

Thrombin Functions as an Inflammatory Mediator through Activation of Its Receptor

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Summary

A rat model of inflammation was used to investigate the biological effects of thrombin. The thrombin-specific inhibitor Hirulog™ markedly attenuated the carrageenin-induced edema of the paw of the rat. Injection of thrombin into the paw also produced edema. The effect of thrombin was due to activation of its receptor; a thrombin receptor activating peptide (TRAP) reproduced the effects of thrombin in causing edema. TRAP also increased vascular permeability as demonstrated by extravasation of Evans blue and ¹²⁵I-labeled serum albumin. The release of bioactive amines played an important role in mediating the TRAP-induced edema; the serotonin/histamine antagonist cryproheptadine and the histamine H2 receptor antagonist cimetidine reduced significantly the edema caused by TRAP. Treatment of rats with the mast cell degranulator 48/80 to deplete these cells of their stores of histamine and serotonin abolished completely the ability of TRAP to produce edema. Histochemical examination confirmed that TRAP treatment led to mast cell degranulation. Thus, it has been possible to demonstrate that thrombin acts as an inflammatory mediator in vivo by activating its receptor, which in turn leads to release of vasoactive amines from mast cells.

Thrombin is a trypsinlike serine protease that has a central role in hemostasis and thrombosis. It is generated as a result of the activation of the coagulation cascade. Once formed, thrombin cleaves fibrinogen to produce the fibrin mesh of the blood clot. Thrombin also exerts a positive feedback on its own production by activating factors V and VIII which are essential cofactors in the cascade. Natural anticoagulant mechanisms limit these processes; thrombin is inactivated by antithrombin III, and in a complex with thrombomodulin, it activates protein C which in turn shuts down the coagulation cascade. Thus, under physiological conditions, this balance between pro- and anticoagulant mechanisms allows the local generation of thrombin and prevents systemic coagulation (1).

The main features of the inflammatory response are: (a) vasodilation, allowing increased blood flow to the affected area; (b) increased vascular permeability, permitting diffusible components to enter the site; and (c) cellular infiltration by chemoataxis. All these processes are induced by soluble molecules that act locally at the site of tissue damage or infection; these molecules are known as inflammatory mediators and include molecules such as IL-1, IL-6, and TNF. Inflammatory mediators can also be derived from molecules that are normally present in plasma in inactive

forms, such as peptide fragments of some components of the complement, coagulation, and kinin systems (2).

Fibrin deposition is an integral feature of the inflammatory response to a variety of stimuli including thermal, chemical, or physical trauma (3, 4), indicating activation of the coagulation system and generation of thrombin at the site of inflammation. Besides catalyzing the formation of fibrin, thrombin also induces several of the cellular responses involved in inflammation. Activation of platelets by thrombin causes the release of 5-hydroxytryptamine (serotonin), a potent vasodilator (2). In addition, thrombin stimulates the secretion of other inflammatory mediators; for example, it causes mast cell degranulation with release of histamine (5) and promotes production of IL-1 by activated macrophages (6). Thrombin also directly mediates several inflammatory responses. It increases vascular permeability (7) and promotes transendothelial cell migration by neutrophils (8). This latter effect of thrombin is due to at least two cellular responses to thrombin. Thrombin enhances adhesion of neutrophils to endothelial cells by increasing surface expression of P-selectin (9–11). In addition, thrombin acts as a chemotactic agent for neutrophils (12, 13). Many of the cellular effects of thrombin involve its proteolytically activated receptor. Thrombin activates this receptor by a

unique mechanism; it cleaves within the extracellular domain of the receptor and the newly created NH₂-terminus then acts as a "tethered ligand" (14). Synthetic peptides corresponding to the tethered ligand are able to activate the receptor in the absence of thrombin (14) and are termed thrombin receptor activating peptides (TRAP).¹ The thrombin receptor has been shown to mediate thrombin-induced vasodilation (15), increased vascular permeability (16), and neutrophil-endothelial cell adhesion (11).

Whereas all these observed effects of thrombin suggest a possible role for this protease in mediating the inflammatory response, no direct evidence has been obtained for such a role *in vivo*. In the present study, we have demonstrated for the first time a direct role for thrombin as a mediator of inflammation in a rat model. Moreover, the results indicated that thrombin functions as an inflammatory mediator through activation of its receptor. Thus, thrombin provides an important link between the initial response to injury (blood coagulation) and the later cellular responses required for healing (inflammation). Thrombin may also be important in inflammation not related to healing where infiltrating leukocytes often express cell-associated procoagulant activity that leads to thrombin generation (17).

Materials and Methods

Materials. TRAP (SFLLRNPNDKYEPF), the control TRAP peptide (SFLLANPNDKYEPF), and Hirulog (Biogen, Cambridge, MA) were prepared as previously described (18, 19). Human thrombin, compound 48/80, λ carrageenin, mepyramine, and cyproheptadine were purchased from Sigma Chemical Co. (St. Louis, MO). Cimetidine was a gift of Dr. Calignano (University of Naples, Naples, Italy).

Measurement of Edema Formation in the Rat Paw Edema. Male Wistar rats (Charles River, Milan, Italy) weighing 120–150 g were divided in groups of five and lightly anesthetized with ether. Each group of animals received subplantar administration of 0.1 ml of a solution containing thrombin (1 or 10 U), TRAP (1–500 μ g), or carrageenin (1% wt/vol) in PBS. Paw volume was measured using a hydroplethysmometer (Ugo Basile, Milan, Italy) immediately before the subplantar injection, each 15 min during the first hour, and at different time intervals thereafter for up to 5 h. The increase in paw volume was evaluated as difference between the paw volume at each interval and the basal paw volume. All edema experiments were repeated at least twice with similar results.

For the evaluation of the effects of drugs on edema formation, groups of animals were pretreated with cimetidine (50 mg/kg, *i.p.*), mepyramine (5 mg/kg, *i.p.*), or cyproheptadine (5 mg/kg, *i.p.*) 30 min before TRAP (500 μ g/paw) administration. Indomethacin was given at a dose of (5 mg/kg, *i.p.*) 15 min before TRAP administration. Hirulog (1–5 mg/kg, *i.v.*) was injected immediately before the subplantar injection of carrageenin or TRAP.

Depletion of Histamine and 5-Hydroxytryptamine. Groups of five rats were depleted of their mast cell stores of histamine and 5-hydroxytryptamine by repeated injection of the compound 48/80 as described by Di Rosa et al. (20). Briefly, a solution (0.1% wt/vol) of compound 48/80 in physiological saline was injected

intraperitoneally morning and evening for eight doses, starting with an evening dose. The doses employed were 0.6 mg/kg for the first six injections and 1.2 mg/kg for the last two administrations. The efficacy of the treatment was shown by the lack of edema for 1 h after the injection of carrageenin in the depleted animals. TRAP (100 or 500 μ g) was injected 5–6 h after the last injection of compound 48/80.

Measurement of Extravasation of Evans Blue in the Rat Paw. Evans blue solution (2.5% wt/vol; 25 mg/kg) was injected intravenously immediately before the subplantar injection of TRAP (1–500 μ g) or the control TRAP peptide (500 μ g). After 15 min, rats were killed; the paw was removed, homogenized, and stored in formamide (10 ml) for 72 h at 37°C to allow Evans blue extraction. Each solution was then filtered and the absorbance of the filtrate assessed at 619 nm (21). Each treatment group consisted of six rats.

Measurement of Local Edema Formation in Rat Dorsal Skin with ¹²⁵I-labeled Albumin. Local edema formation was measured in male Wistar rats (150–200 g) as the local accumulation of ¹²⁵I-labeled human serum albumin into skin sites (22, 23). Rats were anesthetized with sodium pentobarbitone (45 mg/kg, *i.p.*). The dorsal skin was shaven and a solution of Evans blue (0.5 ml/kg of 2.5% wt/vol) containing ¹²⁵I-labeled human serum albumin (2 μ Ci/kg) was injected intravenously. TRAP (10 or 100 μ g) or the control TRAP peptide (100 μ g) was injected intradermally in a final volume of 100 μ l into the shaved dorsal surface using a balanced site pattern of six injections, that is, three control and three treatment injections were symmetrically arranged on opposite sides of the dorsal midline. Six replicates were performed. Blood samples (1 ml) were taken into heparin by cardiac puncture after 15 min. The rat was decapitated and the dorsal skin was removed. The injection sites were punched out (5-mm diameter) and the radioactivity of both the skin and the plasma sample were determined. Edema formation at each site was expressed as plasma volume calculated on the basis of radioactivity in 1 ml of plasma.

Histochemistry. Rats were given a 0.1-ml subplantar injection of TRAP (100 or 500 μ g) or PBS and killed after 15 min. The injected paws were removed and fixed in neutral buffered formalin before being embedded in paraffin. Sections (4 μ m) were stained with Alcian blue and counterstained with hematoxylin.

Statistical Analysis. All results are reported as mean \pm SEM. The unpaired Student's *t* test was used for statistical evaluation of the results when two groups were compared. When more than two groups were compared, analysis of variance followed by Dunnett's test was used. A value of *p* < 0.05 was taken as significant. In some cases, the extent of the edema was evaluated as area under the curve of increase in paw volume versus time according to the trapezoidal rule.

Results

Role for Thrombin in the Acute Inflammatory Response to Carrageenin. To investigate a possible role for thrombin in the acute inflammatory response, the effect of the thrombin inhibitor Hirulog on carrageenin-induced edema was examined. Hirulog is a specific inhibitor of thrombin, binding to the active site and anion-binding exosite of the protease (18, 24). At all doses tested (1, 2, and 5 mg/kg), treatment with Hirulog suppressed the normal response to subplantar injection of carrageenin at the earliest time point (1 h; Fig. 1). However, the edema induced by carrageenin at later times (>3 h) was only significantly reduced by the higher

¹Abbreviation used in this paper: TRAP, thrombin receptor activating peptides.

doses of Hirulog (2 and 5 mg/kg). At the highest dose (5 mg/kg), edema formation was largely suppressed over the entire time course of the experiment (5 h; Fig. 1).

The results obtained with Hirulog suggested that thrombin is a mediator of the acute inflammatory response to carrageenin. The ability of thrombin to induce edema was confirmed by subplantar injection of thrombin into the hind paw. At the lower concentration (1 U/paw), thrombin caused a transient edema; the paw volume peaked at 15 min and returned to baseline by 60 min (Fig. 2 A). With the higher concentration (10 U/paw), the edema also peaked at 15 min, but had not returned to baseline by 90 min (Fig. 2 A).

Thrombin-induced Edema Is Mediated by Activation of the Thrombin Receptor. Peptides that activate the thrombin receptor were used to investigate the mechanism by which thrombin produced edema. Thrombin activates its receptor by cleavage and the newly generated NH₂ terminus acts as a tethered ligand for the receptor. Peptides corresponding to the tethered ligand can activate the receptor in the absence of cleavage (14). A TRAP reproduced the effects of thrombin (Fig. 2 B). A dose-dependent increase in edema was observed. Lower concentrations of TRAP (1 or 10 μg/paw) produced edema by 15 min that had returned to baseline by 45 min. With higher concentrations (100 or 500 μg/paw), greater edema was observed at 15 min, and with the highest concentration, the edema persisted until 90 min. The edema induced by TRAP was not due to activation of the coagulation cascade. Hirulog (5 mg/kg) did not reduce the edema in response to 500 μg TRAP (data not shown). The response to TRAP was specific. Previous studies have shown that replacement of the arginine in position 5 of TRAP by alanine reduces markedly the agonist activity of TRAP (19, 25). Injection of the peptide with alanine substituted in the position five (500 μg) produced significantly less edema than the equivalent dose of TRAP ($p < 0.01$; $n = 5$; Fig. 2 B).

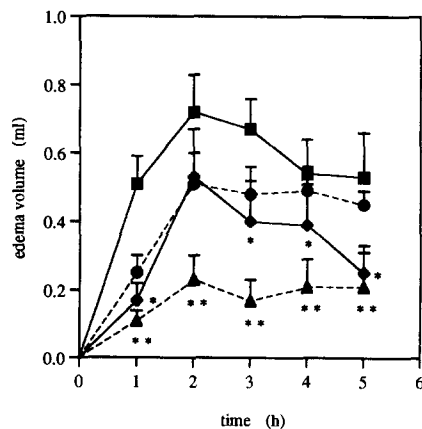


Figure 1. Inhibitory effect of Hirulog on carrageenin-induced paw edema. Edema was induced in the rat hind paw by a subplantar injection of 0.1 ml 1% (wt/vol) carrageenin and the increase in paw volume measured as described in Materials and Methods. Hirulog was given as an intravenous injection immediately before carrageenin at the following doses: 0 (■), 1 (●), 2 (◆), or 5 mg/kg (▲). * $p < 0.05$, ** $p < 0.01$ ($n = 5$).

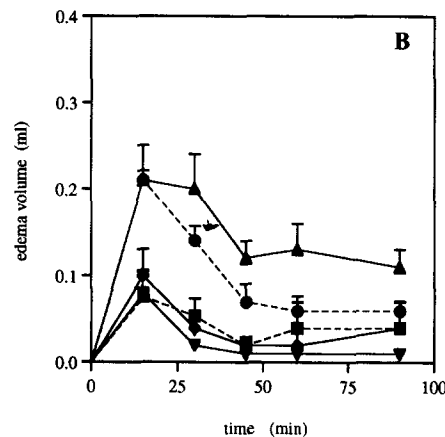
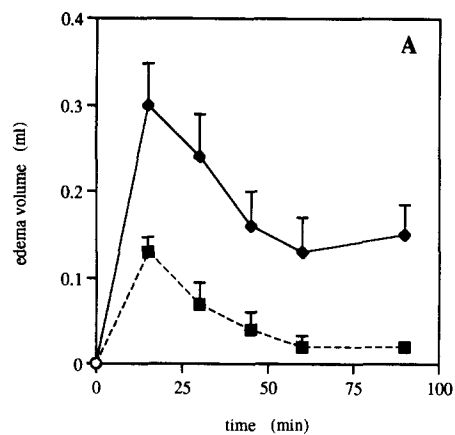


Figure 2. Thrombin and TRAP cause paw edema. Edema was induced in the rat hind paw by a subplantar injection of 0.1 ml solution of either thrombin (A) or TRAP (B); the increase in paw volume was measured as described in Materials and Methods. (A) Thrombin was administered at the following doses: 1 (◆) or 10 U/paw (■). (B) The TRAP doses used were: 1 (◆), 10 (■), 100 (●), or 500 μg/paw (▲). The control TRAP (SFLLANPNDK-YEPF) at a dose of 500 μg/paw (▼) produced significantly less edema ($p < 0.01$; $n = 5$) at all time points than the equivalent dose of TRAP.

The ability of TRAP to induce components of the acute inflammatory response was confirmed in two other experimental models. An increase in vascular permeability leading to extravasation of plasma proteins is an important component of inflammation. TRAP could induce this extravasation. Intravenous injection of either Evans blue or ¹²⁵I-labeled serum albumin was used to evaluate increases in vascular permeability. Subplantar injection of TRAP into the hind paw led to an increase in the amount of Evans blue leaking into the paw (Fig. 3). Significant increases in the amount of Evans blue in the paw were observed with 100 and 500 μg TRAP compared with 500 μg control TRAP. In addition, intradermal injection of TRAP into dorsal sites led to an accumulation of serum albumin at these sites. TRAP (100 μg) led to the accumulation of the equivalent of 48 ± 4 μl plasma/site compared with 24 ± 11 μl/site for the control TRAP (100 μg; $p < 0.05$, $n = 6$).

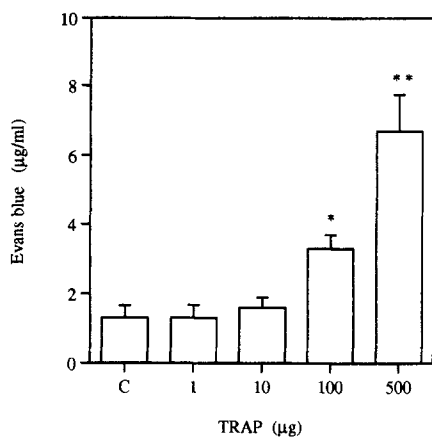


Figure 3. TRAP induces increases in vascular permeability. The effect of TRAP at different doses ($\mu\text{g}/\text{paw}$) on vascular permeability was evaluated by the accumulation of Evans blue in the paw after subplantar injection as described in Materials and Methods. (C) Response to the control TRAP (SLLANPNDKYEPF) at a dose of 500 $\mu\text{g}/\text{paw}$. The responses to 100 and 500 μg TRAP were significantly evaluated compared with the control; * $p < 0.05$, ** $p < 0.01$ ($n = 6$). Data similar to those shown were obtained on two other occasions.

Bioactive Amines Are Involved in TRAP-induced Edema.

The edema induced by TRAP was rapid in onset and of short duration. The most important mediators of the early phase of the inflammation response involving vasodilation and increase in vascular permeability are the bioactive amines histamine and 5-hydroxytryptamine (serotonin) secreted by mast cells and platelets after activation. Thus, two mechanisms for the TRAP-induced edema seemed possible: either (a) TRAP acted directly on endothelial cells to increase vascular permeability; or (b) the effects were due to stimulation of mast cells and/or platelets to release vasoactive amines. It was possible to distinguish between the two possibilities by using histamine/serotonin receptor antagonists and by depleting mast cells of endogenous amines. Treatment of rats with the serotonin/histamine antagonist cyproheptadine and the histamine H₂ receptor antagonist cimetidine significantly reduced the edema caused by TRAP. Edema formation was measured as the area under the curve for data similar to those shown in Fig. 2 B after subplantar injection of 500 μg TRAP. Cyproheptadine and cimetidine reduced the TRAP-induced edema by $57 \pm 8\%$ and $47 \pm 12\%$, respectively ($p < 0.05$; $n = 5$). Cyproheptadine has been shown to cause a similar decrease in serotonin-induced edema at an equivalent dose (26, 27). In contrast, the histamine H₁ receptor antagonist mepyramine did not affect significantly the extent of the edema due to 500 μg TRAP; a $27 \pm 14\%$ reduction was observed. Thrombin has also been shown to stimulate the production of the vasoactive eicosanoids thromboxane A₂ and prostacyclin (28). These compounds do not, however, seem to be involved in TRAP-induced edema. Blocking their synthesis with indomethacin had little effect on the edema in response to 500 μg TRAP (indomethacin treatment caused a $20 \pm 16\%$ decrease in edema).

The results presented in Fig. 4 established that bioactive amines produced by mast cells were the principal mediators of the edema caused by TRAP. Rats were treated with the mast cell degranulator 48/80 to deplete these cells of their stores of serotonin and histamine. Complete depletion of these stores was confirmed by the absence of edema in response to carrageenin in treated rats. Treatment with compound 40/80 did not affect significantly the numbers of circulating platelets, neutrophils, and lymphocytes in comparison with saline-treated controls. As shown in Fig. 4, pretreatment of rats with 48/80 completely abolished the ability of TRAP to cause edema.

Histochemical examination indicated that TRAP treatment had caused mast cell degranulation (Fig. 5). Injection of TRAP led to the loss of tissue organization characteristic of edema (Fig. 5 B). The granules of mast cells contain heparin as well as histamine and serotonin. In sections from the paws injected with buffer control, mast cells stained positively with Alcian blue because of the presence of heparin in the granules (Fig. 5 A). After TRAP treatment, however, mast cells failed to take up Alcian blue because of the release of their granule contents (Fig. 5 B).

Discussion

The thrombin-specific inhibitor Hirulog attenuated greatly the edema produced by carrageenin (Fig. 1). Higher doses of Hirulog dampened both the initial and later responses to carrageenin. These results suggest a role for thrombin in both phases of the carrageenin-induced edema. The initial phase of the response to carrageenin is largely due to the release of histamine and serotonin from mast cells and the later phase occurs when polymorphonuclear cells migrate into the site (29). Thus, the results with Hirulog suggest

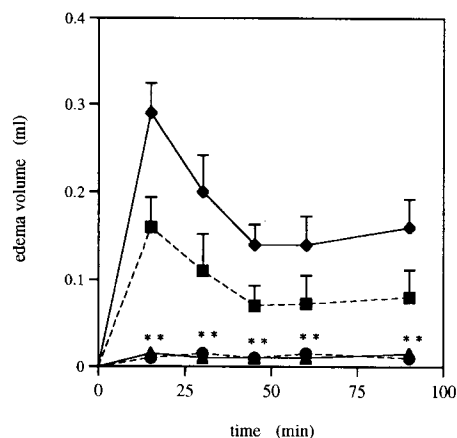


Figure 4. Depletion of bioactive amines abolishes TRAP-induced edema. Rats were treated with 48/80 to deplete their mast cell stores of histamines and serotonin, and TRAP was given by subplantar injection after completion of the treatment. Two groups received saline instead of 48/80 before administration of TRAP at doses of 100 (■) or 500 $\mu\text{g}/\text{paw}$ (◆). The groups treated with 48/80 failed to respond to TRAP at doses of 100 (●) or 500 $\mu\text{g}/\text{paw}$ (▲). ** $p < 0.01$ ($n = 5$). The complete inhibition of the TRAP response by treatment with 48/80 was also observed when the experiment was repeated on two further occasions.

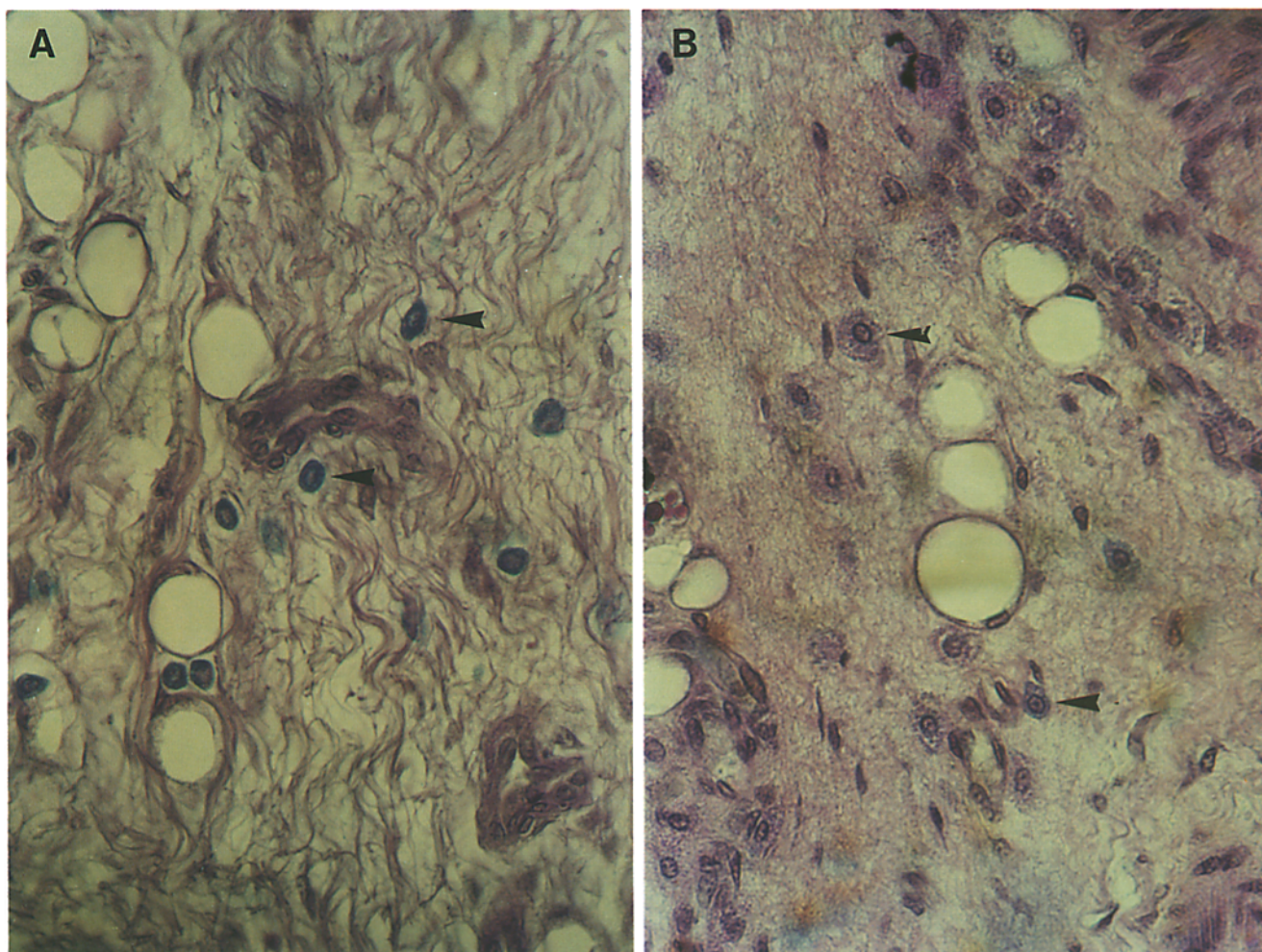


Figure 5. TRAP treatment causes mast cell degranulation. Rats were given a 0.1 ml subplantar injection of PBS (*A*) or TRAP (500 µg in PBS; *B*). Rat paws were processed for histochemical examination as described in Materials and Methods and sections were stained with Alcian blue and counterstained with hematoxylin. In the control section (*A*), mast cells stained blue (arrows). After TRAP treatment, mast cells (arrows) failed to stain with Alcian blue, indicating degranulation. $\times 600$.

that thrombin acts both by stimulating the release of bioactive amines from mast cells and as a chemotactic agent for polymorphonuclear cells. The effect of thrombin in the initial phase of edema formation appears to involve the activation of the thrombin receptor; low concentrations of TRAP (1 or 10 µg/paw) caused a transient edema and more persistent edema was observed with higher concentrations (100 or 500 µg/paw; Fig. 2 *B*). The edema caused by TRAP appeared to be mediated by the release of bioactive amines from mast cells. Histamine/serotonin antagonists reduced the TRAP-induced edema and depletion of stores of bioactive amines in mast cell granules by compound 48/80 abolished completely the ability of TRAP to cause edema (Figs. 3 and 4). Moreover, histochemical examination confirmed that treatment with TRAP caused mast cell degranulation (Fig. 5). The complete suppression of TRAP-induced edema by chronic treatment with compound 48/80 was particularly striking (Fig. 4). Although it is difficult to exclude that compound 40/80 does not affect other cells in-

involved in the inflammatory response, previous studies indicate that the major effect of this compound is to deplete mast cells of their stores of bioactive amines, whereas endothelial cells, platelets, and leukocytes are not markedly affected (30–32). From the results obtained in the present study, it is not possible to evaluate whether TRAP directly induces mast cell degranulation and release of bioactive amines. Alternatively, this degranulation may be mediated by the effect of TRAP on other cells which in turn release substances capable of promoting mast cell degranulation. However, a previous *in vitro* study has shown that thrombin can cause degranulation of mast cells with the concomitant release of serotonin and histamine (5). In conclusion, our results indicate that TRAP (and thrombin) cause edema by stimulating directly or indirectly mast cells to release bioactive amines which in turn cause the increase in vascular permeability that is necessary for edema formation (33).

Although the results obtained with TRAP suggest a role for activation of the thrombin receptor in the initial phase

of edema formation, the transient response to TRAP indicates that activation of the receptor may not be involved in the later phase of the response. The ability of Hirulog to attenuate this later phase suggests that thrombin may be acting independently of its receptor in this phase. The involvement of the thrombin receptor in the later phase cannot, however, be ruled out since TRAP may be short-lived at the site of inflammation; TRAP is rapidly degraded in plasma by aminopeptidase M (34). Nevertheless, there is evidence that thrombin-induced neutrophil chemotaxis does not involve the receptor activated by TRAP. Inactive thrombin, which will not activate the thrombin receptor, stimulates neutrophil chemotaxis (12, 35). Thus, it seems possible that thrombin could stimulate the accumulation of leukocytes at the site of inflammation through activation of a distinct (chemotactic) receptor. The ability of Hirulog to attenuate the later response to carrageenin may be related to its ability to bind to sites on thrombin other than the ac-

tive site; the results of Rowand et al. (13) indicate that neutrophil chemotaxis would be blocked by Hirulog's binding to the anion-binding exosite of thrombin.

Overall, our results indicate that thrombin may be an important mediator of the acute inflammatory response. Thrombin will be formed as a result of vascular injury. Alternatively, exposure of endothelial cells and macrophages to inflammatory stimuli, such as LPS and IL-1, leads to the expression of cell-associated procoagulant activity with resultant thrombin generation (17). Once formed, thrombin could stimulate release of vasoactive amines from mast cells (5) and act as chemoattractants for neutrophils (12, 13, 35). Thrombin may also cause the secretion of additional inflammatory mediators (6, 36, 37) and recruit other cells to the site of inflammation (38). The results indicate synthetic thrombin inhibitors, such as Hirulog, may be useful in controlling inflammation.

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