5-Hydroxytryptamine (5-HT)\textsubscript{2} Receptor Involvement in Acute 5-HT-Evoked Scratching but Not in Allergic Pruritus Induced by Dinitrofluorobenzene in Rats

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Received January 15, 2003; accepted April 4, 2003

ABSTRACT

We investigated the role of serotonin (5-hydroxytryptamine; 5-HT)\textsubscript{2} and 5-HT\textsubscript{3} receptor subtypes in acute itch-associated scratching behavior as well as in an allergic pruritus model in rats. Intradermal 5-HT evoked hind limb scratching directed toward the injection site in naïve rats. Scratching behavior was significantly reduced by pretreatment with the 5-HT\textsubscript{2} receptor antagonist ketanserin. Intradermal injection of α-methylserotonin, a 5-HT\textsubscript{2} receptor agonist, also elicited scratching behavior in a dose-dependent manner, indicating that acute 5-HT-induced scratching is mediated via peripheral 5-HT\textsubscript{2} receptors. To produce a model of allergic pruritus, skin was sensitized by topical application of 5% dinitrofluorobenzene (DNFB). One month later, repeated challenge of the skin with 0.2% DNFB at weekly intervals elicited scratching as part of the immediate allergic response. Scratching was not affected by ketanserin or by the 5-HT\textsubscript{3} receptor antagonist ondansetron, indicating that neither 5-HT\textsubscript{2} nor 5-HT\textsubscript{3} receptors is involved in itch-associated scratching behavior caused by allergic skin dermatitis in rats.

Although histamine is a major mediator of itch in humans, antihistamine medication is often ineffective in reducing itch associated with systemic disorders such as cholestatic liver disease, or local skin conditions such as psoriasis or allergic dermatitis caused by insect bites or contact sensitizers (Wahlgren et al., 1990; Jones and Bergasa, 2000). The mechanisms and mediators of chronic itch under these conditions are poorly understood. Serotonin (5-HT) seems to be an important endogenous mediator of acute itch. It produces a sensation of itch when applied to the human skin (Weisshaar et al., 1997) and has been suggested to be involved in the pruritus of polycythemia vera (Fjellner and Hagermark, 1979; Fitzsimons et al., 1981) and cholestasis (Schworer et al., 1995; Weisshaar et al., 1999; Jones and Bergasa, 2000). 5-HT has been used widely as a representative pruritogen in experimental models with rodents because it induces itch-associated scratching behavior when applied intradermally, regardless of strain differences (Inagaki et al., 2001). In contrast, intradermal histamine induces scratching behavior in only a few strains of mice (Inagaki et al., 2001) and induces little if any scratching in rats (Thomsen et al., 2001; Jinks and Carstens, 2002). Because a considerable amount of spontaneous scratching is observed in untreated naïve mice, whereas naïve rats exhibit little or no spontaneous scratching, the rat seems to be a suitable animal model to quantify acute pruritogen-evoked scratching behavior. One goal of the present study was therefore to investigate which 5-HT receptor subtypes are involved in acute scratching elicited by intradermal 5-HT in the rat. Although 5-HT\textsubscript{3} receptors have been implicated in itch associated with cholestasis, uremia, and spinal opioids (Schworer et al., 1995; Balaskas et al., 1998; Kyriakides et al., 1999), 5-HT\textsubscript{3} receptor antagonists did not affect scratching elicited by intradermal 5-HT in mice, in contrast to the blockade of scratching by 5-HT\textsubscript{2} antagonists (Yamaguchi et al., 1999). These data argue against a role for 5-HT\textsubscript{3} receptors in acute evoked scratching in mice. In the first part of this study, we have investigated the role of the 5-HT\textsubscript{2} receptor in mediating acute scratching behavior in the rat.

A second goal of the present study was to develop a rat model of contact dermatitis and to investigate the potential role of 5-HT in allergic pruritus. Contact dermatitis is an allergic reaction induced by skin contact with irritants such as haptens and metals. It is characterized by clearly demarcated areas of rash at sites of exposure and is frequently accompanied by intense itching. The rash and itch improve upon removal of the offending agent. However, there is as yet no effective medication for this type of pruritus. Rodent mod-

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); DNFB, dinitrofluorobenzene; ANOVA, analysis of variance.
els of allergic pruritus have recently been developed (Tahara et al., 1999; Inagaki et al., 2000; Ohtsuka et al., 2001; Laidlaw et al., 2002). The mouse or guinea pig is passively sensitized with a murine monoclonal IgE antibody specific for the dinitrophenyl group and is later challenged with 2,4-dinitrofluorobenzene (DNFB) (Tahara et al., 1999; Inagaki et al., 2000) or repeated epicutaneous application of dinitrochlorobenzene (Laidlaw et al., 2002). In the present study, we have developed a rat model of allergic pruritus by first exposing the animals to DNFB and then quantifying the itch-associated scratching elicited by subsequent repeated challenge with DNFB. Finally, we investigated a role for 5-HT$_2$ and 5-HT$_3$ receptors in scratching using this model.

Materials and Methods

Animals. Adult male Sprague-Dawley rats, weighing 480 to 570 g, were used. The experimental protocol was approved by the University of California Davis Animal Use and Care Advisory Committee. Rats were housed in a room under controlled temperature (20 ± 2°C) and light (light on from 8:00 AM to 8:00 PM). Food and water were freely available.

Drugs. Serotonin hydrochloride (5-HT), $\alpha$-methylserotonin malate, ketanserin tartrate, and DNFB were purchased from Sigma-Aldrich (St. Louis, MO). Ondansetron hydrochloride was a gift from Professor Y. Kuraishi (Toyama Medical and Pharmaceutical University, Toyama, Japan). All agents except DNFB were dissolved in physiological saline (0.9% NaCl). The 5-HT and $\alpha$-methylserotonin solutions were prepared freshly on each experimental day. Ketanserin was also freshly prepared daily in a dose (3 mg/kg) that was intermediate between 1- and 5-mg/kg doses that have been used previously (Pourgholami and Goshadrou, 1995; Kettle et al., 1999). Solutions of DNFB at concentrations of 5% (398 mM) or 0.2% (16 mM) were prepared by dilution with acetone. Ketanserin and ondansetron were injected intraperitoneally in a volume of 0.1 ml/100 g body weight, 30 min before intradermal injection of 2% 5-HT or application of 0.2% DNFB.

Acute Scratching Elicited by Intradermal Injection. At least 3 days before receiving an intradermal injection, fur over the nape of the neck was gently grasped with forceps and the injection needle was advanced into the neck was gently grasped with forceps and the injection needle was advanced into the nape of the neck. Skin at the nape of each rat was cut carefully with scissors. To sensitize the animals to DNFB and then remove the fur over the rostral portion of the nape of each rat’s back was cut carefully with scissors. To sensitize the skin, a solution of 5% DNFB in a volume of 80 $\mu$l was applied to the middle portion of the exposed skin on the rostral back. Several days after the initial DNFB treatment, eroded skin was observed at the site of application in all rats. Approximately 1 month later, after the skin had recovered, 0.2% DNFB in a volume of 80 $\mu$l was applied to the same area that had previously been sensitized by DNFB. The 0.2% DNFB was reapplied in the same manner three additional times at 1-week intervals. Animals were videotaped immediately after each DNFB challenge and then removed. The challenge was repeated again for 90-min periods at 4 and 24 h after the fourth DNFB challenge to check for the presence of spontaneous scratching.

Because scratching was consistent across the 4 DNFB challenges (see Results; Fig. 4), we additionally tested the effects of ketanserin and ondansetron on DNFB-evoked scratching in three additional challenges given at weekly intervals. Thirty minutes before each challenge with 0.2% DNFB, each animal received either saline (control), ketanserin, or ondansetron i.p.

Observation and Scoring of Scratching Behavior. Procedures for observing and scoring scratching behavior were described previously (Jinks and Carstens, 2002). Briefly, all animals were acclimated to the observation chamber (30 × 30 × 40 cm) in three daily sessions before testing, and on test days for 30 min before treatment. After intradermal injection or application of DNFB, the rat was then quickly put back the observation chamber and videotaped from above using a digital video camcorder (12 bit, OPTURA Pi; Canon, Irvine, CA) for 40 (intradermal injections) or 60 min (DNFB application). Four rats were treated and videotaped simultaneously in separate chambers. Experimenters left the room during videotaping.

Analysis of videotapes was done by an experimenter blinded as to treatment condition. A scratching bout consisted of a series of one or more scratching movements by the hind paw that was directed toward the injection site, and ended when the rat either licked its hind paw or placed its hind paw back on the floor. The duration of each scratching bout was timed with a stopwatch. The total number of scratching bouts, and cumulative duration of bouts, was counted at 2-min intervals throughout the 40- or 60-min observation period. The mean duration of individual bouts was calculated by dividing total cumulative bout duration (40 or 60 min) by the number of scratching bouts (40 or 60 min) for each treatment group.

Statistical Analysis. Statistical comparisons were made using Student’s $t$ test, or one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison. A $p < 0.05$ was considered to be significant. The mean values of data are presented together with S.E.M.

Results

Acute Scratching by Intradermal 5-HT. Intradermal injection of 2% 5-HT elicited hind limb scratching directed toward the injection site after a lag time of several minutes (Fig. 1A). The total number of scratching bouts, and cumulative duration of bouts recorded over the 40 min observation period, were both significantly higher in 5-HT-treated animals compared with controls receiving intradermal saline (Fig. 1, B and C, respectively). No scratching was observed in three of eight control rats. The mean individual bout duration in 5-HT-treated rats of $\sim 2$ s was approximately double that observed in controls (Fig. 1D).

5-HT$_2$ Receptor Involvement in Acute Scratching. To investigate the involvement of the 5-HT$_2$ receptor subtype in acute 5-HT-induced scratching, two experiments were undertaken. In the first, we assessed the effect of pretreatment with the selective 5-HT$_2$ receptor antagonist ketanserin on acute scratching behavior elicited by intradermal 5-HT. Ketanserin at a dose of 3 mg/kg markedly reduced 5-HT-induced scratching (Fig. 2A). Two of the eight rats pretreated with ketanserin exhibited no scratching at all, although these animals did not show any signs of motor impairment. The total number of scratching bouts, as well as the cumulative duration of bouts over the 40-min observation period, were...
both significantly lower in animals pretreated with ketanserin compared with controls receiving saline followed by intradermal 5-HT (Fig. 2, B and C, respectively). However, the mean individual bout duration was not significantly different between treatment groups (Fig. 2D). No scratching was observed after i.p. administration of ketanserin before intradermal 5-HT.

To confirm the involvement of the 5-HT₂ receptor subtype, a second experiment was undertaken to assess scratching elicited by intradermal injection of the selective 5-HT₂ receptor agonist 5-HT₆-methylserotonin. Intradermal injection of 2% 5-HT₆-methylserotonin elicited hind limb scratching directed toward the injection site with a lag time of around 10 min (Fig. 3A). Lower concentrations of 5-HT₆-methylserotonin elicited scratching in a concentration-dependent manner (Fig. 3, A and B). The total number of scratching bouts, as well as mean cumulative duration of bouts over the 40-min observation period, were significantly higher at all concentrations of intradermal 5-HT₆ compared with controls receiving intradermal saline (designated as 0% 5-HT₆-methylserotonin) (Fig. 3, B and C, respectively). The mean individual bout durations of ~1.5 to 2 s were not significantly different among groups receiving different concentrations of intradermal 5-HT₆-methylserotonin, although the mean individual bout duration for each of these groups was significantly greater compared with controls receiving intradermal saline (Fig. 3D).

**Scratching in Allergic Pruritus Model Using DNFB.** Rats were first sensitized by a topical application of 5% DNFB to skin on the rostral back. This initial application of DNFB elicited a very low number of scratching bouts during the 60-min observation period [Fig. 4A, top; 6.1 ± 1.3 (S.E.M.) bouts/60 min, n = 10], although this was significantly greater compared with control topical application of vehicle (acetone), which elicited negligible scratching (0.5 ± 0.4 bouts/60 min, n = 10). One month later, the skin was challenged by application of 0.2% DNFB to the same skin area previously treated with 5% DNFB. Each of the 10 rats exhibited pronounced hind limb scratching directed toward the treatment area (Fig. 4A, middle). Scratching began after a lag time of several minutes, peaked at about 10 min, and subsided within 30 min after DNFB application (Fig. 4A, middle).

The skin treatment area was repeatedly challenged with 0.2% DNFB at 1-week intervals. All rats exhibited scratching
to the second, third, and fourth DNFB challenges over a time course that was similar to that observed with the first DNFB challenge. Figure 4A, bottom, shows mean scratching bouts/2 min to the fourth DNFB challenge, which is similar to the first challenge (Fig. 4A, middle). To determine whether any spontaneous scratching occurred in these animals after the fourth DNFB challenge, they were again videotaped for 90 min at 4 and 24 h after the fourth DNFB challenge; the mean number of scratching bouts was 1.1 ± 1.0 bout/90 min and 0.8 ± 0.4 bouts/90 min, respectively. At the conclusion of each videotaping session, visual observation of the treated skin area did not reveal obvious edema.

The mean total number of scratching bouts and mean cumulative bout duration per 60 min elicited by the series of DNFB challenges are plotted in Fig. 4, B and C, respectively. These values did not vary significantly across the four challenges with 0.2% DNFB but in each case were significantly greater compared with these measures of scratching elicited by the initial application of 5% DNFB. Likewise, the mean individual bout duration of ~2 to 2.5 s did not vary across repeated challenges with 0.2% DNFB but was significantly greater compared with the initial application of 5% DNFB (Fig. 4D).

DNFB-Elicited Scratching Is Not Reduced by 5-HT<sub>2</sub> or 5-HT<sub>3</sub> Antagonists. To investigate the involvement of endogenous 5-HT<sub>2</sub> or 5-HT<sub>3</sub> receptor subtypes in scratching elicited by DNFB challenge, the effects of the selective antagonists ketanserin and ondansetron were tested. One, 2, and 3 weeks after the fourth DNFB challenge, each rat received i.p. injection of either saline, ketanserin, or ondansetron, followed 30 min later by skin challenge with 0.2% DNFB as before. Rats receiving i.p. saline followed by challenge with DNFB (fifth challenge) exhibited scratching bouts over a time course (Fig. 5A, top) that was indistinguishable from that observed after each of the first four DNFB challenges (Fig. 4A). In the rats treated with 3 mg/kg ketanserin followed by DNFB (sixth challenge; Fig. 5A, middle) or ondansetron followed by DNFB (seventh challenge; Fig. 5A, bottom), the time course of scratching bouts was similar to that of the i.p. saline controls (Fig. 5A, top). The mean total number of scratching bouts (Fig. 5B), as well as mean cumulative bout duration (Fig. 5C), were not statistically different across treatment groups, although there was a trend toward longer cumulative bout duration with ketanserin (Fig. 5C). Likewise, the mean duration of individual scratching bouts did not differ significantly across treatment groups (Fig. 5D).
Discussion

5-HT₂ Receptor Involvement in Acute Scratching Elicited by Intradermal 5-HT. In naive rats, intradermal injection of 5-HT elicited intermittent bouts of hind limb scratching directed toward the injection site at the nape of the neck. Scratching affords mild noxious mechanical counterstimulation at the site of pruritic stimulation to presumably reduce itch sensation. The number of scratching bouts elicited presently by intradermal 5-HT was markedly suppressed by systemic administration of the selective 5-HT₂ receptor antagonist ketanserin. Furthermore, intradermal injection of the selective 5-HT₂ receptor agonist α-methylserotonin also elicited scratching bouts whose number varied in a concentration-dependent manner. At the highest α-methylserotonin concentration used (2%), the mean number of scratching bouts and cumulative bout duration, as well as the prolonged time course of scratching, were comparable with the parameters of scratching elicited by 2% 5-HT (Figs. 1 and 3). The present findings confirm a previous report that itch-associated scratching induced by intradermal 5-HT is mediated via the 5-HT₂ receptor subtype in mice (Yamaguchi et al., 1999).

In rats, 5-HT seems to be one of the most consistent and effective scratch-inducing agents. Intradermal injection of other agents that induce itch in humans, such as histamine, compound 48/80, substance P, and proteases, elicited little or no scratching in Sprague-Dawley rats (Thomsen et al., 2001; Jinks and Carstens, 2002). Rodent mast cells contain relatively high concentrations of 5-HT (Gustafsson, 1980; Graziano, 1988; Purcell et al., 1989), consistent with a role for 5-HT as an itch mediator in rodents.

The average duration of individual scratching bouts was fairly constant at ~1.5 to 2 s across all treatment conditions, with the exception that the mean bout duration was shorter for the infrequent scratching bouts that occurred after control intradermal saline injections or first-time application of DNFB. We have separately observed that the within-bout scratching frequency remains constant at a mean of ~8 Hz across a variety of different pruritic (intradermal 5-HT) and antipruritic (systemic naltrexone) treatment conditions.
Given that both the mean duration of scratching bouts, and the within-bout scratching frequency, are fairly constant, we conclude that the number of scratching bouts over time is the most valid measure of the intensity of itch-related scratching behavior.

**Allergic Pruritus Model.** Previous studies have used repeated application of haptens such as DNFB to the ear of rodents as a model of contact dermatitis (Tahara et al., 1999; Inagaki et al., 2000; Natsuaki et al., 2000; Laidlaw et al., 2002). Most of these studies have focused on hapten-induced allergic skin reactions but have not extensively examined pruritus. For this reason, we presently determined whether exposure of skin to DNFB might induce an immune response such that subsequent challenge with DNFB elicits an allergic reaction that includes pruritus as assessed by scratching. Although the initial application of DNFB (5%) to the skin elicited very little scratching, subsequent challenges to the treated skin area with a much lower concentration of DNFB (0.2%) indeed elicited significant scratching, suggesting that itch occurs as part of the allergic response. It was previously reported that exposure of guinea pigs to another hapten, 2-4 dinitrochlorobenzene, resulted in a significant increase in scratching behavior over a 15-h postexposure period (Laidlaw et al., 2002). Although we observed significant scratching in response to the DNFB challenge in the sensitized rats, we did not observe any large degree of spontaneous scratching at 24 h after the fourth DNFB challenge, which corresponds to the late phase of allergic skin reactions (Tahara et al., 1999; Natsuaki et al., 2000). Our data thus suggest that sensitization with DNFB did not in itself produce a condition of chronic itch, but rather sensitized the animal so that a subsequent challenge elicited an immediate allergic response that includes itch.

It is of interest to compare the present results with those of a related study of scratching elicited by mosquito bites (Ohtsuka et al., 2001). In the latter study, mice were initially exposed to mosquito bites or received intradermal injection of mosquito salivary gland extract. They were subsequently challenged with mosquito bites (or salivary gland extract) twice weekly. There was a progressive increase in the number of scratching bouts that became significant after 7 and 2 weeks, respectively, for mosquito bites and injections of salivary gland extract (Ohtsuka et al., 2001). Scratching elicited by mosquito bites was not affected by an H1 histamine an-
It was concluded that the scratching is part of an immediate allergic reaction to a constituent of the mosquito salivary gland extract that is independent of histamine release from cutaneous mast cells. In the present study, the scratching elicited by acute challenge with DNFB in previously sensitized rats may also represent an immediate allergic reaction. This scratching is unlikely to be mediated by histamine release because Sprague-Dawley rats do not exhibit significant scratching after intradermal histamine (Thomsen et al., 2001; Jinks and Carstens, 2002), nor was it affected by selective 5-HT$_2$ or 5-HT$_3$ receptor antagonists (Fig. 5). The shorter time course and lack of 5-HT$_3$ involvement differentiate the scratching response in this allergic pruritus model from the more prolonged time course of acute scratching elicited by intradermal 5-HT via the 5-HT$_2$ receptor subtype (Figs. 1–3). The important question of which mediators cause itch during an acute allergic reaction remains to be answered.

The scratching presently observed after each DNFB challenge was fairly consistent in terms of the time course of scratching bouts, the total number of scratching bouts, and both individual and cumulative bout duration (Fig. 5). This result was fortuitous because it afforded us the possibility to investigate the effects of 5-HT antagonists on DNFB-evoked scratching. Ketanserin at a dose that significantly suppressed scratching elicited by acute intradermal injection of 5-HT (Fig. 2) did not affect scratching elicited by the DNFB challenge in sensitized rats (Fig. 5). We additionally investigated a possible role for 5-HT$_3$ receptors, because antipruritic effects of 5-HT$_3$ receptor antagonists, including ondansetron have been reported for patients with cholestatic and uremic pruritus (Schworer et al., 1995; Balaskas et al., 1998). Moreover, the selective 5-HT$_3$ receptor antagonist tropisetron reduced itch elicited by application of 5-HT to skin in which mast cells had been previously depleted (Weisshaar et al., 1999). However, ondansetron did not affect the scratching elicited by DNFB challenge in sensitized rats (Fig. 5). These results therefore indicate that endogenous 5-HT acting through 5-HT$_2$ or 5-HT$_3$ receptors is not importantly involved as a mediator of itch-associated scratching behavior in the present model of allergic pruritus. This further suggests that the mechanism underlying allergic pruritus in this model differs from acute itch-related scratching via 5-HT$_2$ receptors. Nevertheless, future studies using such newly devel-
oped rodent models will hopefully shed light on the mediators of itch associated with allergic reactions and other skin disorders, thereby providing a rational basis for the development of new antipruritic treatments.

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