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The Bee Venom Test:
A New Tonic-Pain Test

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Abstract

The present study describes a new test of tonic pain in rats which can be used as an animal model of persistent pain. In the first experiment, the response to subcutaneous injection of various doses of bee venom into the hind paw of the rat was quantified. The second experiment investigated the effect of morphine and aspirin on the response to an intermediate dose of bee venom. Finally, the third experiment examined the response to concurrent injections of bee venom and formalin. Subcutaneous injection of bee venom produced local inflammation, marked edema, and tonic pain responses. Increasing doses of bee venom produced higher mean pain scores and increased durations of responding. Pain responses lasted up to approximately one hour and the inflammation and edema were virtually gone by 8 hours with the lower doses of bee venom tested and by 2 days with the two highest doses tested. Analgesia was produced by morphine and aspirin, indicating that the bee venom test can be used to test analgesic drugs. Concurrent administration of bee venom and formalin produced responses similar to formalin alone, with an increased duration of responding at higher intensities. The data suggest that the bee venom test is a valid animal model of experimental tonic pain.

Résumé

Cette étude décrit un nouvel examen de la douleur tonique dans le rat qui peut être utilisé comme modèle animal de la douleur persistante. D'abord, la réaction à des injections souscutanées de dosages variables de venin d'abeille dans la patte arrière du rat a été quantifiée. Ensuite, l'effet de la morphine et de l'aspirine sur la réaction à un dosage intermédiaire de venin d'abeille a été examiné. De plus, la réaction à des injections simultanées de venin d'abeille et de formol a été examinée aussi. L'injection souscutanée de venin d'abeille produit de l'inflammation locale, de l'oedème prononcé, et une douleur tonique. Des dosages plus élevés augmentent la moyenne des résultats de douleur, et prolongent aussi la durée de la réaction. Les réponses de la douleur durent jusqu'à approximativement une heure et l'inflammation et l'oedème sont presque disparues après 8 heures avec des dosages plus bas et après 24 heures avec des deux dosages plus hauts. La morphine et l'aspirine ont un effet analgésique, indiquant que le test de venin d'abeille peut être utilisé pour examiner les drogues analgésiques. L'administration simultanée de venin d'abeille et de formol a un effet semblable à la réaction à l'administration de formol seulement, avec une augmentation de la durée de réponses aux hautes intensités. Les données suggèrent que le test de venin d'abeille est valable comme modèle animal de la douleur tonique expérimentale.

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Introduction

The concept of pain has changed as researchers and clinicians attempt to understand the mechanisms of chronic pain which are not helped by available treatments. Our recent concept of pain has changed from specificity theory to pattern theory. Despite this change. pain and analgesia research continues to utilize ideas and tools that originate in specificity theory.

Pain Theories

According to specificity theory, an injurious event activates pain receptors which produce nerve impulses that travel along pain fibres and pathways to a pain centre in the brain (Melzack and Wall, 1988). An early formulation of specificity theory by Descartes viewed the human body as a machine which reacted to noxious stimuli by the flow of "animal spirits" (Foster, 1970, p.261) through nerves which served as tubes that ended in the pineal gland (Foster, 1970). Injury by a noxious stimulus such as a flame produced pain perceived by the mind, which resided in the pineal gland, the way pulling a rope at the bottom of a church tower rings the bell at the top (Melzack and Wall, 1988). As such, the human body was no different from hydraulic automata in a garden that can play music and move in response to an approaching spectator.

Approximately two hundred years later, Müller's doctrine

of specific nerve energies, and von Frey's deduction that free nerve endings could be identified as pain receptors (Boring, 1942), expanded specificity theory even further, and initiated a search for pain receptors, pain fibres, and a pain centre in the brain. The search proved to be fruitless. For example, the cornea of the human eye has only free nerve endings yet it can respond to painful stimuli as well as to a variety of innocuous stimuli such as touch, cold, and warmth (Lele and Weddell, 1956). Furthermore, C-fibres which respond to noxious stimuli can be stimulated and fired without producing the experience of pain (van Hees and Gybels, 1981). Moreover, an area in the brain that is specifically associated with pain has not been found (Melzack and Wall, 1988). Despite these negative data, many researchers and textbooks still ascribe to specificity theory.

A major shortcoming of specificity theory is that the importance of previous sensory experience is not acknowledged. According to specificity theory, pain experience requires the activation of pain receptors. Thus, specificity theory has difficulty explaining clinical cases of causalgia in which the patient experiences pain after gentle innocuous stimulation. Nor can it explain the delay between stimulation and the onset of pain, as in cases of post-herpetic neuralgia (Melzack and Wall, 1988).

Pattern theories, such as the gate-control theory (Melzack and Wall, 1965; 1988) and Melzack's neuromatrix

theory (1989; 1990; 1992), explain anomalous cases of pain more adequately than specificity theory. According to these theories, patterns of nerve impulses are actively integrated by the nervous system to produce the experience of pain. Hence, the nervous system is not a passive responder to noxious stimuli. Rather, the ongoing activity of the nervous system is actively integrated over time, and the production of pain depends on the state of the nervous system at the moment as well as on previous activity. In addition, the integrating substrates are modulated by sensory input and ongoing activity.

The gate-control theory of pain (Melzack and Wall, 1965; 1988), proposes that transmission cells (T-cells) in the spinal cord integrate activity of afferent C-fibres that respond to noxious input with the activity of afferent A-fibres that transmit information about innocuous input; they are also influenced by descending inputs from the brain. The integrated input determines and modulates the activity of the T-cells so that the likelihood of impulses being transmitted to more central structures is variable. The gate-control theory is characterized by plasticity and the integration of sensory inputs as well as internal neural factors.

Research has shown that the gate-control theory is a good predictor of pain as a function of certain conditions. For example, pain produced by innocuous stimulation can be explained by the integration of impulses from A-fibres which

normally have a net inhibitory effect on T-cells but, in certain pathologies, have a net excitatory effect. The descending effect on the 'gate' also explains how intense stimulation at a distance from a painful locus can nevertheless diminish the pain (Melzack and Wall, 1988).

The neuromatrix (Melzack, 1989; 1990; 1992) is also a concept of an active, plastic nervous system responsible for pain perception. In this model of the nervous system, the neuromatrix--a network of circuits in the brain--integrates peripheral and central activities which determine the activity of the integrating structures and, hence, their influence on other parts of the nervous system. Certain structures, such as the hypothalamus, have an essential role but none are the sought-after pain centre or pain pathways that can be removed to eliminate pain perception entirely or permanently. In this model, it is the ongoing activity of circuits within the nervous system, as well as past and present sensory experience, that determines whether the experience of pain exists at a given time.

Clinical cases suggest that the neuromatrix is a more accurate model of our pain production mechanisms than a straight-through sensory system. For example, specificity theory cannot account for the incidence of phantom limb pain in patients that have had a limb amputated. According to specificity theory, surgical removal of the somatosensory pathways in phantom limb patients should lead to complete and

permanent relief, but experience has demonstrated that it does not (White and Sweet, 1969). The neuromatrix provides a possible explanation for phantom limbs and the pain often associated with them. The neuromatrix continuously generates a 'neurosignature' that indicates the body is intact. It can do so without sensory input, as in cases of phantom limbs in congenitally limb-deficient patients, but it also integrates sensory information that often is exhibited later as a painful phantom which resembles a previously experienced pain in the intact limb (Melzack 1989; 1990; 1992).

One important feature of pattern theories is the acknowledgement that both present and past neural activity determines whether or not pain is perceived. The integration of noxious input can affect the structures receiving that input and can determine the context in which further noxious input is perceived. Thus, perception of a brief painful injury has very different properties from those of a longer-lasting painful event. (This fact will be discussed shortly.) Because pattern theories provide a mechanism by which previous painful experiences can influence present perceptions, they are able to explain clinical cases of onset of pain that do not have an apparent immediate organic cause or injury, such as delayed onset phantom limb pain. Despite this, many investigators still ascribe to the inadequate, simplistic views of specificity theory.

Pain Tests

In studies of pain and analgesia using animal subjects, experimentally-induced pain is used to engage the mechanisms that are thought to be active in clinical pain states. The fact that phasic-pain tests are most commonly used to evoke experimental pain reflects the predominant, specificity-derived view of pain. Phasic-pain tests, such as the hot-plate (Woolfe and MacDonald, 1944) or tail-flick (D'Amour and Smith, 1941) tests, involve the application of a noxious mechanical, electrical, or thermal stimulus that lasts a brief period of less than a minute, usually only seconds, and is terminated at threshold level by the subject's response (Dennis and Melzack, 1979a; Dubner, 1994; Melzack and Wall, 1988).

Consider the tail-flick test, originally described by D'Amour and Smith (1941) and later modified, as an example. The rat is restrained as its tail is placed on a hot plate or in hot water, or a beam of light is focused on the tail. As the stimulus intensity increases, the animal responds by removing its tail from the source of heat and the stimulation is terminated. The time to respond is the measure of pain sensitivity. Other methods of producing phasic pain responses include mechanical stimulation (paw-pressure test) and electrical stimulation (painful shocking of the tail or foot) (Dubner, 1994; Vyklický, 1984).

Phasic-pain tests can be run in a relatively short period

of time and are therefore convenient for the experimenter. This fact is possibly the source of reluctance to stop using them. Implicit in the use of phasic-pain tests, and in accordance with specificity theory, is the idea that pain, whether it is a clinical syndrome or experimentally-induced, is due exclusively to activation of peripheral receptors.

Tonic-pain tests are also used to induce experimental pain and they differ in several respects from phasic-pain tests. The formalin test (Dubuisson and Dennis, 1977; O'Keefe, 1964), the most commonly used tonic-pain test, consists of a subcutaneous injection of a formaldehyde solution usually given in the hind paw of a rat. The response is biphasic: the first phase occurs within 5-10 minutes and, following a 5-10 minute decrease in pain scores, the second phase begins approximately 20 minutes after the injection of formalin, lasting up to 60 minutes (Dubuisson and Dennis, 1977; Vaccarino and Melzack, 1992). The evoked behaviour includes favouring, elevating, and licking, biting, or shaking the injected paw. The second phase is the most useful since it is more like persistent clinical pain than the first phase (Vaccarino and Melzack, 1989).

Tonic-pain tests involve different neural substrates than those in phasic-pain tests. For instance, Vaccarino and Melzack (1989) have demonstrated that injection of lidocaine into the anterior cingulum bundle of the rat significantly lowered formalin pain scores but had no effect on the rat's

latencies to flick its paw out of 50°C water in the foot-flick test. Other research (Ryan et al, 1985) has shown that bilateral lesions of the dorsolateral funiculus spinal pathway in the rat have differential effects on morphine analgesia, eliminating systemic morphine analgesia when measured in the tail-flick test but not affecting morphine analgesia in the formalin test. Abbott et al (1982b) also demonstrated that lesions of the bulbar raphe nuclei or of the caudal periaqueductal gray attenuate morphine analgesia in the tail-flick test but not in the formalin test. Dennis and Melzack (1979a) and Melzack (1986) have even proposed that, based on neuroanatomical observations, there are two distinct spinal cord sensory systems, one whose transmission is fast and especially suited for phasic pains, and one slower for tonic pains.

Another respect in which tonic-pain and phasic-pain tests differ is that certain pharmacological agents, such as cholinergic agents, dopaminergic agents, and antisympathetic drugs, have different effects depending on the pain test used. Dennis and Melzack (1983) demonstrated analgesia in the formalin test following administration of choline, atropine, mecamyline, and apomorphine. At the same doses, these drugs did not produce any significant analgesia in two phasic-pain tests, the hot-plate test and the tail-flick test, except that apomorphine produced analgesia in the tail-flick test. The same drugs also facilitated morphine analgesia in the formalin

test but not in the hot-plate or tail-flick tests (Dennis and Melzack, 1983). Coderre et al (1984) found that two other drugs, guanethidine and FLA63, produced analgesia in the tail-flick test but not in the formalin test. Together, these results suggest that different underlying mechanisms with different pharmacological bases subserve tonic and phasic pain.

Support for the use of tonic-pain tests over phasic-pain tests is the fact that pain in the formalin test more closely resembles clinical pain than does pain in phasic-pain tests. The duration and intensity of the pain in the formalin test is more similar to that of clinical pain (Dennis and Melzack, 1979a; Dubuisson and Dennis, 1977) since in clinical cases, the patient may experience pain that lasts for hours, days, or even years. In phasic-pain tests, the subject terminates the stimulus shortly after it reaches the pain perception threshold. Also, phasic-pain tests permit the subject to control the pain by responding, while in tonic-pain tests the subject must cope with the (time-limited) pain, just as in clinical pain syndromes. It should be noted that although the animal does not have control of the formalin-induced irritation, there is a defined pain end-point, approximately 90 minutes after formalin injection, after which the animal shows no pain behaviours.

Further evidence that tonic-pain tests are more relevant to clinical pain is provided by Abbott et al (1981; 1982a) who

examined morphine analgesia and tolerance in tonic-pain and phasic-pain tests. Abbott et al (1981) gave rats daily injections of 7 mg/kg morphine for 5 days and 20 days, respectively. The next day, the rats were administered 7 mg/kg morphine 10 minutes prior to the formalin test. All of the rats showed the same degree of morphine analgesia as rats that received daily injections of saline for 5 days prior to testing. That is, the rats did not exhibit significant tolerance in the formalin test.

In another study, Abbott et al (1982a) exposed rats daily to morphine and tonic or phasic pain. They injected rats with 8 mg/kg for 8 days followed by 16 mg/kg for 12 days before exposing them to phasic-pain in the tail-flick test or 3-5 minutes tonic-pain from subcutaneous hypertonic saline injection into the paw. After 20 days of morphine exposure, tail-flick-experienced rats were tested in the tail-flick test and displayed a 2.69-fold increase in the ED_{50} compared to morphine-injected, tail-flick-tested rats with no prior pain test experience. Hypertonic-saline-experienced rats were tested in the formalin test and showed no further decrease in the effectiveness of morphine compared to morphine-injected, formalin-tested rats with no prior tonic-pain experience. The rats received daily hypertonic saline injections instead of formalin injections since formalin produces local tissue necrosis (Abbott et al, 1982b; Dubuisson and Dennis, 1977; Rosland et al, 1990). It is possible that situation-specific

tolerance (Siegel, 1976) did not develop when rats received the formalin test because the context differed in some way from the hypertonic saline injection. At the very least, it can still be concluded that prior daily exposure to morphine and tonic pain does not produce tolerance when tested in a tonic-pain test. The lack of morphine tolerance in the formalin test resembles the clinical situation since, after titration to the appropriate dose, there is little or no tolerance to the analgesic effects of opioids with prolonged use by cancer patients (Mount et al, 1976; Portenoy, 1995).

Finally, the affective quality of tonic experimental pain is more like that of clinical pain. Since clinical pain has an affective component (Melzack and Wall, 1988), and the anterior cingulum bundle is involved in formalin-induced pain (Vaccarino and Melzack, 1989), this suggests that tonic pain has a strong affective component since limbic structures are involved in emotional processing (Papez, 1937). In a study examining the affective component of different experimental pains (Chen and Treede, 1985), human subjects completed the McGill Pain Questionnaire after they were administered phasic pain evoked by intracutaneous electrical stimuli and tonic ischemia pain evoked by exercising an arm whose circulation was obstructed by an inflated sphygmomanometer cuff. The number of words chosen to describe the pain in the affective dimension was significantly higher for the tonic ischemia pain than the phasic pain, indicating that the ischemia pain is

more similar to clinical pain. Indeed, the score for the affective dimension for the phasic pain was 1.3 compared to 4.9 for the ischemia pain, which is closer to the range for cancer pain (2.3-4.0), lumbalgia (3.5), phantom limb pain (3.2), and headache (4.3-4.6) (Chen and Treede, 1985). Rainville et al (1992) also provide support for this claim. They have shown that two tonic pains, the pain of ischemic exercise and cold-pressor pain, are rated as more unpleasant by human subjects--and thus more like clinical pain--than the pain from contacting a hot thermode and painful electrical shock, both phasic pains.

To summarize, tonic pain and phasic pain involve different neural substrates and pharmacological bases. In addition, the lack of tolerance to morphine in situations of tonic pain, and the duration, intensity, and affective component of tonic experimental pain resemble clinical pain more than the properties of phasic pain do. Therefore, tonic-pain tests are more appropriate to use in experiments that study mechanisms of pain perception relevant to clinical cases of persistent or prolonged pain.

In addition to the formalin test, other animal models of experimentally-induced inflammation and tonic pain are available. For example, acute inflammation can be induced by local injection of irritants such as kaolin and carrageenan into the knee joint, evoking behavioural changes within 60 minutes (Schaible et al, 1987). Chronic inflammatory

arthritis can be produced by injection of complete Freund's adjuvant (De Castro Costa et al, 1981) or of sodium urate crystals (Coderre and Wall, 1987; Okuda et al, 1984) into an animal's ankle or knee joint, evoking behaviour indicative of pain within a few hours which persists for several days to 3-4 weeks. However, the utility of the inflammation models is limited by the delay of an hour or more prior to the appearance of significant effects. Also, if a procedure or mechanism under study is thought not to involve processes that require the subject to experience pain for long periods, then the persistent pain between observation periods would be unnecessary and should be avoided.

In the search for an alternate test to the formalin test, the administration of several noxious substances has been studied by Wheeler-Aceto et al (1990). They examined the responses of rats to subcutaneous injection of acetic acid, carrageenan, formalin, kaolin, platelet-activating factor, serotonin, and yeast, and topical mustard oil application. Only formalin and acetic acid produced pain behaviours that were prolonged and relatively intense. Acetic acid produced a response with a peak in frequency of flinches at approximately 30 minutes and a peak in time spent licking at approximately 15 minutes. They concluded that formalin injection is the best alternative since it produces the most vigorous response, and it is preferred because it produces a biphasic response, allowing for "the study of acute [first

phase] and tonic [second phase] pain" (p.238). Unfortunately, the first phase necessarily precedes the phase of tonic pain, prohibiting the exclusive study of tonic pain in the formalin test.

Models using thermal stimulation to evoke tonic pain are also available but are not favoured. For example, Meyer and Campbell (1981) exposed the skin on the hand of monkeys and human subjects to 53°C for 30 seconds. Hyperalgesia developed within minutes and persisted for 2 hours. The stimulation causes tissue damage and, therefore, precludes retesting, thereby diminishing the value of this procedure.

Models of persistent pain that involve nerve injury or deafferentation in rats (Dennis and Melzack, 1979b) exist and result in scratching, biting, and autotomy of the affected area. Although the behaviour has been shown to be a response to pain and not a response to the insensitive limb as a foreign object (Coderre et al, 1986), the method has a few shortcomings. First, the model involves a technical surgical procedure which makes it inconvenient for frequent and routine use. Second, it is uncertain how the affected nervous system compares to the normal unaffected nervous system and therefore this type of model is best suited for studies of pain resulting from deafferentation, such as that due to brachial plexus avulsion.

Paradigms also exist which assess pain in animals using unlearned behaviours such as an escape response, or learned

behaviours such as bar-pressing (Dubner, 1994). These methods still require that pain be produced using available pain tests. The discussion here is not concerned with the assessment of pain as such, but instead with methods of inducing pain in experimental animal subjects.

Purpose

The purpose of this study is to develop a new method of evoking pain that meets as many of the following requirements for testing pain mechanisms in animals as possible. These requirements are based on suggestions proposed by Dubner (1994), Vierck and Cooper (1984), and Vyklický (1984), with slight modifications.

Regarding the stimulus:

1) The stimulus should be natural and quantifiable.

2) There should be little or no tissue damage with repetitive stimulation.

Regarding the response:

3) The response produced should be discernible from the response to innocuous stimuli and from normal behaviour without stimulation.

4) At least some of the responses should not be simple reflexes.

5) The response should have a clearly identifiable pain

end-point after which no pain behaviours are observed.

6) Restraint of the subject during responding should be avoided.

7) A relationship should exist between the intensity of the stimulus and the intensity of the pain experienced.

8) The response evoked should be susceptible to pharmacological manipulations that alter the perceived intensity of noxious stimuli.

Regarding the method as a whole:

9) The method should be easy to use.

10) The method should be applicable to humans and animals.

Phasic-pain tests fulfil most of these requirements (Dubner, 1994; Vierck and Cooper, 1984; Vyklický, 1984). The thermal, electrical, and mechanical stimuli are readily quantifiable, and thermal and mechanical stimuli are natural, but electrical stimuli are not. Little or no tissue damage occurs with repetitive stimulation, but only because limits are imposed on the duration of exposure to the stimuli. The responses evoked, such as tail-flicks, foot-flicks, and escape responses, are distinguishable from responses to innocuous stimuli and from normal behaviour, but in most cases the responses are simple reflexes. No pain behaviours are exhibited after the stimulus is terminated, satisfying the

suggestion of a clear pain end-point. In phasic-pain tests, "the magnitude of the response cannot be related to the stimulus intensity in most applications" (Chapman et al, 1985, p.3), but the response is usually sensitive to analgesic agents. The methods are relatively simple to use, with some technical knowledge requirements of the stimulation apparatus, and in most cases, the methods are applicable to humans and animals alike since precautions, such as the one to prevent tissue damage, are taken. One drawback of these methods is that, in most cases, in order to administer the stimuli the animal must be restrained during testing, the stress of which may influence the animal's response (Dubner and Bennett, 1983). The most serious shortcoming is that the pain produced is phasic.

In addition to the above suggestions, and of great importance, the response evoked should be one of tonic pain. That is, it should persist for a sufficiently long time to bear some resemblance to longer-lasting forms of pain in human subjects suffering persistent pain. Although this would preclude the animal from having control of the noxious stimulus, it is necessary to evoke tonic pain instead of phasic pain so that the findings from use of the test are more relevant to clinical pain for the reasons discussed earlier. To compensate for lack of control, the evoked response should be of a limited duration and have a clear end-point.

This study describes the development of a new,

potentially useful tonic-pain test, the bee venom test. A bee sting results in a "sharp prick which is followed by pain lasting for a few minutes up to 1/2 hour. The pain is usually slight but can be severe" (Keele and Armstrong, 1964, p.228). Hence, bee venom injection is a prime candidate for investigation as a tonic-pain test for use in animals. In Experiment 1, the response to subcutaneous injection of various doses of bee venom was quantified. In Experiment 2, the response to an intermediate dose of bee venom was examined after administration of morphine and aspirin. Finally, Experiment 3 was performed in an attempt to induce a moderately intense pain that lasts longer than the pain in the bee venom test or the formalin test by concurrent administration of both bee venom and formalin. The results of these experiments show that the bee venom test is a valid test for use in research on the basic mechanisms of pain and analgesia.

General Methods

Subjects

Subjects were male Long-Evans hooded rats, weighing 255-405 g. The rats were housed two to three per cage, with food and water available *ad libitum*, and they were kept on a 12 hour light/ 12 hour dark cycle with the lights on from 7 AM. All testing was performed between 10 AM and 4 PM. Only one rat was tested at a time, and each rat was tested only once.

Procedure

The rats were habituated to the 30cm x 30cm x 30cm transparent plexiglass observation box in the testing room for 30-60 minutes on a day prior to testing. On the day of testing, each rat was placed in the observation box for 30-60 minutes before it was removed from the box and received a 0.05 ml subcutaneous injection of bee venom, formalin, or saline under the plantar surface of one hind paw. In each group of ten rats, five rats received the injection in either hind paw.

Bee venom was lyophilized whole venom of *apis mellifera* purchased from Sigma Chemical Company and dissolved in saline. The bee venom is obtained by placing bees on a 6 mm wire grid, which is electrically pulsed. The bees then produce venom which drops to a glass plate beneath. The venom is collected from the glass, freeze-dried, and packaged for sale. Formalin was 10% buffered formalin acetate diluted in saline to produce 2.5% formalin. Sterile, non-pyrogenic 0.9% saline was used.

Immediately after injection, the rat was placed in the

observation box. A mirror mounted at a 45° angle below the floor of the box and a large mirror behind the box facilitated observation of the injected paw. The behaviour of the rat was scored using a method similar to the method described by Dubuisson and Dennis (1977). The behaviour was scored as a '2' if the rat licked, bit, or shook the injected paw; as a '1' if the rat elevated the paw without biting, licking, or shaking it; and as a '0' if any part of the paw, other than the tips of the digits, was in contact with the floor of the box. The behaviour was scored as it occurred, using a computer program that recorded the score once every half second.

Statistical Analyses

A mean pain score was calculated for the entire observation period and for individual 5-minute time bins as the sum of the scores divided by the number of scores summed. This is mathematically equivalent to the weighted-scores technique described by Dubuisson and Dennis (1977). An overall between-within repeated measures analysis of variance (ANOVA) was performed to test for group effects, and to test for group x time interactions. ANOVAs, without repeated measures, were performed to test for significant group differences between mean pain scores of individual time bins. Post-hoc Tukey HSD tests were performed, when necessary, to identify which group means were significantly different. An effect was determined to be significant if the p value was

less than, or equal to, 0.05.

Despite the fact that a similar scale used has been demonstrated to be an ordinal scale (Coderre et al, 1993), parametric analyses were performed instead of nonparametric analyses for several reasons (Harris, 1995). First, the scale is not purely ordinal. Although the relative distances between values of the scale are unknown, the calculated mean pain scores are meaningful since they indicate the relative amounts of extreme values ('0's and '2's) that were observed. Second, parametric analyses were necessary to investigate the group x time interaction of the repeated-measures data. Finally, although parametric tests are usually more powerful than nonparametric tests, two comparisons--in Experiment 2 with morphine pretreatment at 40 and 45 minutes--which were not significantly different with parametric analyses were found to be significantly different with a Kruskal-Wallis one-way ANOVA for nonparametric data. All other one-way ANOVAs demonstrated significant differences with both types of analyses.

Experiment 1: Dose/Response Relationship

Methods

Six groups of ten rats were used. After habituation, each rat received a 0.05 ml injection in the hind paw of either saline or bee venom at one of five doses: 0.01 mg, 0.05 mg, 0.1 mg, 0.2 mg, or 0.3 mg. One bee sting is estimated to contain 0.1 mg of dried bee venom (Barker et al, 1963; Habermann 1971; Habermehl, 1981). The behaviour of each rat was recorded for 60 minutes.

Results

Injection of bee venom into the hind paw of the rat produced local inflammation, marked edema, and continuous pain responses. The pain responses are shown in Figure 1. There was a significant overall effect of dose of bee venom on the mean pain scores ($p < 0.001$) and a significant time x dose interaction ($p < 0.001$).

Figure 2 shows the mean pain scores for the 60-minute period following injection of various doses of bee venom or saline. There was a significant effect of dose of bee venom ($p < 0.001$) and all but three pairwise comparisons were significantly different: 0.3 mg compared to 0.2 mg of bee venom, 0.05 mg compared to 0.01 mg of bee venom, and 0.01 mg of bee venom compared to the saline group were not significantly different. The relationship between dose and mean pain score for the 60-minute period fits a logarithmic curve with an $R^2 = 0.88$. Although the relationship fits a

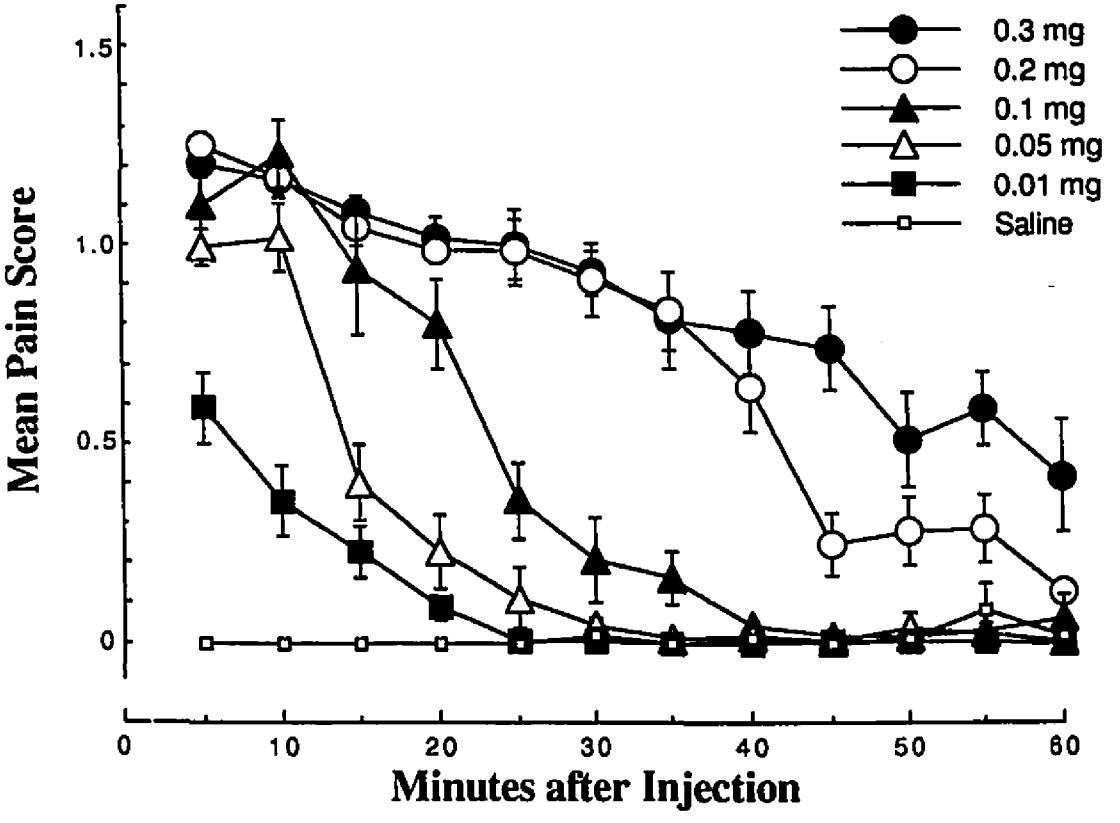

Figure 1. Responses of rats to subcutaneous injection of various doses of bee venom or saline (n = 10 per group). Each data point is the mean pain score for the preceding five-minute period. Error bars indicate SEM. There is a significant overall effect of dose of bee venom on the mean pain scores ($p < 0.001$) and a significant time x dose interaction ($p < 0.001$).

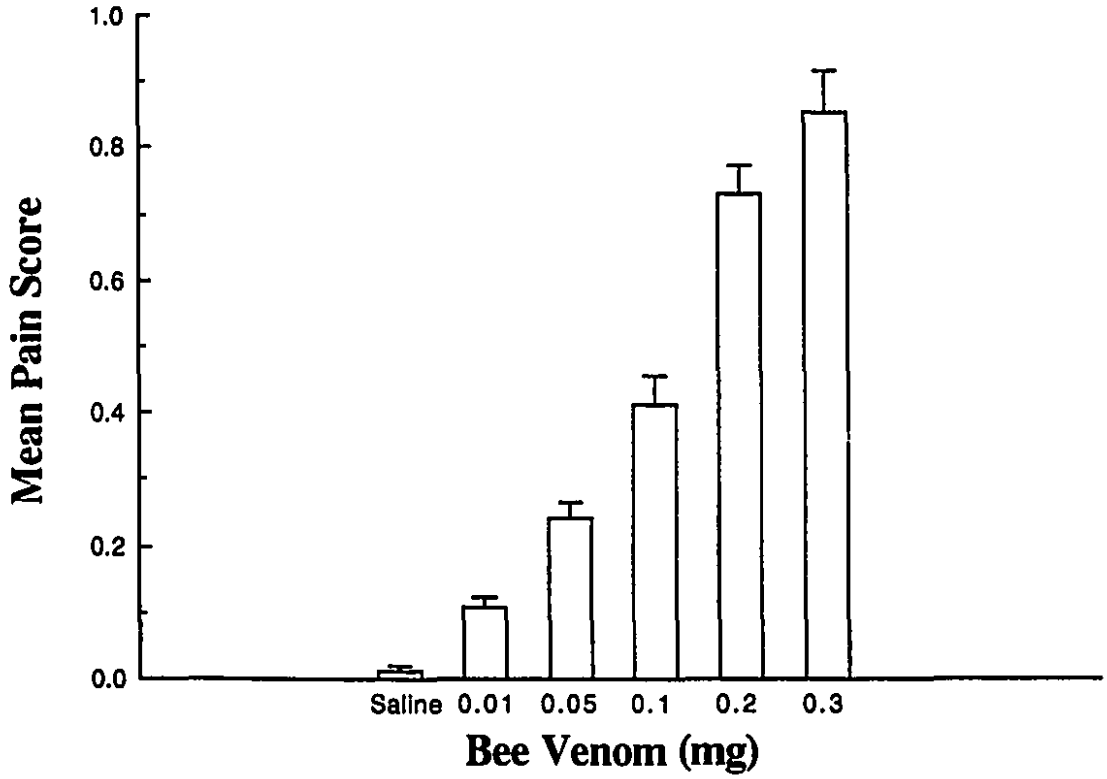

Figure 2. Mean pain scores for the 60-minute period following subcutaneous injection of various doses of bee venom or saline (n = 10 per group). Error bars indicate SEM. There is a significant effect of dose of bee venom ($p < 0.001$). Nonsignificant differences ($p > 0.05$): saline versus 0.01 mg; 0.01 mg versus 0.05 mg; and 0.2 mg versus 0.3 mg.

linear curve with an $R^2 = 0.97$, it is highly unlikely that a linear relationship would hold with higher or lower doses than those tested. According to the law of mass action (Goldstein, 1990), increasing doses would increase the amount of receptor-ligand complexes and/or the amount of reaction products, which would tend to limit further reaction of bee venom components. With lower doses, the tendency would be for the components to bind to receptors or react to form products since receptor-ligand complexes and/or reaction products would be low in number. Hence, the relationship should be logarithmic.

Figure 1 shows the response to injection of saline or bee venom as 12 5-minute time bins following injection. Saline injection produced little or no behaviour indicative of pain (mean pain score ≤ 0.02 for 11 of 12 time bins, 0.09 for the remaining bin). In contrast, increasing doses of bee venom produced higher mean pain scores and increases in the duration of responding. After reaching peak intensity within ten minutes after injection, the mean pain scores decreased continually until there was no significant difference from the saline-injected rats at 15 minutes with 0.01 mg, 20 minutes with 0.05 mg, 30 minutes with 0.1 mg, and at 55 minutes with 0.2 mg of bee venom. The response to injection of 0.3 mg of bee venom was still significantly different from the saline group at the end of 60 minutes. Demonstrating ceiling effects is the lack of significant differences in the first 10 minutes between the groups receiving 0.05 mg, 0.1 mg, 0.2 mg, and 0.3

mg of bee venom and in the first 40 minutes between the groups receiving 0.2 mg and 0.3 mg of bee venom.

Careful observations of the inflammation and edema revealed that by 5 minutes the paw is red and swollen. The edema reaches maximal levels at approximately 15 minutes after injection, and remains relatively constant for about 2 hours. With doses of bee venom up to 0.1 mg, the injected paw appears almost normal by 6-8 hours, with no redness and only slight edema apparent at the knuckles; and at 24 hours after injection, there are no apparent differences between the injected and the contralateral paws. With injection of 0.2 mg and 0.3 mg of bee venom, there was a substantial amount of inflammation at 8 hours; at 24 hours, the injected paw had only slight redness and appeared slightly less bony than the contralateral paw; and at 48 hours after injection, only slight redness remained. No overt necrosis developed in response to bee venom injection. Furthermore, no rat showed signs of an allergic reaction, even when retested in pilot studies within one month after the first testing.

Discussion

The optimal dose of bee venom for research purposes appears to be between 0.1 mg and 0.2 mg. This dose produces a continuous response that decays slowly while avoiding ceiling effects apparent with the highest doses tested.

A lack of correlation between the time course of the pain and the time course of the edema is particularly noticeable

with the lower doses of bee venom since the pain responses decrease while the edema increases, and the pain responses are absent in the latter portion of the 60 minutes while the edema is still near maximal levels. This suggests that different mechanisms are responsible for the edema and the pain in the bee venom test. Quantification of these observations and comparison with the pain scores will be carried out in a future study.

The main ingredients of bee venom are melittin, histamine, mast-cell degranulating peptide, apamin, phospholipase A and hyaluronidase (Habermann, 1971; Habermehl, 1981; Tu, 1977). Other less potent components are found in smaller amounts (see Appendix I). The substances responsible for the inflammation and edema are: histamine; melittin, which causes the release of histamine and serotonin from mast cells, erythrocytes, and thrombocytes; apamin, which increases capillary permeability; mast-cell degranulating peptide, which causes the release of histamine and serotonin from destroyed mast cells; phospholipase A, which potentiates melittin's effects; and hyaluronidase, which increases the permeability of interstitial ground substance (Habermann, 1971; Habermehl, 1981; Tu, 1977). Histamine and serotonin have been demonstrated to be inflammatory mediators as reviewed by Bach (1982), Larson and Henson (1983), and Owen (1987).

The substances that are assumed to be responsible for the pain are: histamine from bee venom and the above mentioned

endogenous sources; serotonin, released by the effects of the bee venom; and apamin, which has neurotoxic effects, especially in the spinal cord, where it produces prolonged hyperexcitability and augments polysynaptic reflexes (Habermehl, 1981; Keele and Armstrong, 1964; Minton, 1974; Tu, 1977). Histamine and serotonin can be identified as responsible for the pain since it has been demonstrated that intradermal injection of histamine produces transient pain in humans (Keele and Armstrong, 1964), and application of serotonin to the human blister base produces pain (Bleehen and Keele, 1977) as does intraplantar injection of serotonin in the rat (Hong and Abbott, 1994). The role of hyaluronidase in the production of pain is uncertain.

Experiment 2: Effect of Morphine and Aspirin

Methods

In the first part of this experiment, two groups of ten rats were given either morphine or a control injection. The rats were habituated, after which one group received 6 mg/kg morphine (10 mg/ml saline) subcutaneously in the neck. The control animals received an equivalent volume of saline. The rats were then placed back in the observation box for 10 minutes after which 0.05 ml of saline containing 0.1 mg of bee venom was injected in the hind paw. Behaviour was recorded for 45 minutes.

In the second part of this experiment, two groups of ten rats were given either aspirin or a control injection. After habituation, one group received 300 mg/kg aspirin (16.7 mg/ml tris buffer) via intraperitoneal injection. Control animals received an intraperitoneal injection of an equivalent volume of tris buffer (0.1 M, pH 7.4-7.6), the vehicle in which aspirin was dissolved. They were then placed back in the observation box for 30 minutes after which 0.05 ml containing 0.1 mg of bee venom was injected in the hind paw. Behaviour was recorded for 45 minutes.

Results

Figure 3 shows the response to injection of 0.1 mg of bee venom following subcutaneous injection of morphine or saline. There was a significant effect of group ($p < 0.001$) and a significant group x time interaction ($p < 0.001$). Rats that

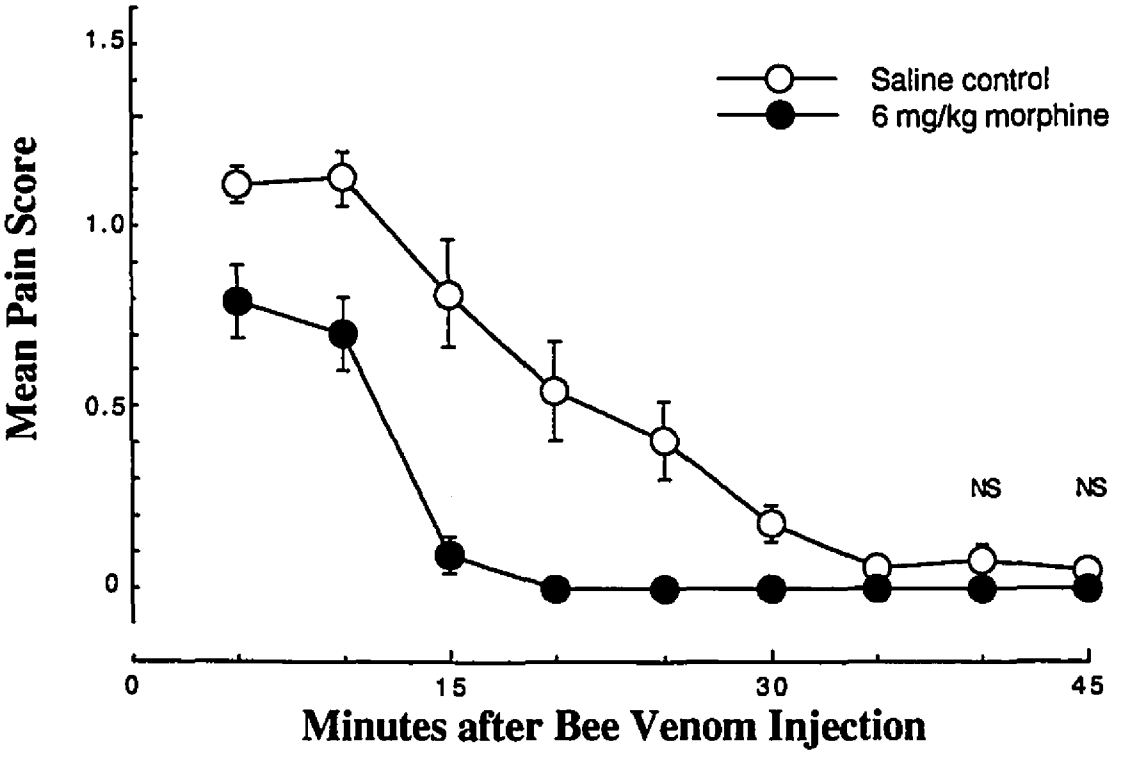

Figure 3. Responses of rats to injection of 0.1 mg of bee venom following subcutaneous injection of morphine or saline (n = 10 per group). Error bars indicate SEM. There is a significant effect of group ($p < 0.001$) and a significant group x time interaction ($p < 0.001$). NS indicates no significant difference between groups ($p > 0.05$).

received morphine had significantly lower mean pain scores compared to control animals for all 5-minute time bins until 40 minutes after the bee venom injection. The mean pain scores ± standard error of the mean (SEM) for the entire 45-minute period were 0.17 ± 0.03 for the morphine group and 0.49 ± 0.05 for the saline control group, which were significantly different ($p < 0.001$).

Figure 4 shows the response to injection of 0.1 mg of bee venom following intraperitoneal injection of aspirin or tris buffer. There was a significant effect of group ($p < 0.001$) and a significant group x time interaction ($p < 0.001$). Rats that received aspirin had significantly lower mean pain scores compared to control animals for all 5-minute time bins of the 45 minute observation period. The mean pain scores ± SEM for the entire 45 minutes were 0.11 ± 0.02 for the aspirin group and 0.54 ± 0.06 for the tris buffer control group, which were significantly different ($p < 0.001$).

There were no significant differences among the morphine control group (saline injected), the aspirin control group (tris buffer injected), and the group that received 0.1 mg of bee venom in Experiment 1.

Discussion

Morphine and aspirin both produce analgesia in the bee venom test, indicating that the test can be used to study analgesic drugs. It remains to be investigated whether the test is sensitive enough to demonstrate dose-dependent effects

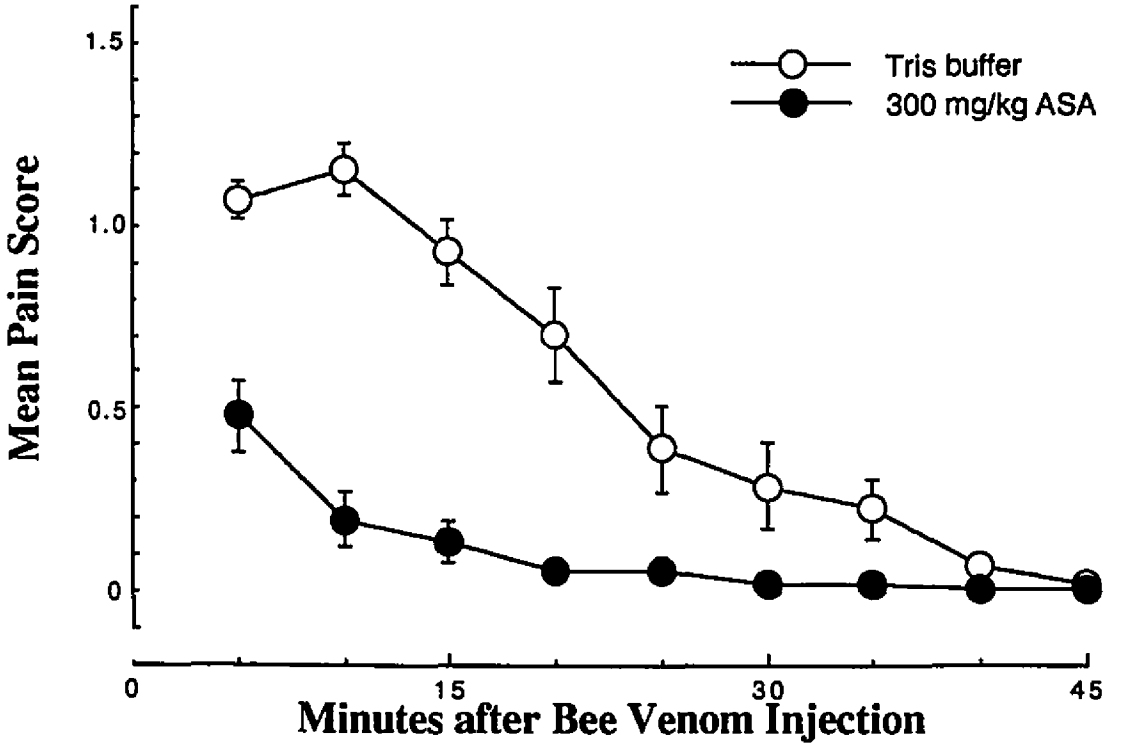

Figure 4. Responses of rats to injection of 0.1 mg of bee venom following intraperitoneal injection of aspirin (ASA) or tris buffer (n = 10 per group). Error bars indicate SEM. There is a significant effect of group ($p < 0.001$) and a significant group x time interaction ($p < 0.001$). All points are significantly different between groups ($p < 0.05$).

of analgesic drugs, as has been demonstrated for morphine in the formalin test (Coderre et al, 1993), aspirin in the formalin test (Hunskaar et al, 1986b), morphine in the tail-flick test (Abbott et al, 1982b), and for morphine and aspirin in the hot-plate test (Hunskaar et al, 1986a).

Morphine has an effect on the central nervous system at various sites in the brain and spinal cord (Millan, 1986), and it also has peripheral effects on inflammation (Gyires et al, 1985; Joris et al, 1990; Planas et al, 1995; Stein, 1993) which could be partly responsible for the analgesia observed. Similarly, the analgesia due to aspirin pretreatment is probably due to a peripheral effect on prostaglandins (Ferreira, 1972), and an effect on the nervous system, modulating nociceptive processing directly (Hunskaar and Hole, 1987; McCormack, 1994).

Experiment 3: Combining Bee Venom and Formalin

Methods

Three groups of ten rats were used. All rats were habituated as described earlier prior to receiving one of three treatments. One group, the BV/F group, received an injection of 0.1 mg of bee venom and, immediately before or after, received an injection of formalin. The second group, the BV/S group, received an injection of 0.1 mg of bee venom and an injection of saline. The third group, the F/S group, received an injection of formalin and an injection of saline. All injections had a volume of 0.05 ml, and each pair of injections was administered in the same hind paw. Within each group, half of the rats received the injections in the reverse order of the other half of the group. The rats were then placed in the observation box, and the behaviour was recorded for 60 minutes.

Results

There was a significant effect of group on the mean pain scores for the 60-minute observation period ($p < 0.001$). The mean pain scores ± SEM were: 1.11 ± 0.05 for the BV/F group; 0.83 ± 0.05 for the F/S group; and 0.32 ± 0.04 for the BV/S group. Each of the three groups was significantly different from the others. There was no effect of order of injections on the mean pain scores for the 60-minute period.

Figure 5 shows the time course of the response to concurrent injections of bee venom and formalin. There was

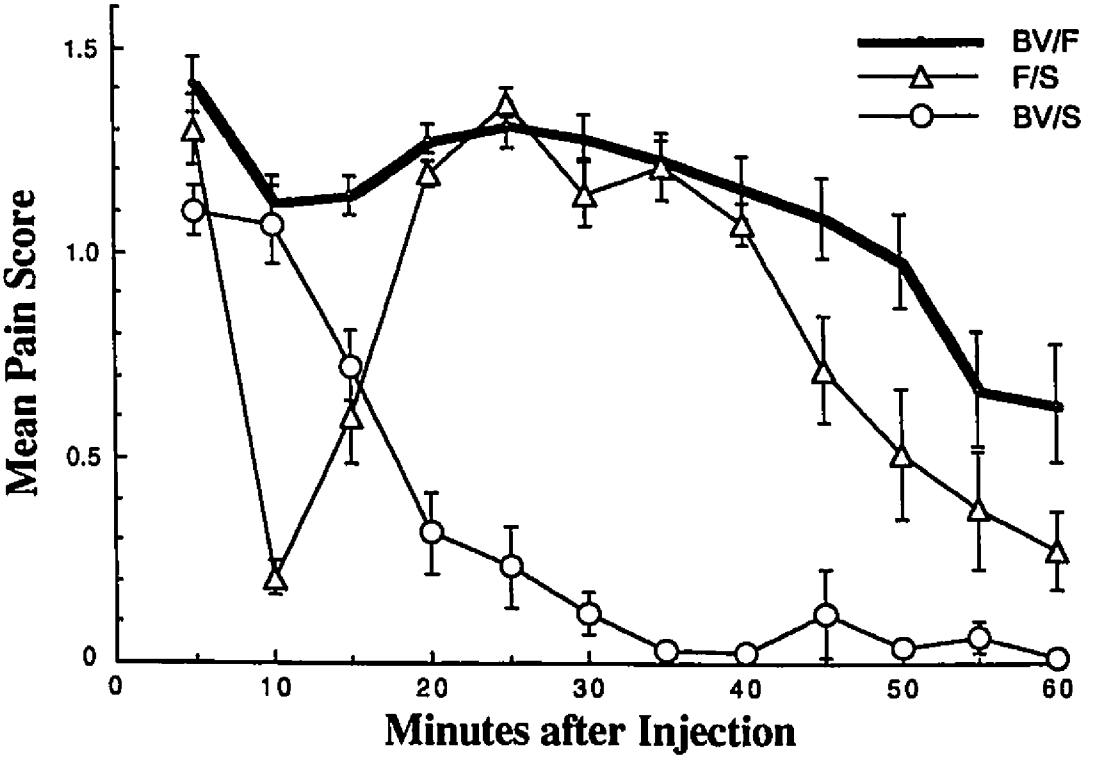

Figure 5. Responses of rats to concurrent subcutaneous injection of 0.1 mg of bee venom and 2.5% formalin (BV/F), 2.5% formalin and saline (F/S), and 0.1 mg of bee venom and saline (BV/S) (n = 10 per group). Error bars indicate SEM. There is a significant effect of group ($p < 0.001$) and a significant group x time interaction ($p < 0.001$).

a significant effect of group ($p < 0.001$), a significant group x time interaction ($p < 0.001$), and no significant effect of order of injections.

A comparison of the response of the BV/S group to the response of the BV/F group showed that the mean pain scores of the BV/S group were significantly lower at all times, except at 10 minutes when the BV/F response entered an interphase depression.

A comparison of the response of the BV/S group to the response of the F/S group showed that the first significant difference was apparent at 10 minutes, with BV/S having a higher mean pain score as the F/S group displayed an interphase depression. There was no significant difference at 15 minutes as the F/S response entered the second phase while the BV/S response continued to decline in intensity. The F/S response was higher from 20 to 50 minutes, and at 55 minutes the second phase of the F/S response had diminished and was no longer significantly different from the BV/S response.

A comparison of the response of the F/S group to the BV/F group revealed no significant difference for the first 5 minutes, but a significantly lower interphase depression for the F/S group at 10 and 15 minutes. There was no difference between groups for the portion of the second phase from 20 minutes to 40 minutes. In the latter portion of the second phase, the F/S group had significantly lower pain scores compared to the BV/F group, except at 55 minutes where there

was no significant difference between the two groups.

Discussion

Concurrent injections of bee venom and saline results in a continuous pain response as seen in Experiment 1. Concurrent injections of formalin and saline results in a biphasic response with an interphase depression of responding characteristic of the formalin test. Concurrent injections of bee venom and formalin results in a biphasic response with a less profound interphase depression than the F/S group, and a slower decline in responding at the end of the 60 minutes.

Concurrent administration of bee venom and formalin results in behaviour similar to the behaviour following administration of formalin and saline, and dissimilar to the behaviour following administration of bee venom and saline. The chemical properties of formaldehyde provide a possible explanation. "Formaldehyde is one of the most reactive organic chemicals" (Walker, 1964, p.206), which forms polymers especially with proteins and other formaldehyde molecules (Raphael, 1976; Walker, 1964). Exemplifying the reactivity of formaldehyde is its equilibrium constant for hydration of 2.2×10^{3} in comparison with 8.3×10^{-3} for benzaldehyde (Loudon, 1988). This high reactivity, may be responsible for the effects of formalin being most predominant when combined with bee venom.

The administration of bee venom in combination with formalin produces a tonic response of relatively high

intensity which lasts longer than either the bee venom test or the formalin test. Therefore, it can be used to engage the systems involved in tonic pain perception for a longer time than either test alone. The mechanisms that would be engaged by the combination are unknown. It is unknown whether the mechanisms that have been found to be involved in the formalin test would be engaged in addition to those specifically engaged in the bee venom test or whether different mechanisms would be involved due to the maintenance of responding at higher intensities for a longer duration or due to a different source of stimulation. In any event, combining the two, and using lower doses of formalin, may reduce the extent of necrosis induced by formalin injection.

General Discussion

The bee venom test satisfies virtually all of the requirements for testing pain mechanisms in animals that were described in the Introduction. Honey bee venom, the stimulus, is a natural substance that an animal in the wild and humans could encounter from a bee sting; thus, the pain mechanisms involved in the experimental context are not unnatural. Also, the amount of bee venom administered is easily quantified as the mass of dried venom dissolved in a volume of saline, allowing for strictly controlled experiments. Although the exact concentration of bee venom components varies from bee to bee (Minton, 1974), the effect is minimized by the preparation of injectable solutions. For example, to make a 10 ml solution of 0.1 mg bee venom per 0.05 ml, 20 mg, the equivalent of 200 bee stings, is used. Furthermore, bee venom does not produce any obvious tissue damage after a single injection of the doses tested, although inflammation did persist for up to two days with the two highest doses tested. From this study, no conclusion can be made regarding repetitive stimulation since it was not examined.

The evoked response meets the requirements as well. The response produced is discernible from the response to innocuous stimuli, such as the intraplantar isotonic saline injections in Experiment 1, and from normal behaviour without stimulation, which rarely includes elevation of the hind paw for extended periods as is seen in the bee venom test. Normal

grooming includes licking but it rarely includes the shaking or biting of the hind paw that is seen in the bee venom test. Moreover, since "spinal reflex responses do not measure critical higher central nervous system functions involved in the experience of pain" (Chapman et al, 1985, p.3), it is desirable that some of the behaviours evoked not be simple reflexes. Some of the behaviours evoked, such as licking, biting, or shaking of the paw, are not simple reflexes, nor is elevating the paw for extended periods. Therefore, the response evoked engages central nervous system structures above the spinal cord. Also as suggested, the response has a definite end-point after which the rat displays no pain behaviours. Thus, even though the rat does not have control of the painful stimulus, the rat no longer displays pain behaviours after approximately one hour. Since it is gone by approximately one hour, the bee-venom-induced pain, like most experimental pain, differs from clinical pain. Nonetheless, an advantage of the disappearance of the pain is that the animal no longer suffers after the observations are made. In the bee venom test, the animal is not restrained while responding. This eliminates any extraneous source of stress that may influence the response, and allows for examination of normal complex behaviour of the animal and how it is affected by various procedures.

Furthermore, there is a relationship between the intensity of the stimulus and the intensity of the pain

experienced. The relationship is logarithmic. Concomitantly, we see that the variability among individual Long-Evans rats is low enough to detect a dose/response relationship. Moreover, the response evoked is susceptible to pharmacological manipulations, as demonstrated by the analgesia produced by pretreatment with morphine and aspirin in Experiment 2. Again, the variability is low enough to detect the effectiveness of two analgesic drugs.

The bee venom test is an easy method to use. Bee venom is relatively inexpensive to purchase and is available from chemical retailers such as Sigma Chemical Company and ICN Biomedicals Canada Ltd.. It is easy to administer by injection since it is readily soluble in isotonic saline; subcutaneous injection in the paw is a simple procedure; a transparent plexiglass observation box is easy to make; and recording the behaviour requires little training and simple computer software and hardware. The method is not applicable to both animals and humans since, although the rats used in this study did not show allergic reactions, the risk of a dangerous allergic reaction (Habermann, 1971; Habermehl, 1981; Minton, 1974) prohibits its use in humans.

In addition to these suggestions, and of utmost importance, the response is one of tonic pain, engaging pain mechanisms more relevant to clinical pain than phasic pain does. Furthermore, the response is continuous and monophasic and as such may be preferable in many situations in lieu of

the biphasic response of the formalin test. Although the presence of two distinct phases in the formalin test allows for the study of both phasic and tonic pain, it is not entirely preferable since the second phase is influenced by the first phase (Coderre et al, 1990; Vaccarino et al, 1992; Vaccarino and Melzack, 1992). The tonic pain induced by bee venom injection is better for many experiments since it is not preceded by a phase of phasic pain. Also, the response follows immediately and, therefore, it can be studied from the time of injection. Experimenters do not have to wait for the onset of tonic pain as is required with the formalin test. In fact, many researchers who are interested only in tonic pain follow the suggestion of Dubuisson and Dennis (1977) and begin observations only after the first phase has ended (Vaccarino and Melzack, 1989, for example). If an experimenter is only interested in tonic pain, then the unobserved, unnecessary pain of the first phase can be avoided altogether.

Another advantage of using bee venom to evoke tonic pain is the large amount of existing research on bee venom and its biochemical activity. This research has provided a considerable understanding of the physiological responses to injection of bee venom, including mechanisms involved in the production of pain and inflammation. This knowledge should prove useful in experiments involving procedures that have an effect on the elements of pain production in the bee venom test.

One suggestion regarding assessment of pain mechanisms has been ignored intentionally. Dubner (1994) suggested that the appropriate noxious stimulus to evoke experimental pain "should excite a restricted group of primary afferent fibres and activate only receptors preferentially sensitive in the noxious range" (p.294). Honey bee venom contains many components with the collective potential to activate more than one type of primary afferent and receptor. For example, acetylcholine, a minor component of honey bee venom, acts on different kinds of receptors and activates both myelinated and non-myelinated sensory fibres (Keele and Armstrong, 1964). Another minor component, noradrenaline, may have a role in producing pain in the bee venom test by acting on sympathetic afferent neurons (Rang et al, 1991). According to pattern theories, the experience of pain is not the result of activation of a specific set of receptors, fibres, and brain structures. Rather, pain is a result of the simultaneous activity in many types of fibres, receptors, and areas of the brain that summate to produce the multidimensional experience. The production of pain by bee venom, if it involves the activation of many classes of fibres and receptors, resembles the situation that pattern theories state is responsible for the production of clinical pain, adding to the value of the test.

Overall, the bee venom test merits use in studies of basic pain mechanisms. The method satisfactorily fulfils the

recommendations of a method to assess pain mechanisms, and the method evokes tonic pain. As such, it has advantages over other methods of evoking experimental pain that do not meet the recommendations as effectively, and over methods that evoke phasic pain. In conclusion, the bee venom test is a valid and potentially useful model to examine the basic mechanisms of pain perception.

Appendix I

CONSTITUENTS OF VENOM OF *APIS MELLIFERA*[*]

Acetylcholine[1]

Amino acids[1]

Apamin (2%)[1,2,3,4]

Carbohydrates (2%)[1]

Cardiopep (0.7%)[1]

Dopamine[1,4]

Esterase[1]

Histamine (1.5%)[1,2,3,4]

Hyaluronidase (<3%)[1,2,3,4]

Lipids (5%)[1]

Mast-cell degranulating peptide (2%)[1,2,3]

Melittin (50%)[1,2,3,4]

Melittin F (<1%)[1]

Minimine[1]

Noradrenaline[1,4]

Phosphatase[1]

Phospholipase A (12%)[1,2,3,4]

Phospholipase B[4]

Procamine[1]

Promelittin[1]

Protease inhibitor[1]

Secapin (1%)[1]

Tertiapin (<1%)[1]

[1]Tu, 1977; [2]Habermann, 1971; [3]Habermehl, 1981; [4]Minton, 1974.

[*]Values in parentheses indicate percent by weight of dry bee venom.

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