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Inhibition of carrageenan induced inflammation in the rat knee joint by substance P antagonist

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SUMMARY The pathophysiology of acute joint inflammation remains unclear. Evidence is available to suggest a neurally mediated component to the inflammatory process. Acute joint inflammation in the rat knee, induced by intra-articular injection of 2% carrageenan, was reduced by 44% in animals whose knee had previously been injected with 1% capsaicin, while chronic joint denervation produced a 37% reduction. These results indicate a significant neurogenic component in this model of acute joint inflammation. Substance P may be the mediator of this response as intra-articular injection of this agent provoked an acute inflammatory response. Pretreatment of the test knee with the substance P antagonist d-Pro^4,d-Trp$^{7\ 9\ 10}$–SP(4–11), however, resulted in a 93% reduction of the inflammatory response to carrageenan. This unexpectedly large effect suggests that this substance P antagonist blocks both neurogenic and non-neurogenic mediators of inflammation. Sympathetic efferent fibres innervating the knee joint were not found to contribute to the neurogenic component of the inflammatory process.

The term neurogenic inflammation has been used to describe the finding that antidromic stimulation of cutaneous nerves leads to vasodilatation and increased vascular permeability in the territory innervated by the stimulated nerve,[1–13] and that chronic denervation of skin abolishes the response to topically applied irritants.[2 8] These effects are mediated by unmyelinated sensory (C) fibres,[14] and there is increasing evidence that substance P contained in these fibres plays an important part.[8 11 12 15]

Neurogenic inflammation is not confined to skin and has been shown in a wide variety of internal structures, such as gall bladder, vagina, oesophagus, trachea, and ureters.[16] More recently, neurogenic inflammation has also been shown in the cat knee joint, where it was found that antidromic electrical stimulation of knee joint nerves produced plasma extravasation into the synovial cavity of the knee.[17] This effect appeared to be mediated by the neuropeptide substance P as prior intra-articular administration of the substance P antagonist d-Pro^4,d-Trp$^{7\ 9\ 10}$–SP(4–11) completely blocked the neurogenically induced plasma extravasation. Additional evidence implicating substance P is that electrical stimulation of the nerve supply to the cat knee joint caused release of substance P from the articular nerves.[18] Thus the potential for neurogenic joint inflammation exists, but it is unclear whether this could significantly contribute to experimentally induced acute joint inflammation. Our experiments were performed to determine whether a neurogenic component could be shown in the carrageenan model of acute inflammation in the rat knee joint.

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Materials and methods

Experiments were performed on male Wistar rats (~300 g) deeply anaesthetised by intraperitoneal injection of urethane (1·13 g/kg) and diazepam (2·5 mg/kg). Evans blue (100 mg/kg) was injected into the external jugular vein. The core procedure entailed injection of 2% λ-carrageenan (Sigma) into the synovial cavity of one knee, the other being injected with 0·9% saline to provide an internal control. These were left in the joint for four hours and anaesthesia maintained, after which the animals were injected with euthatal and exsanguinated. The anterior and posterior portions of the knee joint capsule on both sides were dissected free from each rat. The amount of tissue obtained from each animal was small, necessitating pooling of samples from five

rats. These samples were weighed and Evans blue extracted by a modified dye extraction technique.[19] This entailed cutting the capsules into smaller pieces and mixing them with 14 ml of acetone and 6 ml of a 1% aqueous solution of sodium sulphate in a 30 ml drug bottle. The bottle was capped and placed in a Heidolph electrical agitator for 24 hours at room temperature with continuous mild shaking. Each preparation was then centrifuged for 10 minutes at 2000 rev/min and the supernatant was separated. The amount of dye recovered was calculated by comparing the absorbance of the supernatant at 620 nm (LKB Ultrospec II) with that of a standard curve prepared with known concentrations of Evans blue solution. As Evans blue binds to plasma proteins normally restricted to the vascular compartment its presence in the capsule provides an index of altered vascular permeability. For each experimental procedure five groups of five rats were used, unless otherwise stated.

In 25 animals the nerves supplying the knee joint were transected unilaterally under general anaesthesia (Hypnorm 0·1 mg/kg; diazepam 2·5 mg/kg) and the animals then allowed to recover. Ten days later these animals were assessed for their response to the carrageenan injection into this knee. This period of time was sufficient for substantial degeneration of the nerves to occur, as judged by their electron microscopic appearance.

In a further 25 animals, under general anaesthesia, a 1% solution of capsaicin (0·02 g capsaicin (Sigma) dissolved in 0·1 ml absolute alcohol, 0·1 ml Cremophor (Sigma), and 1·8 ml physiological saline) was injected into the synovial cavity of one knee. Topical application of capsaicin has been shown to deplete nerve endings of substance P.[18] Ten days later these animals were assessed for their response to carrageenan injection into this knee.

In 15 animals bilateral adrenalectomy was performed under general anaesthesia and the animals allowed to recover. One week later these animals were assessed for their response to intra-articular injection of carrageenan preceded by injection of d-Pro^4,d-Trp$^{7\,9\,10}$–SP(4–11).

Results

To establish the control response 0·2 ml saline was injected into both knees, and the mean difference (SEM) in Evans blue content (μg/100 mg tissue) between the two knees was small (1·64 (0·97)) (Fig. 1). When 0·2 ml of 2% carrageenan was injected into one knee the Evans blue content rose relative to the control knee, and the mean difference (22·05 (1·76)) was significantly greater than for the control group (Fig. 1). Injection of the same dose of

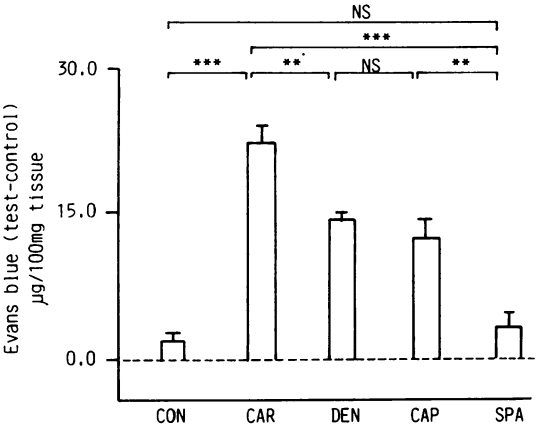

Fig. 1 *Histogram of Evans blue content of rat knee joint capsules (mean (SEM)) under different conditions. The difference between the test knee and the control (saline) knee for five groups of five rats is displayed. CON=control response (bilateral injection of 0·9% saline); CAR=mean difference in dye content between the control knee and the other injected with 0·2 ml of 2% carrageenan; DEN=effect of 2% carrageenan injection in chronically denervated knees; CAP=response to carrageenan injection in knees previously injected with 1% capsaicin; SPA=injection of 10 μg of the substance P antagonist d-Pro^4,d-Trp$^{7\,9\,10}$–SP(4–11) 15 minutes before carrageenan substantially attenuated plasma extravasation. **$p<0·01$; ***$p<0·001$; $n=5$.*

carrageenan into the knees of rats which had been surgically denervated 10 days earlier produced an inflammatory response (13·89 (1·18)), which was ~37% lower than carrageenan in the intact knee. Intra-articular injection of 1% capsaicin 10 days earlier also resulted in a smaller inflammatory response (12·31 (2·12)) to carrageenan injection (~44% lower). These last two means differed significantly ($p<0·01$) from the response to carrageenan alone but did not differ from each other. Sham operated and vehicle pretreated animals showed no attenuation of the inflammatory response. These findings therefore indicate a significant neurogenic component in this model of acute joint inflammation. It was expected that injection of the substance P antagonist d-Pro^4,d-Trp$^{7\,9\,10}$–SP(4–11) into the joint would similarly attenuate the inflammatory response. As Fig. 1 shows, however, intra-articular injection of 10 μg of the antagonist 15 minutes before carrageenan resulted in a much greater (~93%) reduction in the inflammatory response (3·24 (1·64)). This mean differs significantly from all the other means except the value for the control group.

In the dose used in the present experiments, d-Pro^4,d-Trp$^{7\ 9\ 10}$–SP(4–11) did not appear to produce significant vasoconstriction of articular blood vessels. The presence of 10 μg of the substance P antagonist in the synovial cavity of the knee produced little alteration in Evans blue content compared with saline alone (Fig. 2). The mean difference between the two knees was only 0·45 (1·61). Had the antagonist been a potent vasoconstrictor, significant difference between the two knees would have been expected. In addition, assessment of synovial blood flow by a laser Doppler flowmeter in four animals failed to show potent constrictor effects. In two cases transient (<10 minutes) decrease in flow occurred after intra-articular injection of 10 μg of the substance P antagonist, whereas two cases showed transient increase in flow. The difference between the control value in Fig. 1 (saline in both knees) and the value obtained in Fig. 2 (saline in one knee, the other pretreated with substance P antagonist before saline injection) was not significant.

Seven days after adrenalectomy, pretreatment of the test knee of three groups of five rats with 10 μg d-Pro^4,d-Trp$^{7\ 9\ 10}$–SP(4–11) followed by 2% carrageenan resulted in an inflammatory response (−4·08 (1·51)), which was actually lower than that occurring in intact animals pretreated with the antagonist. The negative figure indicates that the mean Evans blue content was lower in the test knee than in the control knees.

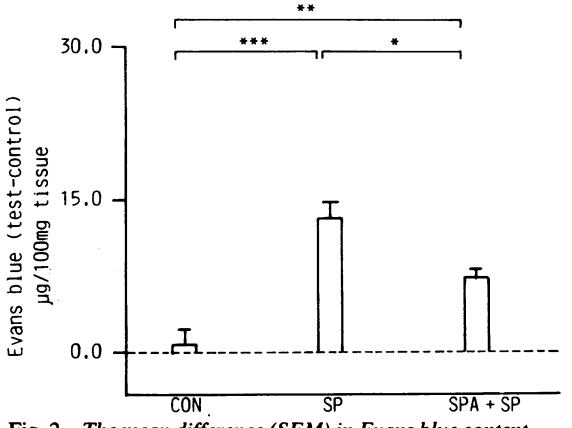

Fig. 2 *The mean difference (SEM) in Evans blue content between knees injected with 0·9% saline against those injected with 10 μg substance P antagonist before saline injection is small (CON). Injection of 20 μg substance P into the knee resulted in a significant inflammatory response (SP), and this was only partially blocked by pretreatment of the knee with 10 μg of the antagonist (SPA+SP). Each histogram represents the difference in Evans blue content between the test knee and its saline injected (control) partner. ***p<0·001; **p<0·01; *p<0·05; n=5.*

Pretreatment of rats with reserpine (1 mg/kg daily) for three days to deplete sympathetic nerve endings of catecholamines[20] did not influence the inflammatory response. This treatment did produce a generalised reduction in Evans blue content in both test and control knees, but in comparison with the control knee, little change was observed. It was found that the inflammatory response induced by carrageenan resulted in a 218% increase in Evans blue content in normal animals, while in the group of rats pretreated with reserpine the Evans blue content was increased by 221% (20 rats in each of the two groups).

Intra-articular injection of substance P (20 μg) resulted in a significant inflammatory response (Fig. 2). This was reduced by pretreatment of the joint with the substance P antagonist (10 μg). It is noticeable that this dose of the substance P antagonist only produced a 46% reduction of the inflammatory response to substance P (6·94 (1·03)), whereas the same dose almost completely blocked the carrageenan induced inflammation.

Discussion

The present experiments have clearly shown that the carrageenan model of acute joint inflammation has a significant neurogenic component. The inhibition of the carrageenan induced inflammatory response by surgical denervation or capsaicin pretreatment of the knee could have been mediated entirely by unmyelinated afferent fibres, or could have been partly mediated by sympathetic efferent fibres which are also present in articular nerves.[21] The results of the experiments performed on reserpinised animals suggest that the neurogenic component of the inflammatory response is mediated in large part by the unmyelinated afferent fibres which supply the joint. There is little evidence here to indicate a role for sympathetic efferent fibres. This is at variance with the findings of Levine *et al*,[20] who observed a contribution of sympathetic efferent fibres to adjuvant arthritis in rats. This variance may arise from the different models of inflammation used in the two series of experiments.

A surprising finding was that the substance P antagonist produced a greater inhibition of the carrageenan induced inflammation than was expected on the basis of the effects of denervation and capsaicin pretreatment. As the same dose of the antagonist produced smaller inhibition of the inflammatory response induced by substance P than the carrageenan induced inflammation, and bearing in mind the smaller inhibition obtained with de-

nervation and capsaicin pretreatment, it is possible that this antagonist inhibits both the neurogenic component and other non-neurogenic mediators of the inflammatory response. There was no evidence to suggest that the greater degree of inhibition of the inflammatory response by d-Pro^4,d-Trp$^{7\ 9\ 10}$–SP(4–11) was due to constriction of the articular blood vessels, though it has been shown that other substance P antagonists are potent vasoconstrictors.[22]

It is unlikely that the near-abolition of the inflammatory response by the substance P antagonist pretreatment was mediated by corticosterone release triggered by this antagonist reaching the blood stream as the antagonist was also found to be effective in chronically adrenalectomised animals. Although the Evans blue content was lower in the carrageenan treated knee than in the control knee, this is probably not a significant effect as the larger series of knees which were bilaterally injected with saline also showed differences in Evans blue content (Fig. 1).

The finding that intra-articular injection of substance P produced an inflammatory response and that this was attenuated by the substance P antagonist suggests that the neurogenic component of the carrageenan induced inflammatory response may be mediated by substance P. It is not possible, however, to ascribe an exclusive role to substance P as it is not known whether other neuropeptides, such as calcitonin gene related peptide, neurokinin A, and neurokinin B, are co-localised in articular nerve fibres, and the selectivity of d-Pro^4,d-Trp$^{7\ 9\ 10}$–SP(4–11) for these other neuropeptides has not been established.

There is increasing interest in the role of neuropeptides in inflammatory processes and particularly whether these may be involved in arthritis. It has been observed that infusion of substance P into the knee joint of rats with adjuvant induced arthritis resulted in more pronounced joint inflammation and destructive changes of bone and cartilage than animals whose joints had been infused with the substance P antagonist d-Pro^2,Trp$^{7\ 9}$–SP.[23] It has also been found that capsaicin reduced the inflammatory response of adjuvant induced arthritis in the rat.[24] Also, substance P has been detected in the synovial fluid aspirated from inflamed joints in a patient with rheumatoid arthritis.[25] In recent years it has been shown that a number of neuropeptides, including substance P, promote inflammatory cell chemotaxis,[26] neutrophil activation,[27] mast cell degranulation,[28] and fibroblast proliferation,[29] all of which are recognised components of the arthritic inflammatory process. In addition, substance P has been shown to activate synoviocytes to secrete prostaglandin E_2 and collagenase,[30] as well as to stimulate secretion of interleukin-1-like activity from macrophages.[31]

In conclusion, the results of our experiments add to the growing body of evidence which suggests that substance P, and perhaps other neuropeptides released from peripheral nerve terminals in joints, could play an important part in the initiation and maintenance of inflammatory articular diseases. If this proves to be the case, inhibition of the local effects of these neuropeptides may be of therapeutic value.

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