Acta Derm Venereol 2001; 81: 250–254

INVESTIGATIVE REPORT

Scratch Induction in the Rat by Intradermal Serotonin: a Model for Pruritus

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Eight substances (histamine, compound 48/80, kallikrein, trypsin, papain, substance P, serotonin and platelet activating factor) were injected intradermally (volume 50 μl) into the rostral back (neck) of rats in order to establish an animal model for peripherally elicited pruritus. While serotonin induced excessive scratching at the site of injection, the other substances were weak or inactive. The dose–response relationship of serotonin was sigmoid, EC_{50} = 2.1 mg/ml (95% confidence interval: 1.0 to 4.3 mg/ml). Injections of serotonin 1 mg/ml into the caudal back elicited no scratching at all, i.e. neither at the site of injection nor elsewhere, so the experiment indicated no systemic effect of serotonin 1 mg/ml intradermally. Scratching was probably elicited histamine-independently, since histamine itself did not elicit scratching. The intra- and inter-observer variations were 3–4%. We conclude that serotonin is a reproducible local pruritogen eliciting scratching in the rat. The model may be useful in research and development of topical antipruritics of the non-histaminic type as well as for various other purposes in pruritus research. ***Key words: histamine; compound 48/80; proteases; substance P; platelet activating factor (PAF).***

(Accepted May 16, 2001.)

Acta Derm Venereol 2001; 81: 250–254.

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Only a few valid animal models for peripherally elicited pruritus have been established. Scratching has been induced by means of injection of chemical substances into the skin (or subcutaneous tissue) in mice (1–8), guinea pigs (9) and rats (5, 10–13), and reactions vary between species. Intradermal injection of prostaglandin E_2 induces a strong itch-scratch response in guinea pigs (9), but does not elicit scratching in mice (2). In humans, histamine is the best characterized pruritogen, but does not induce scratching in guinea pigs (9). In mice, Kuraishi et al. found no participation of histamine in scratching behaviour (8), while Inagaki et al. found participation of both histamine H_1 and histamine H_2 receptors in passive cutaneous anaphylaxis-induced scratching behaviour in ICR mice (14). In research to find new antipruritic drugs, there has therefore been a focus on establishing animal models. However, the large species variation obviously makes it difficult to come up with a relevant human itch model, since screening of different itch inducers in rodents might not be relevant in humans. Consequently, animal models for pruritus comprise scratching in different species as a result of injection of chemical substances all known to induce itching in humans.

We aimed to develop a model for experimental pruritus in the rat by injecting substances already known to be pruritogenic in humans, and at the same time to compare pruritus elicited by test substances in the rat with human observations in order to discuss the relevance of this rat model.

MATERIALS AND METHODS

Animals

The study comprised 168 female Sprague Dawley rats [MOL, Iffa Credo (200 g) – Charles River, France] kept in a quiet room and housed under conventional conditions on wood chip bedding in a Scantainer with two rats per cage (type 3 cages). There was only one rat per cage during the actual study; a 12:12 h dark and light (230 Lux) cycle, and constant temperature 23 ± 1°C. Air humidity was not controlled and relative humidity varied with outer relative humidity (i.e. 35–50%). Conventional food pellets (Altromin® 1324, Brogaarden Denmark) and tap water were provided ad libitum.

Substances

The test substances were prepared from lyophilized crystalline powder of the following chemicals: histamine-dihydrochloride [0.1 mg/ml (low) and 10 mg/ml (high)], serotonin-hydrochloride (10 mg/ml), phosphatidylcholinechloride (synonyms platelet activating factor or PAF) (0.01 mg/ml), substance P (10 mg/ml), compound 48/80 (1 mg/ml), papain [1 mg/ml (activity 10–30 units per mg protein)], bovine trypsin [1 mg/ml (activity 10000–13000 BAEE units per mg protein, chymotrypsin < 0.1 BTEE units per mg protein)] and bovine kallikrein [1 mg/ml (activity minimum 40 units per mg solid)]. These eight chemicals (all from Sigma, St. Louis, USA) were solubilized in sterile physiological saline and injected within 10 min after solubilization.

Study design

Procedures. Test substances (saline, serotonin, trypsin, kallikrein, papain, compound 48/80, substance P, histamine and platelet activating factor (PAF)) (4 rats per substance) were injected intradermally (50 μl) into the rostral back area (defined as the area between the scapulae on a line between the lower angle of each scapula). Each rat was used only once. The syringes used for the injections were 29G insulin injection needles. The injection site had been shaved 2 days prior to injection as pilot studies had shown that this was necessary to eliminate the influence of shaving on background scratching. Immediately after the injection, the rats were placed in individual cages for video-recording of scratching activity (see below for details of equipment). The recording time was 2.5 h.

Serotonin dose response study. A dose–response study of serotonin was performed based on the results of the initial screening study. Initially, serotonin (0.1 mg/ml, 50 μl and 1 mg/ml, 50 μl) was injected intramuscularly and subcutaneously into the rostral and caudal back area in 14 rats to evaluate possible systemic effects of serotonin. The

 ISSN 0001-5555

caudal back area was defined as the skin over the spine 1 cm proximal to the root of the tail. Another 4 rats were injected intradermally in the caudal back with a high dose of serotonin (10 mg/ml, 50 µl). Following this, serotonin was injected intradermally in concentrations from 0.01 mg/ml to 31.6 mg/ml (10 rats in each group). Two rats were given injections of 100 mg/ml.

Evaluation study. In order to validate the model, a blinded study was performed in which the rats were shaved in both the rostral and the caudal back. Each rat was randomly given an intradermal injection of serotonin 1 mg/ml in one of these areas. After registration of scratching, the code was broken. Recordings in the blinded study were viewed a second time 6 weeks after the first view (intra-observer variation). This was done by the most experienced observer, who had viewed more than 300 recordings of rats. The recordings were also viewed by another observer (inter-observer variation), who had about 40 recordings up to that time.

Video recordings

The rats were recorded with 2 black-and-white CCD video cameras (Topica model TP 606) placed above the cages so that the rats could be seen directly from above. Recordings were made in authentic real time speed and videotaped on a Time Laps video recorder (Sony model SVT-L230P). The rats were evaluated visually by an observer at a speed 3 times as fast. Scratching was defined as motoric behaviour when the hindpaws moved up to the site of injection and touched the skin at least once (usually more). Only scratching at the site of injection was scored, and one scratch sequence could comprise one to several frequent touches of the skin. Still, this was registered as only one scratch sequence. A pause of more than 1 sec was defined as the end of a scratch sequence. Movements with the forelimbs were not scored as scratching, but instead were regarded as grooming activity.

Statistics

Number of scratch sequences was normally distributed (as judged graphically), and Student's unpaired *t*-test was used to compare the groups of rats. The dose–response data were fitted to the sigmoid curve by the least squares method implemented in GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). The EC_{50} was estimated with 95% confidence limits. Intra- and inter-observer assessments were compared using Student's paired *t*-test. The intra-and inter-observer variation, the inter-rat variation and the method variation (residual variation) were estimated by analysis of a variance component model with observer, observation number (first or second observation by the first observer) and rat as effects. Variation was expressed as CV% (SD as percentage of the mean). The VARCOMP procedure of SAS (SAS Institute Inc., SAS OnlineDoc®, Version 8, Cary, NC: SAS Institute Inc., 1999) was used for the analysis. $P < 0.05$ (two-tailed) was considered significant.

RESULTS

Screening study

Fig. 1 shows the initial screening of the 8 substances. All injections were given in the shaved rostral back. When serotonin was deemed especially pruritogenic, more rats were studied (as well as in the saline control group) for later blinded comparisons and dose–response investigations. High concentrations (about half the maximal amount of substance possible to solubilize) were used for all substances (except for PAF). Serotonin 10 mg/ml in the initial screening of substances elicited by far the highest number of scratch sequences, and was therefore studied in more detail.

Serotonin dose–response study

When viewing the videotapes, the number of scratch sequences was registered at 5-min intervals. In this way we could describe

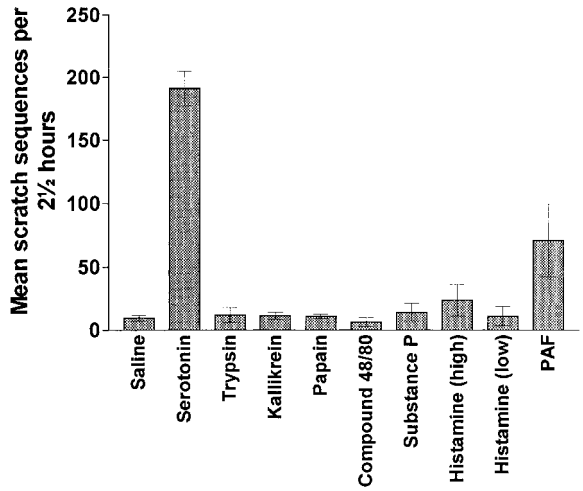

Fig. 1. Number of scratch sequences (mean ± SEM) in response to injections into the rostral back (50 µl) of 8 different substances (recordings are 2.5 h, with saline as a control); $n = 4$ for all groups except saline and serotonin, where $n = 10$. Trypsin, kallikrein, papain and compound 48/80 are at concentrations of 1 mg/ml. Substance P, histamine (high) and serotonin are at concentrations of 10 mg/ml, while histamine (low) = 0.1 mg/ml and PAF = 0.01 mg/ml. (PAF = platelet activating factor.)

a profile of itch intensity for each rat. Profiles for 2 selected concentrations and saline are shown in Fig. 2. The standard observation period was 2.5 h. Concentrations of 0.01 mg/ml, 0.1 mg/ml, 2 mg/ml, 5 mg/ml and 31.6 mg/ml are omitted from the figure, since these show the same profile, i.e. an increasing number of scratch sequences until maximum at about half an hour, followed by a decline

Each concentration of serotonin within the effective range produced a different itch curve. When the concentration of serotonin rose, the area under the curve (AUC) increased. The AUC is plotted against $\log_{10}$ of the concentration injected in Fig. 3, where it can be seen that the data fitted a sigmoid curve. The EC_{50} was 2.1 mg/ml (95% confidence interval: 1.0 to 4.3 mg/ml).

The initial subcutaneous and intramuscular injections of serotonin 0.1 mg/ml and 1 mg/ml revealed a low scratching

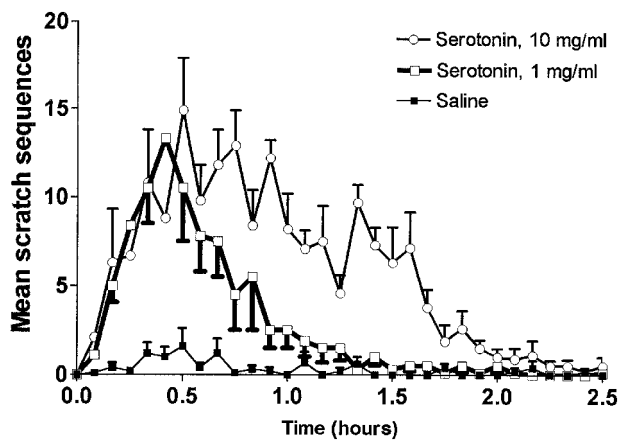

Fig. 2. Different concentrations of serotonin injected into the rostral back, and itch profile curves. Scratching was registered at 5 min intervals. Error bars represent SEM ($n = 10$).

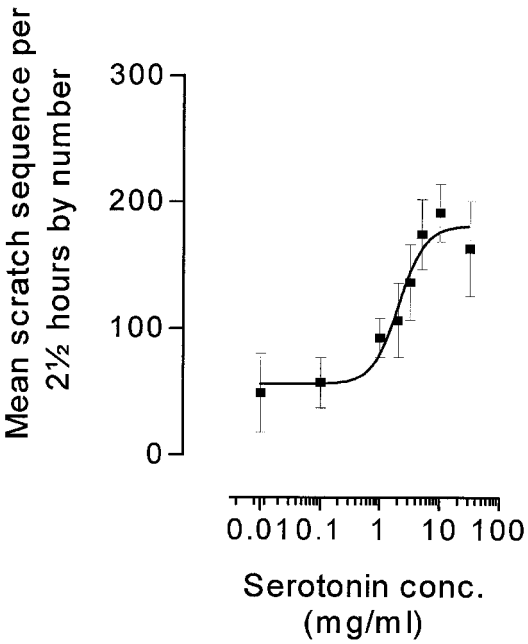

Fig. 3. The dose–response relationship of scratching as a function of the concentration of serotonin injected into the rostral back. The number of scratch sequences (mean scratch) is the area under the curve (AUC) of all the itch curves of serotonin concentrations tested. The data fitted a sigmoid curve as a function of $\log_{10}$ to the concentration injected. Error bars represents ± 2 SEM ($n = 10$).

activity, while injections of a high serotonin concentration (10 mg/ml) in the caudal back resulted in many scratch episodes outside the rostral back (primarily the ears and nose) (results not shown).

Evaluation study

To assess whether the shape of the itch profile curves was a consequence of rapid absorption of serotonin into the blood stream, or whether scratching was due to a sensation of the skin, 24 rats were shaved in both the rostral and caudal back. Injections were given randomly at either site and serotonin 1 mg/ml was chosen since there was no indication of a systemic effect at this concentration. Scratch movements with the hindpaws were counted and registered as 1) scratching of the rostral back, 2) scratching of the caudal back, or 3) scratching outside these two areas (Table I). When serotonin was injected into the rostral back, the number of scratch sequences (79.7 ± 43.4; mean ± SD) directed toward the site of injection was significantly greater ($p < 0.001$) than the number directed towards the rostral back when serotonin was injected into the caudal back. When serotonin was injected into the caudal back, the lower number of scratch sequences was not compensated by more scratching outside the area. In fact, serotonin injected into the rostral back also elicited significantly ($p = 0.027$) more scratching outside the 2 shaved areas compared to injections in the caudal back. No scratching of the caudal back area was observed when serotonin was injected into either the rostral or the caudal back; the rats probably could not reach this area.

The intra- and inter-observer variations were also assessed, and 6 weeks after viewing the recordings in the blinded study, the 12 recordings of rats injected in the rostral back were reviewed in random order (intra-observer variation). Results of scratching the rostral back are given in Table II (upper third). Mean difference is 4.7 scratch sequences (SD = 9.7), but the results are not significantly different from each other. The inter-observer variation is assessed in the lower two-thirds of Table II. There was a significant difference between the scoring of the second observer and the first observer's first results, but not his second results. In all cases the mean observer difference was below 10 scratch sequences. The intra- and inter-observer variations were 3–4% (SD as percentage of the mean), the inter-rat variation about 50% and the method variation about 10% (estimated by analysis of variance components).

DISCUSSION

In the first part of this study (the screening study) 8 different substances, all known to be pruritogenic in humans, were screened for their pruritogenic potential in rats. Since scratching decreased after daily repeated injections of serotonin in the same rat (results from pilot studies not shown here), each rat received only one injection. The rostral back was used as the site of injection, because the rat can reach this skin area only with the hindpaws. The screening study identified two candidate substances for further studies – serotonin and PAF. In higher concentrations, PAF did not lead to scratching at all (results from pilot studies not shown here) why serotonin was chosen for a more detailed study.

In a dose–response study, serotonin was injected intradermally in concentrations from 0.01 mg/ml to 31.6 mg/ml. The intradermal injections of very high concentrations (100 mg/ml) in 2 rats led to necrosis (and only very few scratch sequences) and was not investigated further. When the concentration of injected serotonin was increased from 0.01 mg/ml, the AUC of the itch profile curves also increased, primarily due to a longer duration of scratching. The AUC of each itch profile curve was plotted against $\log_{10}$ of the serotonin concentration and the dose–response relationship was found to be sigmoid, with an EC_{50} of 2.1 mg/ml. Usually, there was a lag time of 5–10 min before scratching started. The lag could be

Table I. *Injections into the rostral back frequently elicit scratching at the site of injection, but also outside this site. Recordings (mean ± SD) represent scratch sequences scored in the first hour following injections (n = 12)*

	Injections into rostral back	Injections into caudal back	*p*-value
Scratch sequences related to rostral back	79.7 ± 43.4	6.7 ± 9.2	< 0.001
Scratch sequences related to caudal back	0	0	
Scratch sequences, other sites	31.0 ± 16.6	14.3 ± 11.5	0.027

Table II. *Intra- and inter-observer variation studies (registered scratch sequences per 1 h; mean ± SD)*

Observer A Assessment 1	Observer A Assessment 2	Observer B	Difference ± SD	Paired *t*-test (*p* value)
79.7 ± 43.4	75.0 ± 39.8		4.7 ± 9.7	0.124
79.7 ± 43.4		72.0 ± 39.6	7.7 ± 8.4	0.009
	75.0 ± 39.8	72.0 ± 39.6	3.0 ± 11.0	0.36

The upper third of the table represents the intra-observer variation study. The video recordings were assessed by the same observer (A) a first and a second time with a 6-week interval. Another observer (B) independently assessed the recordings and the results were compared with the findings of observer A (inter-observer variation). There is a statistically significant difference between the results of observer B and those of the first assessment by observer A (middle third of the table). However, a second assessment by observer A is not statistically different from the second assessment of B (lower third of the table).

the time for local distribution and absorption of serotonin into the blood stream, or simply a transient neuronal disturbance due to the injection trauma. It could also be a result of metabolism of serotonin (i.e. serotonin in the skin was metabolized into a more active pruritogenic substance).

The blinded evaluation study was then carried out in order to detect any systemic effects of serotonin and to assess the intra- and inter-observer variation. Injections of serotonin 1 mg/ml in the rostral back induced scratching, while injections into the caudal back elicited no scratching at all (see Table I). Site dependency of scratching with the hindpaws allowed the conclusion that scratching of the rostral back was due to a local stimulus from the injected skin area, and was not a response to systemic absorption of serotonin into the blood stream. When serotonin was injected into the caudal back, the rats probably could not reach this area with the hindpaws. However, a surrogate behaviour was observed. Rats could be seen sitting and biting the skin at the injection site. The intra-observer variation study revealed no significant difference in the number of scratch episodes when the videotapes were viewed the first ($n = 12$) and second ($n = 12$) time by the same observer. When another person assessed the recordings and the results were compared with the first observer's results (inter-observer variation), a statistically significant difference in the number of scratch sequences was found. However, the intra- and inter-observer variations were only 3–4%.

In Berendsen & Broekkamp's rat model with subcutaneous injection of serotonin in the rostral region (11), any scratch movement with the hind limbs was counted (not just scratching at the injection site). Like us, they found increased scratching intensity until about half an hour after injection followed by a decline, but contrary to us they concluded that scratching with the hindpaws in their model was due to systemic absorption of serotonin.

Serotonin is a known histamine liberator, but neither histamine itself nor the histamine-releasing compound 48/80 induced scratching in the present study. Thus scratching in the rat due to injections of serotonin is probably elicited in a histamine-independent way. In humans, too, serotonin is a local pruritogen and has its own pruritogenic potency not only acting over histamine containing mast cells (15).

It is very difficult to determine whether scratching is a result of itch, pain or some other sensation. The question is central to itch research in animals, but unfortunately we cannot ask the animal. Nevertheless, in a study by De Castro-Costa et al., both morphine and acetylsalicylate, but not the antihistamine drug astemizole, depressed scratching behaviour in arthritic rats, and it was concluded that scratching was due to pain (10). On the other hand, Kuraishi et al. induced scratching behaviour in mice with pruritogenic (compound 48/80 and substance P) but not algesiogenic agents (capsaicin and dilute formalin) (8). In the present study, we found no indications of histamine being important to scratching behaviour in rats, not even at a concentration of 10 mg/ml, so H_1-receptor antagonists are hardly able to reduce scratching behaviour in rats. Furthermore, scratching activity was greatly reduced in the present study when necroses developed at the site of injection. If serotonin-induced scratching in rats was due to pain, then one would expect necrosis of the skin to lead to intense scratching rather than little scratching. We therefore believe that scratching in the present study was due to a pruritic sensation.

In conclusion, this animal model is a candidate for pruritus research in dermatology and could be useful in screening programmes and for further investigations into topically applied antipruritic (non-histaminic) test compounds in drug development. The model appears relevant since serotonin is already recognized as a weak local pruritogen in humans.

ACKNOWLEDGEMENTS

The Dermatological-Pharmacological Research Centre (Chairman: Torkil Menné, project coordinator Eva Benfeldt) was established in 1997 comprising Clinical Pharmacological Unit, (head Jesper Sonne), and the Department of Dermatology, both at Gentofte Hospital, and the Department of Dermatological Research, Leo Pharmaceutical Products. The Research Centre is supported by a grant from the Danish Medical Research Council and is part of Centre for Drug Design and Transport (Chairman Sven Frokjaer).

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