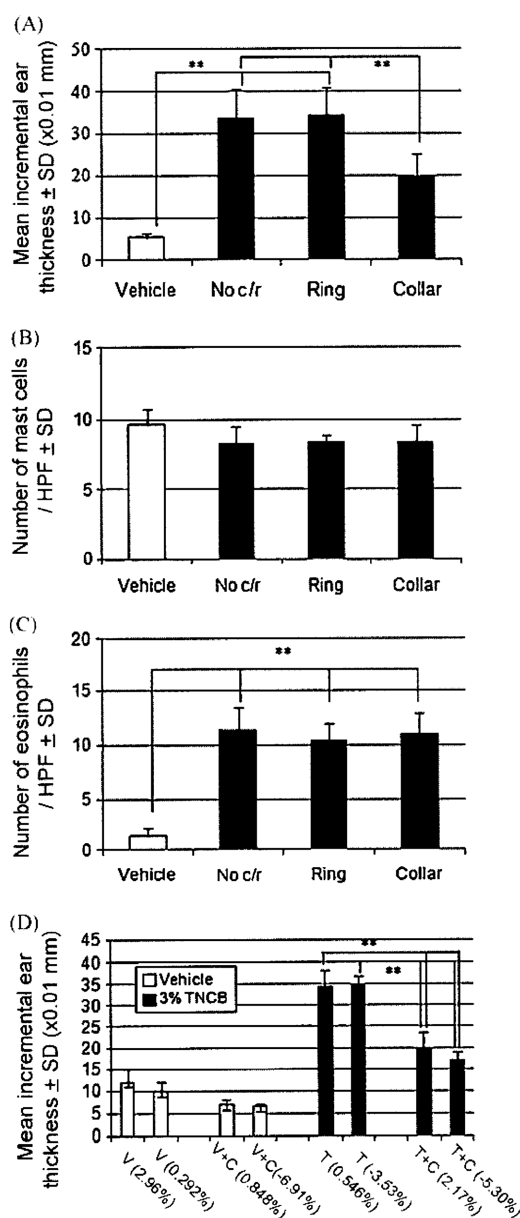

Fig. 5. Ear swelling of CH decreased only when collars were used (A). The number of mast cells and infiltrating eosinophils was unchanged among sensitized groups regardless of whether they were wearing collars and/or rings (B and C). Note that mean body weight change did not seem to affect ear swelling in control sensitized mice or the decrease of ear swelling in collared mice (D). HPF: high power field, No c/r: no collars or rings, SD: standard deviation. Note that some description of statistical analysis, such as those between vehicle-treated groups and sensitized groups were omitted in graph 5D for clarity. $^{**}p < 0.01$.

(Fig. 4C). The ear swelling in collared mice did not change at 6 h, but decreased at 12 and 24 h after elicitation, compared to that seen in control mice in which the ears continued to thicken. There was a very high correlation between the two measures for the assessment of ear swelling (Fig. 4D). Use of collars did not decrease the degree of dermal infiltration as a whole, as evidenced by equal MPO activity in situ of challenged ears that were either collared or not collared. We further extended these experiments by using plastic rings (collars without the upper part) to determine whether mice that are stressed by wearing the ring but could scratch their ears fully can develop normal CH reactions. Such "ringed" mice showed as much ear swelling of CH as that of non-collared and non-ringed mice, while collared mice showed a significant decrease of CH (Fig. 5A). The number of mast cells and infiltrating eosinophils was no different among sensitized mice regardless of the use of collars or rings (Fig. 5B and C). We also measured the mean weight change and ear swelling during development of CH elicitation and found that weight change did not seem to affect ear swelling of CH (Fig. 5D), suggesting that the decrease of CH did not merely come from dehydration of mice that wore plastic collars.

4. Discussion

The purpose of this study was to determine the effects of scratching on allergic skin reactions using murine contact hypersensitivity (CH) to TNCB as a model and to assess classical "anti-pruritic" agents using this model. Scratching by the mice was found to contribute significantly to ear swelling. This is in keeping with clinical experience wherein scratching exacerbates skin eruptions in pruritic skin diseases such as atopic dermatitis. The contribution of scratching to skin inflammation was first examined using an in vivo LC activation assay. Epidermal LC exhibited increased MHC II expression and elongated dendrites when vehicle (or olive oil) were used, but these LC changes were abolished when collars were used to prevent mice from scratching. LC exhibited these changes (increased MHC II expression and elongated dendrites) when TNCB was used even in the presence of collars. It is not known whether these LC changes in mice painted with vehicle is accompanied by their migration to the regional lymph nodes as occurs in the induction of CH after hapten painting [12,13]. Although we initially suspected that acetone was responsible for LC changes because of its induction of dryness after evaporation [14], we found that olive oil was responsible for the scratching induced by the vehicle. This grooming behavior (scratching) by the hind legs was very rapid. This observation led us to think that the scratching/grooming behavior might possibly contribute to the intensity of skin inflammation. Our hypothesis was that the MHC II increase and elongated dendrites in LC that occurred in olive oil- or vehicle-painted mice might not be due to the chemicals themselves but due to the inflammation caused by scratching/grooming. LC and other dendritic cells (DC) are known to be activated through TNF-α and/or IL-1β [15,16], both of which are upregulated with hapten painting [17]. Although these cytokines are key pro-inflammatory cytokines in the initiation of skin inflammation, we do not know whether the MHC II upregulation and increased dendrites exhibited by LC is caused by cytokine upregulation.

We then hypothesized that the scratching/grooming behavior might possibly contribute to the ear swelling assessed during CH. When collars were used to prevent mice from scratching during the last 12 h of 24 h after elicitation of CH, ear swelling was diminished. Ear swelling in control, non-collared sensitized mice exceeded that of collared mice at 12 and 24 h after elicitation, and the diminished ear swelling appeared to be associated with the degree of tissue edema of the elicited dermis. Interestingly, there was no significant difference between control non-collared mice and collared mice

when MPO activity was assessed (as an index of inflammatory cell infiltration) in situ. This, we think, reflected the equivalent numbers of dermal infiltrating polymorphonuclear cells, the major infiltrates in CH in mice when TNCB is used [18]. Thus, scratching may modulate factors that affect tissue edema significantly in the challenged dermis. Vascular permeability-associated factors are also important in the formation of contact hypersensitivity [19–21] and they can be upregulated by TNF-α [22–24], and/or by minor trauma such as gentle cutaneous rubbing [25] which may be equivalent to scratching.

An important point from a methodological standpoint is that the use of collared mice can dissect out the ear swelling induced by scratching. Thus, there are two components of CH that can be dissected: (1) the allergic reaction and (2) the scratching.

All of the agents used in the current study caused diminished ear swelling-the decreased expression of CH could be due either to the anti-pruritic (anti-scratching) effects, or the antiinflammatory effects or both. Phenobarbital appeared to decrease ear swelling in CH and this was probably due to its effect on scratching because phenobarbital could not suppress CH of collared mice where scratch-enhanced ear swelling was disabled. Other agents including chlorpheniramine appear to have some antiinflammatory effects because they could suppress CH in collared mice as well as in control mice.

In conclusion, we established a model to assess the effects of scratching on allergic skin disease using CH as a model. This enables us to assess scratching as a physiological phenomenon and to dissect out the scratching-enhancement on the expression of CH. In addition, CH-suppressing effects of phenobarbital, possibly through their soporific effects, can be identified using the collared mice. Thus, this in vivo model might be useful in the assessment or development of agents with potential anti-pruritic properties.

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九州大学病院皮膚科におけるアトピー性皮膚炎初診患者のQOLに対する検討

—Skindex-16, DLQIを用いて

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要　旨

九州大学病院皮膚科を2007年1月から2008年12月に初診した2,643名のなかで，湿疹皮膚炎群の患者は28%（740名），そのうちアトピー性皮膚炎患者は8%（224名）であった．Skindex-16とDermatology Life Quality Index（DLQI）を用いたQOLの検討では，湿疹皮膚炎群患者のQOLは他疾患群患者に比べ有意に低下していたが，そのなかでもアトピー性皮膚炎患者のQOLはさらに有意に低下していた．全患者では，Skindex-16の総合，感情，DLQIの総合，症状感情，日常活動スコアで女性のQOLが男性よりも低下していたが，アトピー性皮膚炎患者においては男性が有意に高かったDLQIの治療スコアを除いて，男女間に差はみられなかった．年代別に比較すると，全患者では年代が低いほどQOLが障害されている傾向があったが，アトピー性皮膚炎患者ではその傾向がみられなかった．

はじめに

Skindex-16は皮膚疾患特異的なQOL評価尺度の一つであり，2001年にChrenらにより開発され，Skindex-61からSkindex-29, Skindex-16の短縮版が作成されている[1)~3)]．Skindex-16日本語版は2002年に作成され，日本での信頼性（reliability），妥当性（validity）が確認されている[4)]．症状・感情・機能の3つの下位尺度に属する16項目の質問からなり，患者は過去1週間に最も悩まされた皮膚症状について,「全く悩まされなかった」から「いつも悩まされた」の7段階選択肢から選択する.得点は0～100までのスコアに変換され，スコアが高いほどQOLが低いことを表す．DLQIは1994年に英国のFinlayらによって開発された皮膚疾患特異的QOL尺度である[5)]．日本語版は2002年に福原らによって作成され[6)]，痤瘡患者において計量心理学的な検証も行われている[7)]．10項目の質問からなり，患者はここ1週間で皮膚の状態が生活にどれくらい影響を与えたかを「非常に」=3点，から「全くない」=0点，又は「この質問は私にはあてはまらない」=0点の選択肢から選択する．総合得点と症状・感情，日常活動，レジャー，仕事・学校，人間関係，治療の6つの下位尺度からなり，それぞれの最高得点は30, 6, 6, 6, 3, 6, 3点であり，得点が高いほどQOLが低いことを表す.我々は2007年から九州大学病院皮膚科新患患者に対しSkindex-16とDLQIを用いたQOL調査を行っている[8)9)]．今回は特に2007年度，2008年度に受診したアトピー性皮膚炎初診患者について検討した．

対象と方法

2007年1月から2008年12月までに九州大学病院皮膚科外来を初診で受診し，QOL調査票に自己記入できる16歳以上の患者に対し，皮膚科外来受付で問診票と共にDLQI, Skindex-16の調査票を渡し，各自記入してもらった後，回収箱及び主治医が回収した．回収した質問票のうちすべての項目に記入がある2,643例に対し，主治医の記載とカルテを参照して年齢・性別・主病名一つを調査し解析した．統計解析にはSPSS 16.0Jを用い，Mann-Whitney U test, Spearman's correlation testを行った．有意水準は$P<0.05$とした．

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