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MOBILIZATION OF COLONIC KALLIKREIN FOLLOWING PELVIC NERVE STIMULATION IN THE ATROPINIZED CAT

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SUMMARY

1. Pelvic nerve stimulation (p.n.s.) in cats induces atropine-resistant colonic vasodilatation and colonic contraction. The effects of this on cat colon are mimicked by synthetic bradykinin infusions. The present study examines the effect of p.n.s. on the activation of kallikrein, the kinin-forming enzyme present in colonic tissue and its effects on the plasma kinin system in the atropinized cat.

2. Mean level ($\pm$ S.D.) of mucosal kallikrein was found to be about 37 times higher in unstimulated colonic mucosa (300 ± 100 ng bradykinin equivalents $min^{-1}g^{-1}$) than in the underlying muscle ($8{\cdot}2 \pm 6{\cdot}3$ ng bradykinin equiv $min^{-1}g^{-1}$).

3. After a p.n.s. of 5 min, mean kallikrein level in colonic muscle was $7{\cdot}3 \pm 3{\cdot}5$ ng bradykinin equiv $min^{-1}g^{-1}$, which was not significantly different from the control muscle kallikrein. However, there was an 86 % fall in mucosal kallikrein to $41{\cdot}3 \pm 34{\cdot}7$ ng bradykinin equiv $min^{-1}g^{-1}$ after 5 min p.n.s., indicating a rapid activation and secretion of mucosal kallikrein.

4. Secretion of mucosal kallikrein was paralleled by specific depletion of plasma kininogen, the precursor of active kinin in blood draining the colon. The mean plasma kininogen level fell to 79 and 68 % of the prestimulated value ($3{\cdot}1 \pm 1{\cdot}1$ S.D. μg bradykinin equiv per ml. plasma) after 5 and 10 min p.n.s. respectively. Total plasma protein and haematocrit remained unaltered excluding non-specific changes due to protein extravasation or haemodilution and indicating utilization of the plasma kinin precursor.

5. Following 2 hr p.n.s., raised levels of kallikrein were detected in both colonic muscle ($28 \pm 2{\cdot}0$ bradykinin equiv $min^{-1}g^{-1}$) and mucosa 434 ± 118 ng bradykinin equiv $min^{-1}g^{-1}$). Preliminary studies using a kallikrein inhibitor indicated that the increased kallikrein levels originated from plasma.

6. Direct stimulation of the parasympathetic pelvic nerve in the atropinized cat thus produced activation of the plasma kinin system in the colon and formation of free kinins may be responsible for the mucosal vasodilatation and strong motor contraction which is not blocked by large doses of atropine. The observation that prolonged stimulation causes extravasation of plasma kallikrein, a potential inflammatory mediator, into the tissues may be of clinical significance.

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INTRODUCTION

The reservoir function of the colon is mainly controlled by the parasympathetic nerves, the vagal nerve controlling predominantly the proximal colon and the pelvic nerve controlling the distal colon. Stimulation of the pelvic nerves in the cat causes an expulsive contraction emptying the colon. Concomitant to this powerful contraction there is an abundant mucous secretion and mucosal vasodilatation (Hulten, 1969). Large doses of atropine block the mucous secretion but fail to block the vasodilator and motor responses and it has been suggested that some other mechanism, such as plasma kinin release, may be involved in mediating some of these effects, especially the vasodilator responses (Hulten, 1969). Similar suggestions have been made regarding a possible involvement of kinins in the atropine-resistant functional hyperaemia in other glandular tissues, particularly the submandibular gland (Hilton & Lewis, 1956; Gautvik, Nustad & Vystyd, 1972) and pancreas (Hilton & Jones, 1968). Considerable concentrations of kinin-forming enzyme are present in the colonic mucosae in a variety of species (Amundsen & Nustad, 1965; Burger, Lembeck & Wagner, 1968; Seki, Nakajima & Erdös, 1972; Zeitlin, 1970; Zeitlin & Smith, 1973). Close intra-arterial infusions of synthetic bradykinin in the cat colon mimic the effects of pelvic nerve stimulation, not only on blood flow and capillary permeability (Fasth & Hulten, 1973*a*) but also on colonic motility (Fasth & Hulten, 1973*b*), indicating that kinins may be involved in the neurohumoral control of both colonic blood flow and motility.

The present study examines the changes in kallikrein content of colonic tissue and circulating plasma kininogen during pelvic nerve stimulation in atropinized cats.

Some of the results have been communicated to the Physiological Society (Fasth, Hulten, Johnson & Zeitlin, 1977).

METHODS

Operative procedure

Cats were fasted for 24 hr and anaesthetized with intravenous chloralose (50 mg/kg) after induction with ether. The trachea was cannulated and the femoral artery was connected to a mercury manometer to record arterial blood pressure. The abdomen was opened in the mid-line and the spleen, the greater omentum and small intestine were removed. The sympathetic nerves to the colon were dissected free from the superior and inferior mesenteric arteries and cut. After surgery, the cats were heparinized (300 i.u./kg) and atropinized (1 mg/kg).

Nerve stimulation

The pelvic nerves on both sides were dissected free and divided as near the sacral roots as possible. The rectum was divided approximately 2–3 cm distal to this level. The peripheral cut ends of the nerves were mounted on silver ring electrodes. The pelvic nerves were stimulated at a frequency of 5 Hz with 5 msec pulses and a strength of 8 V, with a grass stimulator model S 5E. In some experiments the nerves were submitted to continuous stimulation for 5 or 10 min as indicated in the results. In experiments to examine the effects of prolonged stimulation, the nerves were stimulated intermittently for periods of 2 min alternating with 1 min rests for a total of 2 hr.

Tissue kallikrein determinations

In twelve cats the effect of pelvic nerve stimulation on colonic blood flow was determined by monitoring the superior mesenteric arterial inflow to the colon (Hulten, 1969). Blood was taken from the left carotid artery using a wide bore polyethylene cannula and passed, via a drop

counter, into the superior mesenteric artery supplying the colon. After 5 min continuous stimulation or 2 hr intermittent stimulation of the pelvic nerves, the colon was perfused *in vivo* with heparinized normal saline at 37 °C via the mesenteric arteries to flush out the remaining blood. Tissue was taken from the distal half of the colon as the vasodilator effect of p.n.s. is mainly restricted to that part (Hulten, 1969). The tissue was dissected into muscle and mucosal layers, snap-frozen in liquid nitrogen, stored at −20 °C and prepared for subsequent assay, as described by Zeitlin & Smith (1973).

Kinin-forming enzyme activity of the prepared tissue extract was determined by incubating 0·2 ml. aliquots with excess stable kinin-forming substrate (0·5 ml.) prepared using a modification (Zeitlin, Singh, Lembeck & Theiler, 1976) of the method of Amundsen, Nustad & Waaler (1963) at 37 °C in the presence of 0·2 ml. orthophenanthroline (0·01 M) to inhibit kininase. The incubation was carried out in barbiturate buffer (0·1 M, pH 8·6), final volume 2 ml.; the enzymic action was stopped by heating in a boiling water-bath (10 min). Where enzyme inhibitor studies were to be carried out, the extract was pre-incubated with inhibitor for 10 min before addition of substrate. The activity of the released kinin was compared with standard synthetic bradykinin (Sandoz) using close bracketing bioassay on isolated oestrous rat uterus (Brocklehurst & Zeitlin, 1967), and the kinin-forming activity was expressed as ng bradykinin equivalent released per min of incubation per gram wet weight of tissue (ng bradykinin equiv $min^{-1}g^{-1}$). Tissue extracts with high enzymic activity were diluted so that the activity fell within the linear part of the rate curve.

Plasma kininogen determinations

In nine cats, cannulation was carried out to permit serial sampling of the blood draining the colon and the effect of pelvic nerve stimulation on over-all colonic blood flow was monitored by recording the superior mesenteric venous outflow. A wide-bore polyethylene tube was inserted into the superior mesenteric vein. The venous outflow from the colon was diverted to a closed Perspex drop counter filled with silicone oil and returned to the animal via the external jugular vein (Hulten, 1969).

At timed intervals of 0, 1, 5, 10 min throughout a 10 min p.n.s., blood samples (3 ml.) were withdrawn with plastic syringes from a side-arm in the tube draining the superior mesenteric vein for plasma kininogen, total protein and haematocrit estimations. The blood for kininogen assay was immediately inactivated by squirting into cold redistilled ethanol and prepared for kininogen assay as described by Brocklehurst & Zeitlin (1967).

Control experiments

Kallikrein levels were measured in seven cats with the operative procedure, blood flow recording and tissue sampling identical to those above, but without p.n.s.

Differences in the results were tested for statistical significance using the Mann-Whitney U test.

RESULTS

1. *Atropine resistance of the blood flow response to p.n.s.*

Atropine infusion (1 mg/kg) did not reduce the initial blood flow increase on p.n.s. (Fig. 1). Owing to the subsequent onset of the very powerful sustained contraction of colonic smooth musle, the blood flow response after this initial increase was very unpredictable (see also Fig. 2) and could not be quantified satisfactorily. This response was not affected by sympathetic α- (phenoxybenzamine, 5 mg/kg, I.V.) and β- (propranolol, 3 mg/kg, I.V.) blocking agents, or guanethidine (5 mg/kg, I.V.).

2. *Effects of pelvic nerve stimulation on colonic venous plasma kininogen*

Within 5–10 sec after commencement of bilateral stimulation of the pelvic nerves there was a marked blood flow increase from 20–40 ml, min^{-1} 100 g^{-1} tissue 'at rest',

to some 100 ml./min^{-1} at peak response. The initial blood flow increase was transient and followed by recurrent increases. This blood flow pattern was maintained during the whole 10 min stimulation period (Fig. 2).

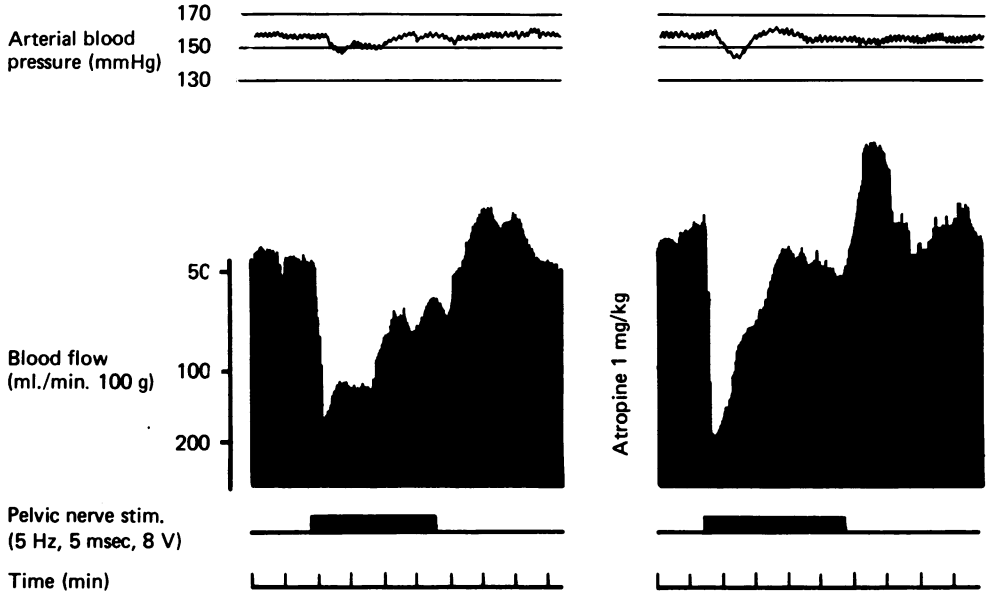
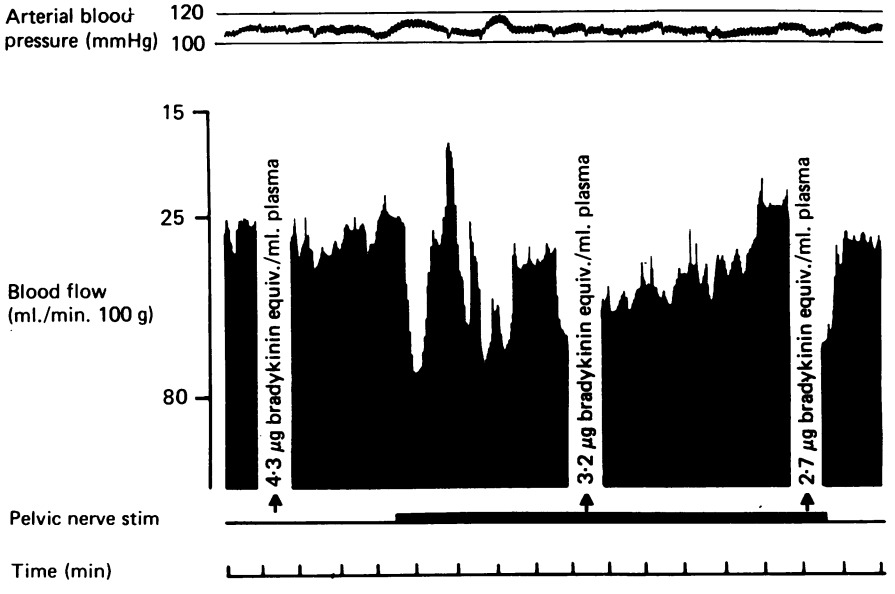


Fig. 1. Comparison of the change in colonic blood flow produced by pelvic nerve stimulation in an anaesthetized cat before and after atropine (1 mg/kg).



▲ Colonic plasma kininogen values at 0, 5, 10 min p.n.s.

Fig. 2. The effect of pelvic nerve stimulation, in the presence of atropine (1 mg/kg), on colonic blood flow and colonic venous plasma kininogen level in a typical cat.

Concomitantly to the increased blood flow, a sustained motor contraction of the colon was observed. Mean plasma kininogen level fell to 79 and 68 % ($P < 0{\cdot}05$) of the prestimulated value ($3{\cdot}1 \pm 1{\cdot}1$ S.D. μg bradykinin equiv/ml. plasma) after 5 and 10 min p.n.s. respectively, following a small rise to 110 % after only 1 min stimulation (Fig. 3). Total plasma protein and haematocrit remained unaltered ($P > 0{\cdot}05$) excluding non-specific changes due to protein extravasation or haemodilution.

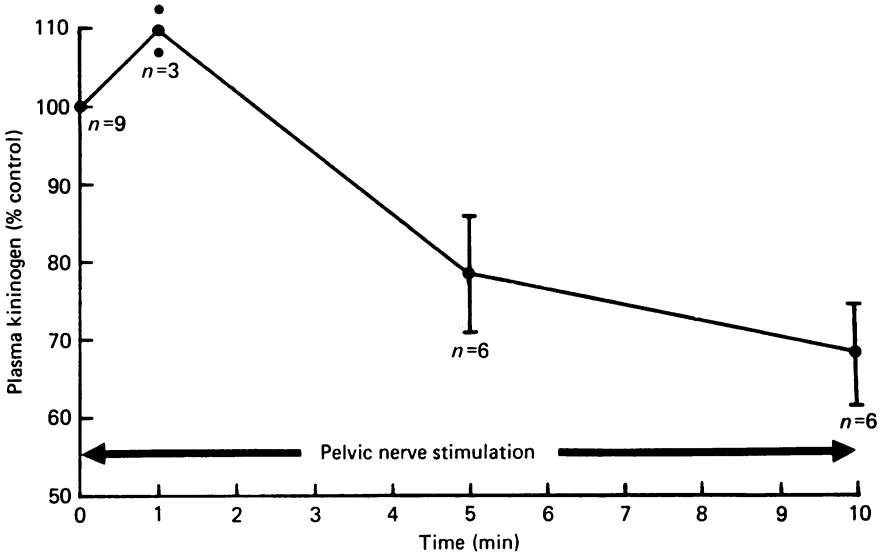


Fig. 3. The effect of pelvic nerve stimulation on colonic venous plasma kininogen levels (means $\pm$ S.D.). The number of animals tested at each time is shown. Mean control, $3{\cdot}1$ μg bradykinin equiv/ml. plasma; S.D. $\pm$ $1{\cdot}1$.

3. *Effects of pelvic nerve stimulation on colonic tissue kallikrein*

In unstimulated control cats mean kallikrein levels in the colonic mucosa were nearly 37 times higher (300 ± 100 ng bradykinin equiv $min^{-1}g^{-1}$) than in the underlying muscle ($8{\cdot}2 \pm 6{\cdot}3$ ng bradykinin equiv $min^{-1}g^{-1}$). After 5 min p.n.s., kallikrein levels in the muscle were unchanged ($P > 0{\cdot}05$) ($7{\cdot}3 \pm 3{\cdot}5$ ng bradykinin equiv $min^{-1}g^{-1}$), whereas there was a marked fall ($P < 0{\cdot}01$) of 86 % in mucosal kallikrein level to $41{\cdot}3 \pm 34{\cdot}7$ ng bradykinin equiv $min^{-1}g^{-1}$ (Fig. 4). Mucosal kallikrein measured in a single cat after only 3 min p.n.s. was also greatly reduced at 45·4 ng bradykinin equiv $min^{-1}g^{-1}$, indicating a rapid onset of kallikrein secretion.

4. *Effects of prolonged pelvic nerve stimulation*

Following 2 hr p.n.s. in six cats, the mean level of kallikrein in colonic muscle was $28{\cdot}3 \pm 2{\cdot}0$ ng bradykinin equiv $min^{-1}g^{-1}$, some 3·5 times that in muscle from control cats ($P < 0{\cdot}01$), while that in the underlying mucosa was 434 ± 118 ng bradykinin equiv $min^{-1}g^{-1}$, some 44 % greater than in the control mucosa ($P < 0{\cdot}05$).

Sufficient tissue remained from four of the 2 hr stimulated colons and four control colons enabling preliminary experiments to be carried out to determine the origin of the large increase in muscle kallikrein. Soybean trypsin inhibitor (SBTI) inhibits plasma kallikrein while having little effect on tissue kallikreins (Webster, 1970). In

the present study, SBTI (100 μg/ml.) had no detectable effect on the kallikrein activity from the unstimulated control muscosae, supporting its identity as a tissue kallikrein. However, an equivalent kallikrein concentration from stimulated muscle was inhibited 94 $\pm$ 3 % by SBTI (100 μg/ml.). Thus the increased kallikrein activity found in the muscle following prolonged neurostimulation was probably due to a plasma kallikrein and not a tissue kallikrein.

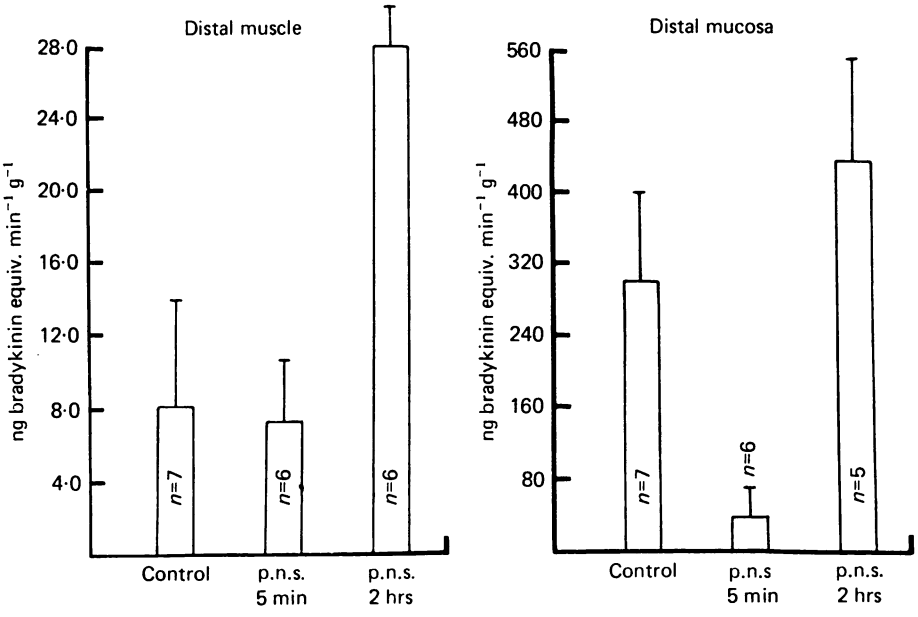


Fig. 4. The effect of pelvic nerve stimulation (p.n.s.) on kallikrein levels in mucosa and muscle from cat distal colons (mean, S.D.).

DISCUSSION

Kallikreins are proteolytic enzymes which release kinin peptides from protein precursors (kininogens). Kallikrein, or kallikrein-like activity has been reported along the entire length of the gut in several mammalian species (Werle, 1960; Amundsen & Nustad, 1965; Burger *et al.* 1968; Zeitlin, 1970, 1971, 1972; Seki *et al.* 1972). In the human colon, Zeitlin & Smith (1973) found that tissue kallikrein is present mainly in the mucosal layer, with little or no activity in healthy colonic muscle. In the present study it is shown that similarly, in cats, colonic kallikrein is found mainly in the mucosa which contained some 37 times the concentration found in the normal muscle.

Stimulation of the parasympathetic pelvic nerve supply to the cat distal colon produces a powerful contraction of both longitudinal and circular muscle together with an increase in mucous secretion and mucosal vasodilatation (Hulten, 1969) and an increase in capillary filtration coefficient (Fasth & Hulten, 1973*a*). The motor and vascular responses to pelvic nerve stimulation are resistant to blockade by both atropine and adrenergic blockers (Hulten, 1969) and other possible non-cholinergic, non-adrenergic mediators of these responses have been sought.

Evidence from the present study indicates that a release of colonic mucosal kallikrein follows pelvic nerve stimulation. Stimulation of the pelvic nerve for 5 min in the presence of atropine produced, concomitant with the motor response and mucosal vasodilatation, a depletion of the mucosal kallikrein store by some 86%. The release of the mucosal kallikrein is accompanied by activation of plasma kinin in the blood draining the colon. This is indicated by a fall of over 20% in the plasma kinin precursor after stimulating the pelvic nerve for 5 min and a fall of over 30% after 10 min stimulation. These changes were not the result of non-specific variations in haematocrit or plasma protein concentration. Intra-arterial infusions of bradykinin in the cat colon cause spasm of both the longitudinal and circular muscle, mucosal vasodilatation and an increase in capillary filtration coefficient all responses also seen as a result of pelvic nerve stimulation (Fasth & Hulten, 1973*a*, *b*, *c*). The release of colonic kallikrein and hence of plasma kinins is thus consistent with a role in the mediation of the response to pelvic nerve stimulation.

In three cats, the colonic venous plasma kininogen level was determined after stimulation for only 1 min and showed a small but consistent increase. The cause of this increase was not clear. The change was unlikely to be due to haemoconcentration, since at this time there was no increase in haematocrit. There is evidence to show that arterial levels of plasma kininogen are greater than venous levels in several species (Haberman, 1970). In the basal state there is possibly a small usage of plasma kininogen as the blood percolates through the colonic capillary beds. In the first stages of the vasodilatation following nerve stimulation, fresh systemic blood flushes through the colonic vasculature. This may more than compensate for any initial usage of kininogen and cause the apparent increase in concentration.

A remarkable finding was seen in the present study during attempts to deplete the colonic tissue kallikrein stores by neurostimulation. In the unstimulated colon, the tissue kallikrein was mainly in the mucosa with very little in the muscularis. After stimulation of the pelvic nerve for 5 min, there was no change in the muscle kallikrein level, while the mucosal kallikrein was depleted. However, after repeated stimulation of the pelvic nerve for 2 min at 1 min intervals for 2 hr, both the muscle and the mucosa contained raised levels of kallikrein compared with those in unstimulated colon. The muscle contained more than three times the control level while the mucosa contained 144% of the control level. Plasma contains large amounts of kallikrein and plasma kallikrein may be distinguished from the tissue kallikreins both in sensitivity to various inhibitors and in substrate specificity (Webster, 1970). Preliminary studies using soybean trypsin inhibitor, which blocks plasma kallikrein while having little effect on tissue kallikreins (Webster, 1970), indicate that the raised levels of kallikrein originate from plasma. The leakage into the interstitial fluid of large amounts of plasma kallikrein together with other plasma proteins is to be expected following prolonged pelvic nerve stimulation, since this is known to cause increased vascular permeability (Fasth & Hulten, 1973*a*).

Kinins are potent inflammatory mediators (Lewis, 1970). The pelvic nerve is stimulated during bowel evacuation and the saturation of colonic tissues with kinin-forming enzyme following prolonged stimulation may well have a clinical significance. It is noteworthy in this respect, that saturation of colonic tissues with kinin-forming enzyme has also been described in patients with ulcerative colitis (Zeitlin & Smith, 1973).

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