Interleukin-31 (L-31) causes alloknesis: pain-stimulation becomes itch-stimulation in mouse skin

Iwao Arai 1, 2, Minoru Tsuji 1, Kazuya Miyagawa 1, Hiroshi Takeda 1, Nobutake Akiyama 2, Saburo Saito 2

1Department of Pharmacology, International University of Health and Welfare, 2600-1, Kitakanemaru, Ohtawara, Tochigi 324-8510, Japan
2Department of Molecular Immunology, Institute of DNA medicine, Jikei University 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan

Correspondence: Iwao Arai
E-mail: arai531206@gmail.com
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We investigated the effect of itchy skin on irritant-induced scratching behavior in relation to itch or pain expression in mice. Itchy skin was caused by either mite-infestation or the intravenous injection of IL-31. Scratching behavior was detected automatically and evaluated objectively using a personal computer. Two kinds of scratching behavior are observed in mice: long-lasting scratching (LLS) and short-lasting scratching (SLS). LLS (scratching duration over 1.5 s) is frequently seen in spontaneous skin-lesioned NC/Nga mice, but not in other strains of non-skin-lesioned mice. We investigated LLS as an indicator of itching induced by histamine, serotonin, compound 48/80, acetylcholine, bradykinin or capsaicin in mite-infested or IL-31-pretreated mice. These irritants caused many counts of SLS and a few counts of LLS for 60 min. Itchy-skin mice showed significant increases in LLS counts compared with a control group. These mice showed sustained scratching behavior, which serves as a measure of the itchy sensation, in response to not only pruritogens such as histamine, serotonin and compound 48/80, but also to algogens such as acetylcholine, bradykinin and capsaicin. These data suggest that pretreatment with IL-31 changes non-selective irritant stimulation into itch-stimulation in mice. The itchy skin in these mice closely resembled the “alloknesis” or “hyperknesis” observed in atopic dermatitis patients. Pain and itch are transmitted through the same nerve fibers, a sensation is perceived as pain or itch depending on the operation of IL-31.

Keywords: interleukin-31 (IL-31); pain; itch; alloknesis; pruritogen; algogen


Introduction

There is a broad overlap between pain- and itch-related peripheral mediators and/or receptors, and there is an astonishingly similar mechanism of neuronal sensitization in the peripheral and central nervous systems [1]. Current studies on itch are based on the human pruriceptive sense, and there are no discernable differences between these nociceptive stimuli. Since itching elicits a strong desire to scratch, the measurement of scratching is useful for evaluating itching [2].

In a previous investigation of spontaneous scratching in NC/Nga mice, an animal model of atopic dermatitis (AD), we designed a method for studying scratching behavior [3, 4]. There are two kinds of scratching behavior in mice: long-lasting scratching (LLS) and short-lasting scratching (SLS). In those studies, LLS was frequently seen in spontaneous skin-lesioned NC/Nga mice, but not in other strains of mice. In contrast, SLS was frequently seen in both skin-lesioned NC/Nga mice and other strains of mice. These results suggested that SLS is a form of social and/or hygiene
behavior, while LLS is the true itching response in these mice. We considered that we could differentiate between the sensory perception of a foreign substance and true itching by objectively dividing the scratching behavior of mice into LLS and SLS.

Interleukin-31 (IL-31) is a possible mediator of itching, and induces both severe pruritus and dermatitis in mice [5]. Furthermore, enhanced IL-31 expression levels have been observed in lesional skin in AD [6, 7]. Itch-associated scratching behavior can be induced by cohousing several strains of mice together with skin-lesioned NC/Nga mice [8]. In a previous study, we found that IL-31 mRNA was expressed exclusively in NC/Nga mice that exhibited LLS [9, 10]. However, the sites of action of IL-31 have not been clarified. Touch- or brush-evoked pruritus around an itching site has been termed ‘itchy skin’ or ‘alloknesis’, whereas pin prick-evoked itching sensations around an itching site have been termed ‘hyperknesis’ [11]. Recent studies in patients with chronic itching have demonstrated that repetitive painful stimuli, such as electrical, noxious heat pain or scratching stimuli distal to an itchy stimuli, may be perceived as an itch [12, 13]. This may also explain why scratching aggravates itching and induces a vicious cycle of scratching-induced itching [14]. In a previous study, we measured the LLS counts before and after mechanical scratching in skin-lesioned NC/Nga mice. While there was no significant overall change, the LLS count was increased immediately after the application of mechanical scratching [15]. This result suggests that alloknesis may be taking place in skin-lesioned NC/Nga mice. In this study, we investigated itching in response to several pruritogens or algogens in BALB/c mice either after they had been cohoused with skin-lesioned NC/Nga mice or after the intravenous injection of IL-31, and we considering a pain and itch expression from the regulatory mechanism of IL-31-induced alloknesis or hyperknesis.

Materials and Methods

Animals

Male 6-week-old BALB/c mice and NC/Nga mice at 15 weeks of age (skin-lesioned NC/Nga) were purchased from SLC Japan (Shizuoka, Japan). In the present study, all experiments were performed with BALB/c mouse. We used the NC/Nga mice only for the development of mite-infestation-induced itching caused by cohoused with skin-lesioned NC/Nga mice. The animals were housed under conditions of controlled temperature (23±3 °C), humidity (50±20 %) and lighting (lights on from 7:00 am to 7:00 pm). All animals were given free access to food and tap water. All procedures for animal experiments were approved by the Committee for Animal Experimentation at the International University of Health and Welfare and were in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006). Histamine (Wako Jyunyaku, Osaka, Japan), serotonin (Sigma-Aldrich, St Louis, MO, USA), compound 48/80 (Sigma-Aldrich), acetylcholine (Wako Jyunyaku), bradykinin (Wako Jyunyaku) or capsaicin (Wako Jyunyaku) were dissolved in saline and intradermally injected on the back and neck of the mice.

Reagents

Mouse IL-31 cDNA spanning amino acids 24 - 163 of IL-31 was cloned in-frame with pET30A (Novagen, Darmstadt, Germany) and the construct was transformed in BL-21 cells (Novagen). After induction with isopropyl-β-D-thiogalactopyranoside, IL-31 protein was purified under denaturing conditions by nickel-chelating sepharose (Qiagen, Benelux B.V. Netherlands), and dialyzed in phosphate buffer solution.

Measurement of scratching counts

Scratching was measured as we described previously [17]. The number of scratches was detected automatically and evaluated objectively using MicroAct [18] (Neuroscience, Tokyo, Japan). The analysis parameters for detecting waves were Threshold: 0.1 V, Event gap: 0.2 s, Minimum duration 1.5 s, Maximum frequency: 20 Hz, and Minimum frequency: 2 Hz. In the present study, two kind of scratching behavior in mice, i.e. LLS and SLS, were measured. The duration of LLS and SLS was prescribed as more than 1.5 s and 0.3 - 1.5 s, respectively.

Induction of Itchy skin

Mite infestation by cohousing with skin-lesioned NC/Nga mice might be one of the most important factors for inducing itch-associated scratching behavior (LLS) in several strains of mice [19]. Stable LLS was induced in BALB/c mice by cohousing them with skin-lesioned NC/Nga mice for 6 days (Arai et al., 2015). LLS was also induced by the local (intradermal, i.d.) or systemic (intravenous, i.v.) injection of IL-31 (1.0 µg/body: 50 µg/kg) in BALB/c mice. This dosage is based on our previous report [20]. The scratch count was measured as described above and compared between groups.

Measurement of compound-induced scratch counts

To elicit scratching behavior, 0.02 mL of histamine (2 µmol/site), serotonin (300 nmol/site), compound 48/80 (160 nmol/site), acetylcholine (1.5 µmol/site), bradykinin (200
nmol/site) or capsaicin (60 nmol/site) were injected 1 h after the administration of IL-31. And naïve or cohoused BALB/c mice with skin-lesioned NC/Nga mice. Immediately after the injection, the mice were placed in an observation chamber and scratching behavior was monitored every 10 min for 1 h.

Data analysis

All data obtained by this study were statistically analyzed using GraphPad InStat software. Experimental values are given as mean and S.E. Statistical comparisons were made using Student’s unpaired t-test or Dunnett’s multiple comparison in scratching counts test. P<0.05 value was considered as having statistical significance.

Results

Scratching behavior under mite-infestation induced by 6 days of cohousing with skin-lesioned NC/Nga mice

The LLS of mite-infestation BALB/c mice that had been cohoused with skin-lesioned NC/Nga mice for 6 days were significantly increased compared to those in non-cohoused BALB/c mice (Fig. 1A). In contrast, statistical differences of SLS counts were not observed between both groups (Fig. 1B). This increase in LLS showed a circadian rhythm: while there was a clear increase at night, the increase during daytime was not so clear, particularly from 9:00 to 14:00 (Fig. 1A). Therefore, we examined the scratching behavior induced by several stimulants from 9:00 to 14:00.

Pruritogen-induced scratching behavior in mice with itchy skin caused by mite-infestation

Fig. 2 shows traces of saline-, histamine-, serotonin- and compound 48/80-induced scratching behavior in mice with itchy skin caused by mite-infestation. These pruritogens elicited many SLS and a few LLS in normal mice (Fig. 2A, left side). In contrast, many LLS were elicited in mice with
itchy skin caused by mite-infestation (Fig. 2A, right side). In normal mice, saline, histamine, 5-HT, and compound 48/80 elicited SLS, but not LLS, which began immediately after injection during the initial 5 min observation period and persisted for at least 30 min. On the other hand, in mice with itchy skin caused by mite-infestation, these pruritogens and saline elicited significant increases in LLS counts (Fig. 2B), but not SLS counts (Fig. 2C). No significant differences in LLS and SLS were observed between each pruritogen- and saline-injected group in IL-31-pretreated mice (Fig. 2B and 2C).

**Effects of algogen-induced scratching behavior in mice with itchy skin caused by mite-infestation**

Fig. 3 shows traces of saline-, acetylcholine-, bradykinin- and capsaicin-induced scratching behavior in mice with itchy skin caused by mite-infestation. These algogens elicited a many SLS and a few LLS in normal mice (Fig. 3A, left side). In contrast, many LLS were elicited in mice with itchy skin...
caused by mite-infestation (Fig. 3A, right side). In normal mice, saline, acetylcholine, bradykinin and capsaicin elicited SLS, but not LLS, which began immediately after injection during the initial 5 min observation period and persisted for at least 30 min. On the other hand, in mice with itchy skin caused by mite-infestation, these pruritogens and saline elicited significant increases in LLS counts (Fig. 3B), but not SLS counts (Fig. 3C). Capsaicin induced LLS and SLS that persisted for 60 min. In addition, significant differences in LLS but not SLS were also observed between saline- and bradykinin or capsaicin-injected group in cohoused mice (Fig. 3B).

Effect of pretreatment with IL-31 on scratching behavior in mice

Intradermal pretreatment with IL-31 (1.0 µg/site, i.d.) increased LLS counts beginning 3 h after intradermal injection, and this returned to the basal level about 30 h after administration (Fig. 4A). On the other hand, systemic pretreatment with IL-31 (1.0 µg/site, i.v.) also increased LLS counts from 0.5 h after intravenous injection, and this returned to the basal level about 30 h after injection (Fig. 4B). In both cases, the increase in LLS counts showed a circadian rhythm: There was a clear increase at night, the increase at daytime is not as clear, particularly from 8:00 to 16:00 (Fig. 4B).
Therefore, we examined the effect of pretreatment with IL-31 on the scratching behavior induced by several stimulants from 9:00 to 14:00.

**Pruritogen-induced scratching behavior in mice with itchy skin caused by pretreatment with IL-31**

Fig. 5 shows traces scratching of saline-, histamine-, serotonin- and compound 48/80-induced scratching behavior in mice with itchy skin caused by pretreatment with IL-31. These pruritogens elicited many SLS and a few LLS in normal mice (Fig. 5A, left side). In contrast, many LLS were elicited in mice with itchy skin caused by pretreatment with IL-31 (Fig. 5A, right side). In mice that were pretreated with vehicle, saline, histamine, 5-HT and compound 48/80 elicited SLS, but not LLS, which began immediately after injection during the initial 5 min observation period and persisted for at least 30 min. On the other hand, in mice with itchy skin caused by pretreatment with IL-31, these pruritogens and saline elicited significant increases in LLS counts (Fig. 5B), but not SLS counts (Fig. 5C). Capsaicin induced LLS and SLS that persisted for 60 min. In addition, significant differences in LLS and SLS were observed between capsaicin- and saline-injection group in IL-31-pretreated mice (Fig. 5B and 5C).

**Algogen-induced scratching behavior in mice with itchy skin caused by pretreatment with IL-31**

Fig. 6 shows traces of saline-, acetylcholine-, bradykinin- and capsaicin-induced scratching behavior in mice with itchy skin caused by pretreatment with IL-31. These algogens elicited many SLS and a few LLS in normal mice (Fig. 6A, left side). In contrast, many LLS were elicited in mice with itchy skin caused by pretreatment with IL-31 (Fig. 6A, right side). In normal mice, saline, acetylcholine, bradykinin and capsaicin elicited SLS, but not LLS, behavior that began immediately after injection during the initial 5 min observation period and persisted for at least 30 min. On the other hand, in mice with itchy skin caused by pretreatment with IL-31, these algogens and saline elicited significant increases in LLS counts (Fig. 6B), but not SLS counts (Fig. 6C). Capsaicin induced LLS and SLS that persisted for 60 min. In addition, significant differences in LLS and SLS were observed between capsaicin- and saline-injection group in IL-31-pretreated mice (Fig. 6B and 6C).

**Discussion**

Many endogenous chemicals, e.g., amines, proteases, growth factors, neuropeptides and cytokines, are locally pruritogenic when injected into the skin [21]. However, there were not development of LLS to mice caused any other treatment except mite-infestation or IL-31 treatment in our experiment. Itchy skin mice caused by mite-infestation showed increased cutaneous IL-31 mRNA expression [9], so that LLS caused by mite-infestation may be regarded as an action of endogenous IL-31. Therefore, some researcher...
monitored the behavioral responses of mice to different stimuli. When histamine or capsaicin was injected into the cheek of the mouse, mice scratched the cheek with a hind limb in response to histamine, but wiped the cheek with a forelimb in response to capsaicin [22, 23]. In this study, we investigated LLS, as an indicator of itching, induced by histamine, serotonin, compound 48/80, acetylcholine, bradykinin or capsaicin on mice with itchy skin caused by either mite-infestation or pretreatment with IL-31. In mice with itchy skin induced by mite-infestation caused by 6 days of cohabiting with skin-lesioned NC/Nga mice, LLS caused by the injection of saline, pruritogens or algogens was significantly increased compared to that in non-mite-infested mice. Especially, algogen-induced LLS was much more clear than that induced by pruritogens, because almost none of LLS was induced by the injection of algogen in non-mite-infested mice. When applied on the human skin, capsaicin produces a burning pain, but not an itch sensation.

Figure 5. Effects of several pruritogens on scratching behavior in mice with itchy skin caused by IL-31. Typical traces of saline-, histamine-, serotonin- and compound 48/80-induced scratching behavior in vehicle- or IL-31-pretreated mice. Traces on the left show scratching behavior in vehicle-pretreated mice. Traces on the right show that in IL-31 (1 µg/body, i.v.)-pretreated mice (A). Effects of IL-31-induced itchy skin on LLS (itch-associated scratching behavior) counts induced by saline, histamine (Hist), serotonin (5-HT) or compound 48/80 (Co48/80) in mice (B). Effects of IL-31-induced itchy skin on SLS (locomotor activity) counts induced by saline, Hist, 5-HT, or Co48/80 in mice (C). Each value represents the Mean±S.E. NS, not significant compared with non-choesed mice. *P<0.05, **P<0.01 and ***P<0.001 compared with the respective values in the normal group. NS, not significant compared with saline-injected cohabited mice (Student’s t-test).
Kuraishi et al. reported that injection of capsaicin or formalin into the rostral back did not elicit scratching behavior in naive mice [2]. Although they measured the number of scratch counts manually using the image which was recorded by a video camera, the total of SLS and LLS counts are equal to our automatic measurements. When intradermal injected into the hind paw of naive mice, these algogens elicit licking of the treated paw [25, 26], a behavior considered to be a pain-related response, indicating that the animals felt pain rather than itch. Considering these previous reports, the preset findings suggest that mite-infestation-induced itchy skin may change pain-stimulation into itch-stimulation in mice. Although algogens reduced both LLS and SLS in non-cohoused mice, neither of these differences was significant. None of the pruritogens or algogens examined, except capsaicin, altered SLS. It is not clear why capsaicin-induced SLS was significantly increased. These results suggest that increasing itching was caused by non-selective stimulation in mice with itchy skin caused by mice-infestation. Since BALB/c mice are not sensitive to the pruritogen histamine a meaningful increase in SLS was not seen, while there was an increase in
LLS. This is the same as the reaction to saline. In IL-31 pretreatment mice, histamine-induced LLS decreased, but no significant different was observed compared with saline injection group (Fig. 5B). Since histamine only causes SLS, the reaction to histamine may be an intracutaneous pressure reaction rather than an original itch in ICR mice that are sensitive to histamine [27].

NC/Nga mice with itchy skin caused by mite-infestation showed increased cutaneous IL-31 mRNA expression [9]. LLS caused by the intravenous (systemic) injection of IL-31 was more sensitive than that caused by intradermal (local) injection. Based on this result, we used the systemic injection of IL-31 in the next experiment. In mice with itchy skin caused by intravenous pretreatment with IL-31, LLS caused by saline, pruritogens (histamine, serotonin and compound 48/80) or algogens (acetylcholine, bradykinin and capsaicin) was significantly increased compared to that in vehicle-pretreated mice. The SLS caused by compound 48/80, acetylcholine and capsaicin was also significantly increased, unlike in mice with itchy skin caused by mite infestation. These results suggest that increasing itching was caused by non-selective stimulation in mice with itchy skin induced by IL-31. In the present study, increase in LLS was also observed in mite-infestation or IL-31-pretreated mice that injected saline as a negative control. Although detailed reasons for this observation are unknown, one possibility is that itchy skin may be sensitive to not only chemical but also physical stimulation, e.g. picked up of epidermal skin with a finger, stings to a skin with a needle or liquid infusion to a skin. Namely, it is suggested that these nonspecific stimulation for naive mice changed in itch stimulation for mite-infestation mice to induce LLS. Further detailed study is necessary to prove this hypothesis. The mite-infestation- and IL-31 injection-induced increases in LLS, especially at night [3,17], including this experiment, may depend on the result of physical stimulation through a form of social and/or hygiene behavior (SLS) in mice (Fig. 1A).

Vogelsang et al. [28] reported that cutaneous sensations significantly differed, since all patients with atopic eczema complained of itching after the injection of acetylcholine, whereas controls reported burning pain. Moreover, Ikoma and co-workers reported that bradykinin, a classic endogenous algogen, can act as a potent histamine-independent pruritogen in lesional skin of atopic dermatitis [29]. These results show that hyperknesis or alloknesis is taking place in eczema in atopic dermatitis patients. Moreover, a recent human study suggested that IL-31 is associated with Th2-driven pruritic skin disease, and that IL-31 may help to cause the itch sensation. IL-31 expression in lesional AD skin is higher than that in non-lesional skin or skin-homing T cells [30]. Serum IL-31 levels are also increased and there is a significant correlation between these levels and disease severity in both adults and children [31]. Increased IL-31 mRNA levels in AD skin are driven by T cells and these cells may be involved in the pathogenesis of AD [32]. On the other hand, there are many reports indicating that IL-31 is not related to the disease of the itch directly [33-35]. Recently, we found that repeated injection of IL-31 gradually induced LLS in NC/Nga mice together with increase in expression of the IL-31 receptor A (IL-31RA) in dorsal root ganglion (DRG), though cutaneous IL-31RA was unchanged. Furthermore, there were close correlation between LLS and DRG IL-31RA, but not cutaneous IL-31 expression. Considering these previous findings, the present results suggest that IL-31 acting on DRG IL-31RA may cause alloknesis or hyperknesis, i.e., it changes non-selective irritant stimulation into itch-stimulation in mouse skin.

For decades, pain and itch were considered to be the same thing, but expressed at different intensities (the so-called “intensity theory”): itch was light pain, and pain was strong itch. However, recent studies have shown that pain and itch stem from a complicated process involving many types of neurotransmitters. While itch and pain can be extreme sensations, they are often originally not classified as itch or pain but rather as some non-harmful contact, such as increased temperature, a feeling of a foreign substance or an insect bite. In many cases we consider a delicate sensation to be an itch without definitely distinguishing it by cutaneous sensation. Moreover, we did not know whether nerves with itch receptors and pain receptors actually send both types of messages to the brain. A recent study showed that itch can be broadly characterized as either histamine-dependent or histamine-independent, both of which are relayed by a subset of C-fibers and by second-order neurons expressing gastrin-releasing peptide receptor and spinothalamic tract neurons in the spinal cord of rodents [36]. However, for pain and itch, neurotransmission involves transmission on the same nerve fibers: a sensation is perceived as pain or itch depending on the operation of IL-31.

In conclusion, pretreatment with IL-31 induced alloknesis or hyperknesis and increased the LLS caused by cutaneous injection of saline or several irritant compounds compared with that in a vehicle-pretreated control. IL-31 can change pain-stimulation into itch-stimulation in mouse skin.

**Conflict of interest**

None of the authors have any conflicts of interest in connection with this study.

**Abbreviations**

Author contributions

Iwao Arai designed the study, conducted the study, collected the data and prepared the manuscript, Minoru Tsuji collected the data and helped in the preparation of the manuscript, Kazuya Miyagawa collected and analysed the data, Hiroshi Takeda conducted the study and helped in the preparation of the manuscript, Nobutake Akiyama collected the data and helped in the preparation of the manuscript, Ikoma A, Fartasch M, Heyer G, Miyachi Y, Handwerker H, Schmelz M. Painful stimuli evoke itch in patients with chronic pruritus: central sensitization for itch. Neurology 2004; 62: 212-217.


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