Release of the Mucosal Mast Cell Granule Chymase, Rat Mast Cell Protease-II, during Anaphylaxis Is Associated with the Rapid Development of Paracellular Permeability to Macromolecules in Rat Jejunum

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Summary

The soluble granule chymase, rat mast cell protease-II (RMCP-II), is abundantly expressed in intestinal mucosal mast cells (MMC) but its function is not known. One hypothesis is that RMCP-II degrades the epithelial basement membrane and promotes the loss of enterocytes typically associated with type I hypersensitivity reactions in the rat. To test this hypothesis more directly, ex vivo perfusion of the cranial mesenteric artery and jejunal lumen was used to monitor the anaphylactic release of RMCP-II and its effects on mucosal permeability and epithelial integrity. Within 2 min of intravascular challenge with soluble adult *Nippostrongylus brasiliensis* worm antigen there was a 1,000-fold ($P < 0.02$) increase in the concentration of RMCP-II in the vascular perfusate from the jejunum of *Nippostrongylus*-sensitized rats but not the controls. Similarly, translocation of RMCP-II into the gut lumen increased 10-fold ($P < 0.02$) after 2 min only in worm antigen–challenged immune rats. Using an identical protocol, but incorporating Evans blue–labeled human serum albumin (EB-HSA) in the vascular perfusate, the timing of the release of RMCP-II into the two compartments was very similar to the first experiment and furthermore the translocation of EB-HSA increased 18-fold ($P < 0.05$) after 4 min in sensitized rats challenged with worm antigen. To examine the effects of RMCP-II more directly 1 mg of the highly purified chymase was introduced into the cranial mesenteric artery in ex vivo perfused normal rats. A significant ($P < 0.05$) 70-fold increase in concentration of RMCP-II in jejunal perfusate occurred after 6 min. In a repeat dose-response experiment, infusion of 0.375, 0.75, or 1.5 mg of RMCP-II, together with EB-HSA, established that the cumulative amounts of RMCP-II and EB-HSA translocated from the vasculature to the gut lumen in each perfusion (during the 10-min period of RMCP-II infusion) were significantly correlated. Analysis of intestinal perfusates by SDS-PAGE and by Western blotting using monoclonal anti–RMCP-II antibody confirmed that there was a concomitant translocation of both the protease and EB-HSA into the gut lumen. Histological evaluation of the mucosa failed to reveal any significant morphological change in any of the experiments. The rapid development of macromolecular leak, its association with the translocation of RMCP-II, and the absence of gross epithelial lesions, suggest for the first time that a mast cell granule chymase increases epithelial permeability via a paracellular route and implies that the substrate may be a protein, or proteins, in the epithelial junctional complex.

Hypersensitivity reactions in the gastrointestinal tract are associated with a secretory response of the epithelium and, frequently, increased permeability to macromolecules (1, 2). Mast cell activation has been implicated as a cause of these changes by a variety of experimental approaches including direct observation of mast cell degranulation supported by measurement of the systemic release of the soluble chymase rat mast cell protease–II (RMCP-II)[1] (3, 4); measurement of jejunal ion transport in rats (5) and mice (6) where the density of mast cells in the mucosa varies; use of mast cell stabilizing agents to block the release of

[1] *Abbreviations used in this paper:* α_1PI, α_1-protease inhibitor; EB-HSA, human serum albumin labeled with Evans blue dye; MMC, mucosal mast cells; RMCP-II, rat mast cell protease–II; we, worm equivalents.

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stored or newly generated mediators (7). While the results of these experiments suggest a role for mast cells in the modulation of mucosal function, the relative importance in vivo of specific mediators has not been clearly established. Of the mediators released by mast cells histamine, adenosine, platelet-activating factor, prostaglandins, leukotrienes and some cytokines have all been shown to alter mucosal function in experimental models (2, 8–16). However, investigation of the interactions between epithelia and the mast cell granule serine proteases tryptase and chymase, which make up a substantial percentage of the total granule protein (17) content, has largely been neglected.

The intestinal mucosa of the rat contains many mast cells which are the major source of the granule chymase RMCP-II. After infection with the intestinal nematode *Nippostrongylus brasiliensis* there is hyperplasia of intestinal mucosal mast cells (MMC) and the concentration of RMCP-II may increase up to 10-fold to reach 2–3 mg RMCP-II/g wet wt of intestine (18). Since RMCP-II containing MMC are confined to the mucosa (19) the concentration of this protease stored in the lamina propria can be conservatively estimated to be 4–6 mg/g wet wt. When *Nippostrongylus*-sensitized rats are challenged intravenously with soluble *N. brasiliensis* antigen systemic levels of RMCP-II reach 400–800 μg/ml serum (3, 20). This represents 4–8 mg RMCP-II in the systemic circulation of a 250–300-g rat. Similarly, 1–2 mg of RMCP-II can be recovered from the lumen of the intestine within 5 min of challenge (20). The massive release of RMCP-II during anaphylaxis is associated with severe damage to the intestinal mucosa and extensive epithelial shedding (3). While these lesions may potentially be a consequence of anoxic damage it has been suggested that proteolysis of basement membrane proteins by RMCP-II is causally involved (4, 20). However, neither the native substrate of RMCP-II, nor the extent to which proteolysis is inhibited by native serpins has been defined, and it is therefore unclear whether RMCP-II plays a significant role.

To examine the relationship between mucosal permeability and the enteric release of RMCP-II in more detail, an ex vivo perfusion model was established. This model involves simultaneous perfusion of the vasculature and lumen of the jejunum in situ, allowing comparison of the precise timing of mast cell mediator release into both compartments. Removal of circulating cells and plasma proteins by perfusion also makes it possible to examine the effect of direct infusion of proteases in the absence of native inhibitors. Using this model we examined the effect on jejunal mucosal permeability of both systemic challenge of sensitized rats with worm antigen and direct infusion of RMCP-II. Our results suggest that RMCP-II is directly implicated in the rapid development of epithelial permeability via a paracellular route.

Materials and Methods

Animals. Male Wistar rats (290 ± 5 g; Bantam and Kingman, Hull, UK) were used for all experimental protocols. Immune rats were infected twice with 3,000 *Nippostrongylus brasiliensis* third-stage larvae by subcutaneous injection (21), 4 and 1 wk before perfusion experiments. Naive control rats were not exposed to the parasite. Soluble worm antigen was prepared from whole adult *N. brasiliensis* worms as previously described (22). The challenge dose of 100 worm equivalents (we) of worm antigen (total protein concentration of 1.1 mg/ml) was administered into the vascular perfusate as a bolus in 0.1 ml of Krebs-Ringer buffer.

Ex Vivo Perfusion. The model of jejunal perfusion has been described previously (23). Briefly, under general anesthesia (intraperitoneal pentobarbitone sodium 84 mg/kg) the vasculature of a 30.8 ± 0.6-cm section of jejunum (starting 5 cm distal to the ligament of Treitz) was isolated, with the intestine remaining in situ, and was then perfused via the cranial mesenteric artery at a rate of 1.5 ml/min with Krebs-Ringer buffer, pH 7.4, gassed with 95% O_2/5% CO_2, containing 5 mM glucose and 10% wt/vol Ficoll 70 (Sigma Chemical Co., Poole, UK). In some experiments (detailed in Results) human serum albumin (10 mg/ml) (Sigma Chemical Co.) labeled with Evans blue (EB-HSA) (Sigma Chemical Co.) was included in the vascular perfusate. The vascular perfusate effluent was collected via a cannula inserted in the hepatic portal vein. The lumen of the isolated section of jejunum was perfused simultaneously with Krebs-Ringer buffer gassed with 95% O_2/5% CO_2 containing 5 mM glucose at a rate of 1.8 ml/min. Once perfusion was established, the rat was killed with an overdose of pentobarbitone sodium.

The vasculature of the isolated tissue was continuously perfused and antigen was introduced via a "Y-piece" over a 10-s period starting at 21 min 50 s after the start of perfusion. Infusion of purified RMCP-II was carried out using a syringe infusion pump (Harvard Apparatus Ltd., Edenbridge, UK) over a 10-min period starting at 22 min after the start of perfusion. Samples were collected for 1 min in every 2 min from the vascular and lumenal outflow during the 20-min basal perfusion period and then continuously at 30-s (vascular) and 1-min (gut lumen) intervals after antigen challenge and 2-min intervals (vascular and gut lumen) after the start of infusion of RMCP-II. At the end of the perfusion period the section of jejunum perfused was measured (weight and length) and samples were immediately fixed or frozen for histological examination and assay of RMCP-II.

Purification of RMCP-II. RMCP-II was isolated from the small intestine of rats infected with *N. brasiliensis* using the method previously reported (24). The stock solution of RMCP-II was finally concentrated and purified by elution from a Mono-S (Pharmacia, Milton Keynes, UK) ion exchange column in 10 mM sodium phosphate, 120 mM NaCl. The protease used for infusion experiments was >90% pure as determined by SDS-PAGE (Fig. 1) and was 96% active when titrated against equine α_1-protease inhibitor of known concentration (25).

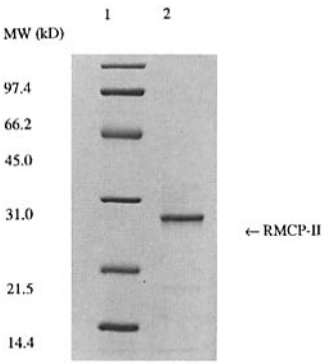

Figure 1. SDS-PAGE analysis of RMCP-II purified from rat small intestine. Proteins separated by 12% PAGE using the Laemmli technique and stained with Coomassie blue. Lane *1*, mol wt standards; lane *2*, RMCP-II.

Measurement of RMCP-II by ELISA. The concentrations of RMCP-II were measured in vascular and lumenal perfusates and in tissue, by ELISA as previously described using a mouse mAb to capture and a horseradish peroxidase–conjugated sheep polyclonal antibody (26). 3,3′,5,5′-tetramethylbenzidine was used as a substrate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). The lower quantifiable limit of detection of the assay was 0.25 ng/ml. 2–3-cm weighed segments of jejunum, frozen after each perfusion, were homogenized on ice in 20 mM Tris/HCl pH 7.5; 1 M NaCl, centrifuged at 8,000 *g* for 30 min, and the supernatant analyzed by ELISA for RMCP-II content which was then expressed in micrograms per gram wet weight of intestine.

Measurement of Albumin in Luminal Perfusates. In experiments investigating the change in permeability to albumin the vascular perfusate was prepared containing EB-HSA. Precipitation of the vascular perfusate before experiment (and samples of gut lumen perfusate after experiment) with 10% TCA demonstrated that all of the dye was protein bound. The amount of EB-HSA present in the luminal perfusates was assessed as a marker of combined endothelial and epithelial macromolecular permeability. A spectrophotometer (DU-650; Beckman Instruments, Inc., Fullerton, CA) was used to measure the optical density of the gut lumen samples and standards at 620 nm. Standard curves were prepared by dilution of preexperiment vascular perfusate with Krebs buffer and the lower limit of detection of the assay was equivalent to 5 μg/ml of albumin. The human serum albumin had no detectable serine proteinase inhibitor contamination.

Histology. Samples of jejunum were opened longitudinally after perfusion and wrapped around the barrel of a plastic pipette with the luminal surface outward. They were immediately fixed in 4% paraformaldehyde in PBS, stored in 70% ethanol, and subsequently embedded in paraffin wax. 4-μm longitudinal sections (27) were cut, rehydrated, and stained with hematoxylin and eosin. Mucosal morphology was assessed in relation to previously described mucosal lesions (28, 29). At least 50 villus/crypt U (27) were examined per section and lesions were scored on a scale 0–5 where 0 = no lesions and 5 = more than 50% of the villi affected. Changes noted and scored were loss of enterocytes from the tips of villi and separation (blebbing) of epithelia from the lamina propria.

Statistical Analysis. Statistical analyses were performed using Minitab software (Minitab Inc., University Park, PA). Flow rate time course data (presented as mean ± SEM) was analyzed using one-way analysis of variance and the Student's *t* test where appropriate to examine differences occurring with time within groups and differences between treatment groups for given time points. RMCP-II and albumin concentration data (presented graphically as median and range) in vascular and luminal perfusates were analyzed in a similar way using the nonparametric tests, Kruskal-Wallis and Mann-Whitney, as appropriate. Tissue concentrations of RMCP-II were also compared using the nonparametric tests. Within the text, overall mean values for RMCP-II concentrations for pre- and postchallenge time periods are quoted for ease of comparison but all probability values refer to statistical analyses as defined above.

Correlations between albumin and RMCP-II translocation into the gut lumen were investigated using linear regression. Results were considered to be statistically significant if a probability value of $P \leq 0.05$ was obtained using any of the tests specified.

Results

The Effects of Mast Cell Activation after Intravascular Challenge with Worm Antigen. To investigate the immunologically mediated release of RMCP-II from MMC and to compare the intravascular accumulation of the protease with its translocation into the gut lumen, ex vivo perfusions of the jejunum were performed in three groups of rats. Naive rats ($n = 5$) were perfused and after 21 min 50 s challenged with 100 we of soluble *N. brasiliensis* antigen via the cranial mesenteric artery. Two groups of rats immune to *N. brasiliensis* were also perfused and after 21 min 50 s rats in one group ($n = 5$) were challenged with 100 we of worm antigen over a 10-s period and, in the other, with BSA ($n = 5$). The following parameters were then measured: output of the vascular perfusate from the portal vein; concentrations of RMCP-II in the vascular perfusate before (first 20 min) and after (the following 10 min) intravascular challenge with antigen; concentrations of RMCP-II in the perfusate from the jejunal lumen before and after challenge.

The outflow rate of the vascular perfusate remained constant for the first 20 min of perfusion for all treatment groups (Fig. 2 *A*). Within 60–120 s after challenge with worm antigen the outflow rate decreased significantly ($P < 0.001$) in immune rats, but not in naive controls or immune rats challenged with BSA, when compared to the basal level. From this time point until the end of the experiment the vascular flow rate was significantly less ($P < 0.05$) in the immune challenge group compared to the other two groups except at 26 and 29 min after the start of perfusion. This result suggests either that there is an immunologically initiated alteration in vascular tone or increased endothelial permeability with subsequent movement of fluid into the tissue within 2 min of specific antigen challenge.

During the initial 20 min of perfusion, baseline secretion of RMCP-II into the vasculature was constant in all groups (Fig. 2 *B*). However, the concentration of RMCP-II measured in the vascular perfusate outflow was significantly greater in the two groups of immune rats (mean values for 20-min period were: immune challenge 18.5 ng/ml; immune control 11.4 ng/ml) when compared to the naive controls (3.9 ng/ml) ($P < 0.05$). Within 30 s of injecting the bolus of worm antigen the RMCP-II in the vascular perfusate of immune rats increased from ~10 ng RMCP-II/ml to ~100 ng/ml ($P < 0.02$) and after a further 60 s to 10,000 ng/ml (Fig. 2 *B*). The concentrations of RMCP-II in the vascular perfusate from the naive controls given worm antigen and the immune controls challenged with BSA were unaltered, although the concentration of RMCP-II (7.5 ng/ml) in the perfusate from immune controls was significantly ($P < 0.05$) higher than from the naive controls (3.2 ng RMCP-II/ml).

Baseline secretion of RMCP-II into the gut lumen remained constant during the initial period of perfusion and, in parallel with the secretion into the vasculature, was significantly greater ($P < 0.02$) in both immune challenge (41.9 ng/ml) and immune control (28.8 ng/ml) groups than the naive controls (0.74 ng/ml) (Fig. 2 *C*). After challenge there was no significant change in the output of RMCP-II from either control group with the concentra-

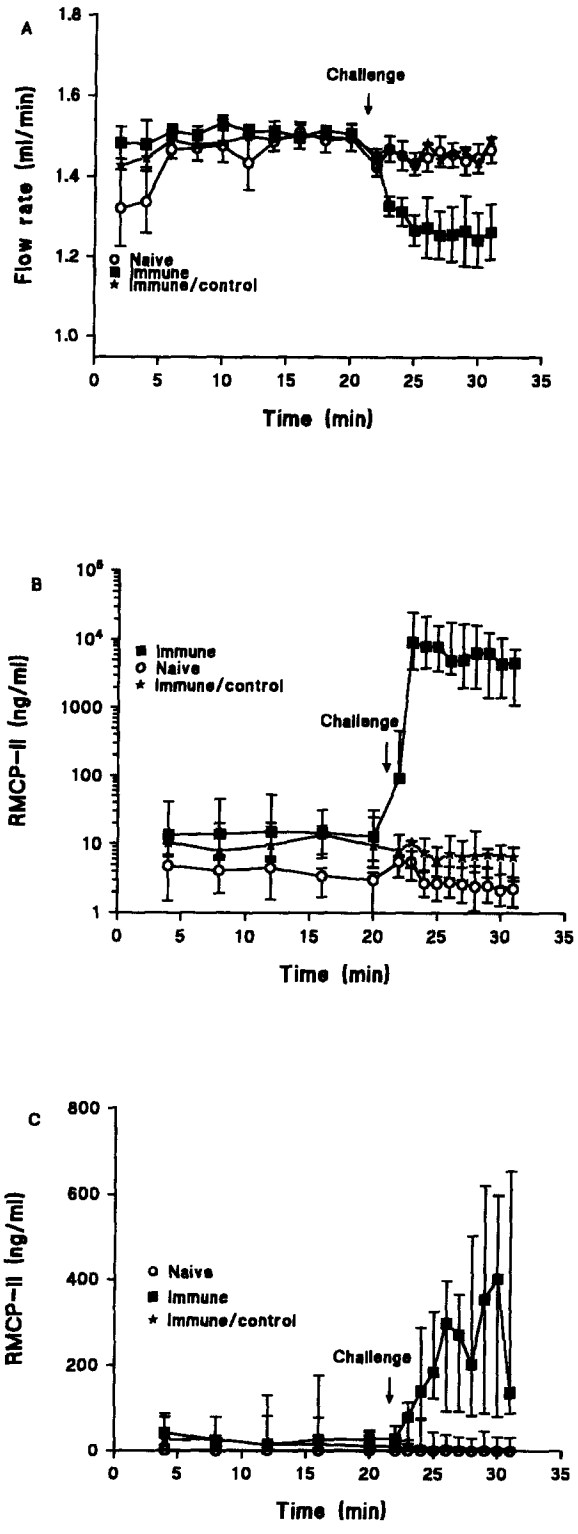

Figure 2. The effects of mast cell activation in ex vivo perfused jejunum after intravascular challenge (↓) with worm antigen (naive ○ and immune ■ rats) or BSA (immune/control ★). (*A*) Outflow rate (mean ± SEM) of vascular perfusate from the hepatic portal vein. Concentration (median and range) of RMCP-II measured by ELISA in (*B*) vascular perfusate outflow and (*C*) perfusate outflow from the lumen of the jejunum.

tion remaining at 13.1 ng/ml in the immune controls and 1.0 ng/ml in the naive rats ($P < 0.02$). 60 s after challenge of immune rats with worm antigen the concentration of RMCP-II in the the jejunal lumen was noticeably higher (70.1 ng/ml) than baseline, further increasing to 148 ng/ml in the next 60 s at which point it was significantly greater than baseline values ($P < 0.02$) suggesting that the jejunal mucosa had become more permeable. From this time point until the end of the experiment significantly more RMCP-II could also be detected in samples from the immune challenged rats compared to the immune controls ($P < 0.02$) (Fig. 2 *C*).

There was no difference in the concentration of RMCP-II in the tissue homogenates from immune or immune control animals (median values 579 vs 553 μg/g perfused tissue), but there were significantly lower levels in tissue from the naive control rats (59 μg/g perfused tissue, $P < 0.01$).

The Effects of Mast Cell Activation on Mucosal Permeability to Macromolecules. A further experiment was performed to assess whether the apparent increase in permeability of the jejunal epithelium to RMCP-II after challenge of primed rats with worm antigen represented a generalized increase in macromolecular permeability. The experimental protocol was similar to that reported above using three groups of rats, naive ($n = 5$), immune challenge ($n = 4$), and immune control ($n = 3$). In contrast to the first experiment EB-HSA was included in the vascular perfusate throughout the perfusion so that alterations in the leak of protein into the jejunal lumen could be monitored. Measurements were made of the perfusate output from the portal vein and concentrations of RMCP-II in the vascular and lumenal perfusates.

As in the first experiment vascular outflow rate from the portal vein was constant for all treatment groups for the first 20 min of perfusion. After challenge of immune rats with worm antigen the outflow rate was reduced compared to baseline ($P < 0.001$), but no change was observed in the flow rate from naive or immune rats challenged with BSA (data not shown). Therefore, after challenge, the flow rate was significantly less in the immune challenge group compared to the other two groups ($P \leq 0.05$).

RMCP-II release into the vasculature followed a pattern similar to that discussed above, with baseline RMCP-II concentrations being significantly higher in immune animals (immune challenge 15.9 ng/ml, immune control 19.4 ng/ml) when compared to naive controls (1.5 ng/ml) before challenge ($P < 0.05$) (Fig. 3 *A*). The increase above baseline levels in protease concentration after challenge of the immune group with worm antigen also followed a similar pattern to the first experiment reaching statistical significance ($P < 0.03$) (~6,000 ng/ml) within 30 s after challenge and increasing to a maximum after 60 s (10,000 ng/ml). The concentration of RMCP-II in the vascular perfusate of the two control groups did not alter significantly after challenge, remaining at 1.4 ng/ml in naive animals and 18.4 ng/ml in the immune control group.

The secretion of RMCP-II into the gut lumen also followed a pattern similar to that seen before with greater concentrations in the gut lumen of immune animals (im-

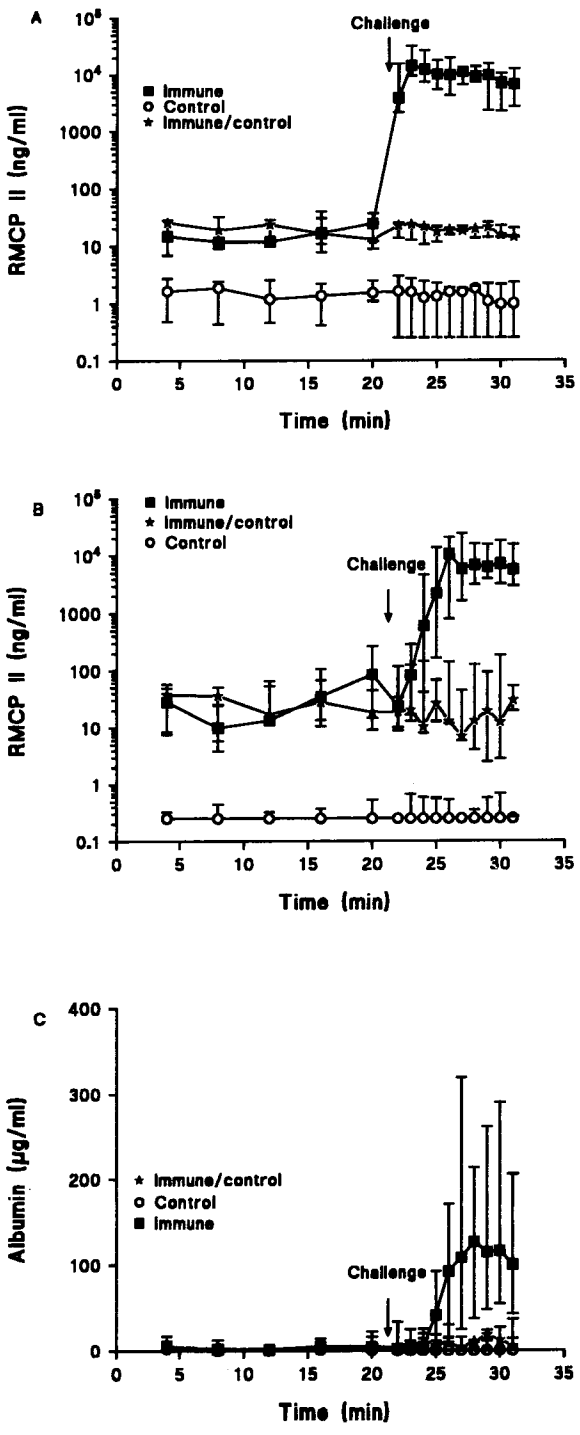

Figure 3. The effect of mast cell activation on the permeability of the mucosa of ex vivo perfused jejunum to macromolecules. The concentration of RMCP-II (median and range) measured by ELISA in (*A*) vascular perfusate and (*B*) luminal perfusate before and after challenge with worm antigen (naive ○ and immune ■ rats) or BSA (immune/control ★). (*C*) Corresponding concentration (median and range) of EB-HSA (optical density measured by spectrophotometer at 620 nm) in luminal perfusate. The time of challenge is indicated by the arrow.

mune challenge 44.8 ng/ml, immune control 31 ng/ml) compared to naive (0.3 ng/ml) before challenge ($P < 0.04$). After challenge of immune rats a trend to increased RMCP-II concentrations above baseline was observed at 60 s (115.7 ng/ml); however, the increase did not achieve significance ($P < 0.03$) until 240 s after challenge (4,600 ng/ml) (Fig. 3 *B*).

In the prechallenge period there were very low levels of translocation of albumin from the vasculature to the gut lumen (Fig. 3 *C*) in all three treatment groups (immune challenge 5.0 μg/ml, immune control and naive <5.0 μg/ml). However from 180 s after challenge of immune rats with worm antigen there was a clear trend to increased concentrations of labeled albumin (45.2 μg/ml) in the gut lumen when compared to both baseline measurements and to the other groups at each time point. The increase (to 90.3 μg/ml) reached statistical significance ($P < 0.05$) when compared to baseline at 240 s. These results show that there is minimal protein leak across the jejunal mucosa in this model before challenge with worm antigen but that after challenge of immune rats with worm antigen there is an increase in permeability to both RMCP-II and albumin.

The spectrophometric assay used routinely for analysis of EB-HSA was 1,000-fold less sensitive than the ELISA used for RMCP-II measurement. Therefore SDS-PAGE and Western blotting were used to see if a closer correlation between the onset of increased RMCP-II and albumin translocation into the gut lumen could be identified. Albumin was detected by Coomassie blue staining and RMCP-II by both Coomassie staining of gels and Western blotting with a specific mAb against RMCP-II. Increased concentrations of EB-HSA and of RMCP-II were both detected by 120 s after challenge. (A representative gel and blot from an immune rat challenged with worm antigen are shown in Fig. 4, *A* and *B*.)

Furthermore, regression analysis of the cumulative amounts of RMCP-II and Evans blue–labeled albumin (Fig. 5) leaking into the gut lumen of immune rats at each time point revealed a strong correlation ($r = 0.97$). Both of these results suggest that the translocation of the two macromolecules was related and probably occurred by the same route.

In agreement with the results of the first experiment no difference in tissue content of RMCP-II was observed between the immune challenge and immune control rats (median value 681.2 vs 631.9 μg/g perfused tissue) but there was significantly less RMCP-II in naive rat tissue (65.3 μg/g perfused tissue, $P < 0.03$).

The Effects of Direct Infusion of RMCP-II into the Vasculature of Ex Vivo Perfused Jejunum. The previous experiments had revealed changes in epithelial permeability related to mast cell activation and also shown a correlation between the translocation of RMCP-II and albumin into the jejunal lumen. In a further series of experiments we aimed to demonstrate whether the protease itself could be responsible for the observed alterations in epithelial permeability by infusing RMCP-II into the vasculature of ex vivo perfused jejunum.

In the first experiment a total of 1 mg of purified

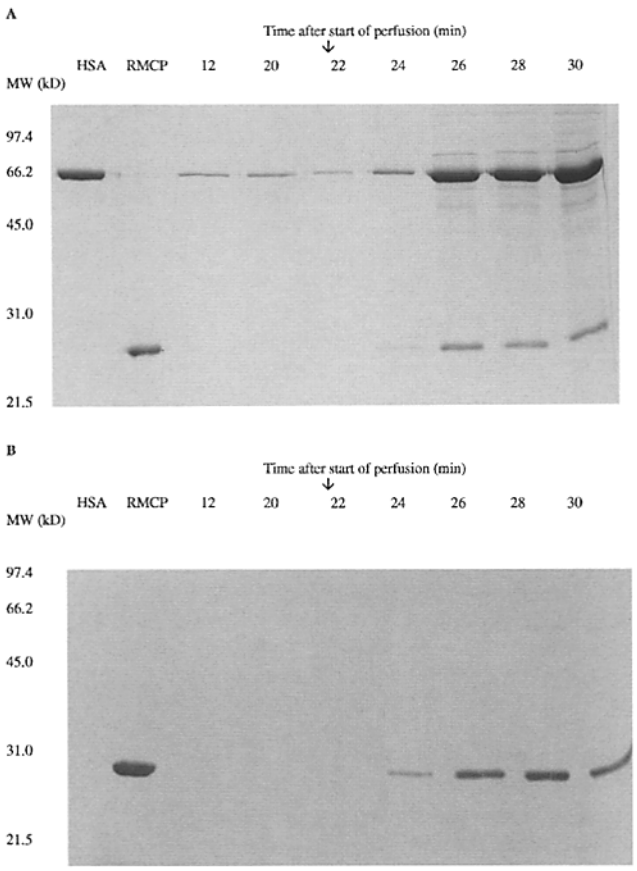

Figure 4. SDS-PAGE and Western blot analysis of timing of changes in permeability to macromolecules in ex vivo perfused jejunum from an immune rat challenged with worm antigen. Duplicate samples of human serum albumin (*HSA*), RMCP-II, and jejunal lumen perfusates taken at different times during the perfusion were separated by 12% SDS-PAGE and then one gel was blotted on to nitrocellulose. The first gel (*A*) stained with Coomassie blue demonstrated increased levels of albumin in the lumen of the jejunum after challenge with worm antigen. (*B*) Western blot from the second gel showing RMCP-II detected with a mouse mAb against RMCP-II and peroxidase-labeled conjugate and diaminobenzidine. Note that the first detectable appearance of RMCP-II by Western blot, at 24 min, is associated with an increased intensity of staining for HSA in the Coomassie-stained gel. Translocated RMCP-II is visible on the Coomassie-stained gel at 24 min and is readily detected at this time point by Western blotting. Arrow (↓) indicates time of challenge with soluble *N. brasiliensis* antigen.

RMCP-II (66.66 μg/ml vascular perfusate) was infused into the vasculature of each of four naive rats over a 10-min period beginning 22 min after the start of perfusion. The perfusion was then continued for a further 10 min. Measurements were recorded of the vascular perfusate outflow rate and concentrations of RMCP-II in the jejunal lumen perfusate. Infusion of RMCP-II had no effect on the flow rate of the vascular perfusate (data not shown). As noted in all previous experiments the basal secretion of endogenous RMCP-II in naive rats during the first 20 min of perfusion was very low (0.8 ng/ml) (Fig. 6). Rising concentrations of RMCP-II (12.5 ng/ml) could be measured in the gut lumen from 4 min after the start of infusion and were significantly ($P < 0.05$) above baseline by 6 min (55.4

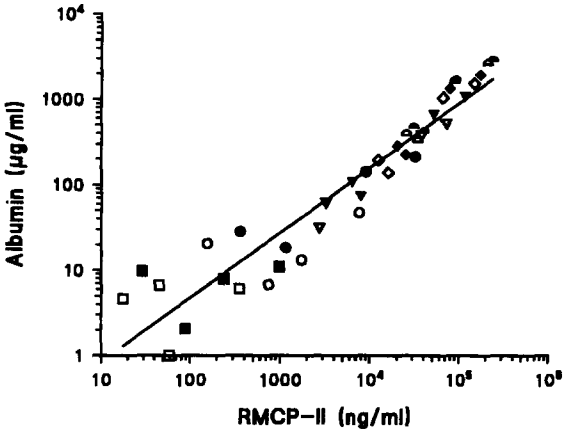

Figure 5. Regression analysis of the relationship between concentration of albumin and RMCP-II translocated into luminal perfusate after challenge of immune rats with worm antigen. Each point represents the cumulative amount of protein for an individual animal at 1 (□), 2 (■), 3 (○), 4 (●), 5 (▽), 6 (▼), 7 (◇), 8 (◆), 9 (☆), 10 (★) min after challenge; $r^2 = 0.90$, $P < 0.001$. log y = 0.76 logx − 0.84.

ng/ml). Therefore in the absence of other mast cell mediators it appeared that RMCP-II could cross both endothelial and epithelial barriers but it was not certain from this experiment that a change in permeability had occurred. Thus, in a further trial, EB-HSA was included in the vascular perfusate throughout the perfusion period.

In the second experiment 0.375, 0.75, or 1.5 mg of RMCP-II (25, 50, and 100 μg/ml of vascular perfusate) were infused over a period of 10 min into the perfused mesenteric arteries of three groups of two naive rats. There was, again, no change in the flow rate of the vascular perfusate during the perfusion. Before infusion of RMCP-II, concentrations of albumin and endogenous RMCP-II in the perfusate from the jejunal lumen were both very low (albumin 7.6 μg/ml, RMCP-II 0.25 ng/ml) (mean values are presented in Fig. 7, *A* and *B*). In all rats, the concentra-

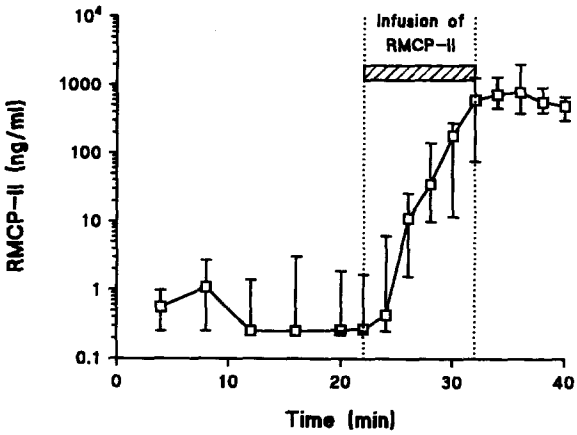

Figure 6. Direct infusion of 1 mg of purified RMCP-II into the vasculature of the ex vivo perfused jejunum of naive rats. Concentration of RMCP-II (median + range) increases significantly ($P < 0.05$) in the perfusate from the lumen of the jejunum when compared to the baseline by 4 min after the start of infusion.

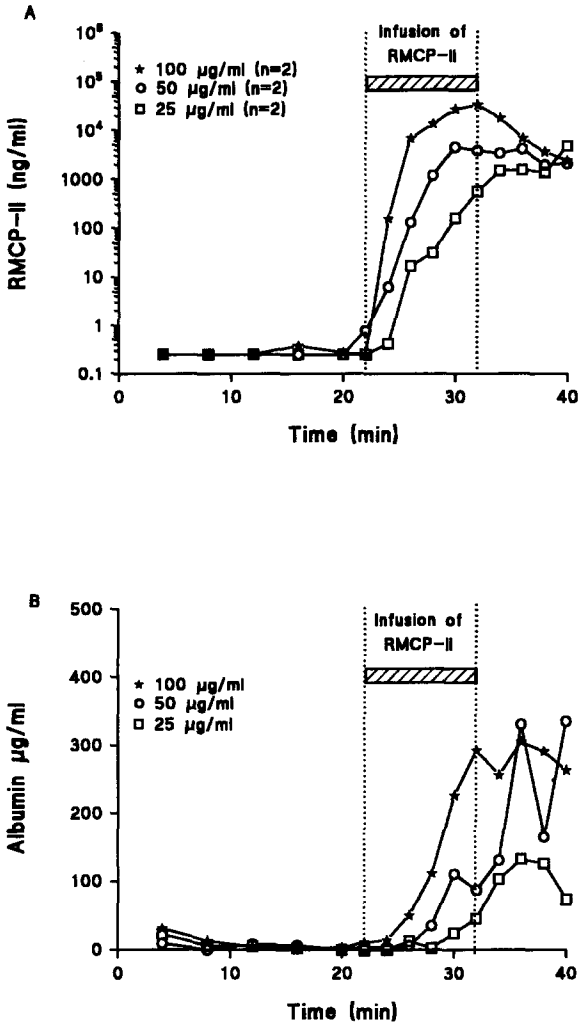

Figure 7. The effect of infusion of 25, 50, and 100 µg/ml of RMCP-II on macromolecular permeability of ex vivo perfused jejunum of naive rats. Mean concentrations of (*A*) RMCP-II and (*B*) albumin in the luminal perfusate before, during, and after infusion of three doses of RMCP-II.

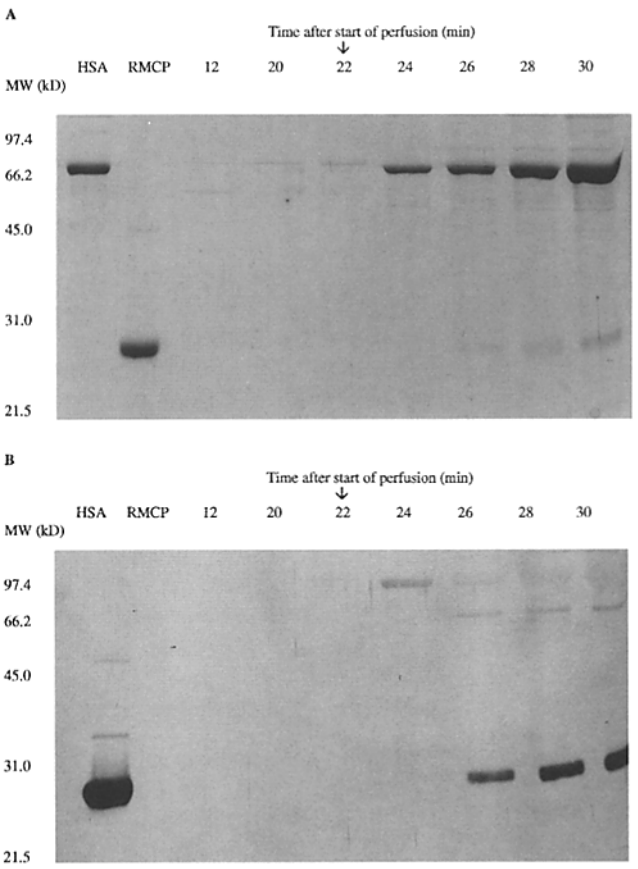

Figure 8. SDS-PAGE and Western blot analysis of changes in permeability to macromolecules in ex vivo perfused jejunum from a naive rat before and during infusion of RMCP-II (100 µg/ml). Duplicate samples of jejunal lumen perfusates taken at different times during the perfusion were separated by 12% SDS-PAGE and proteins from one gel were Western blotted on to nitrocellulose. (*A*) Gel stained with Coomassie blue demonstrating increased levels of albumin in lumen 2 min after the start of protease infusion. (*B*) Western blot of duplicate samples stained as previously described showing appearance of immunoreactive RMCP-II in lumen, first detected as a high mol wt (70-kD) complex 2 min after the start of infusion. Note the presence of a second lower mol wt (59-kD) RMCP-II immunoreactive band at 26–30 min. Arrow (↓) indicates start of RMCP-II infusion.

tion of RMCP-II increased in the jejunal perfusate although the timing of this increase varied in individuals from 1 to 6 min after onset of infusion. Leakage of EB-HSA into the gut lumen also started after infusion of RMCP-II but appeared to be delayed relative to the leak of RMCP-II (Fig. 7, *A* and *B*). As was discussed above, the detection method for EB-HSA was 1,000-fold less sensitive than the ELISA for RMCP-II and luminal perfusates were further investigated by SDS-PAGE and Western blotting. An example in which 1.5 mg of RMCP-II was infused at 100 µg/ml starting at 22 min shows that albumin and immunoreactive RMCP-II could both first be detected at 24 min (Fig. 8, *A* and *B*). The concentrations of albumin and RMCP-II increased over the period of infusion and both were readily detected by SDS-PAGE at 26 min (Fig. 8 *A*). Interestingly, RMCP-II reactivity on the blot shows a higher mol wt (70-kD) band suggestive of a serpin/protease complex. At subsequent time points, free RMCP-II was visible by SDS-PAGE and was strongly stained on Western blot (Fig. 8 *B*), weak staining of an additional immunoreactive band of 59 kD was visible from 26 to 30 min. This result confirms the spectrophotometric and ELISA results. Furthermore when the ELISA and EB-HSA data were corrected for the length of gut perfused, a significant correlation between the total cumulative amounts of RMCP-II and EB-HSA translocated into the gut lumen of each rat during the 10-min period of infusion could be shown ($r = 0.98$, $P < 0.0001$) (Fig. 9).

Histology. There was no gross evidence of mucosal damage in any of the experiments described above. A few minor lesions, including some loss of epithelium from the villus tips or separation of epithelium from the lamina propria, were detected histologically in several of the samples. However there was no overall pattern of epithelial loss or

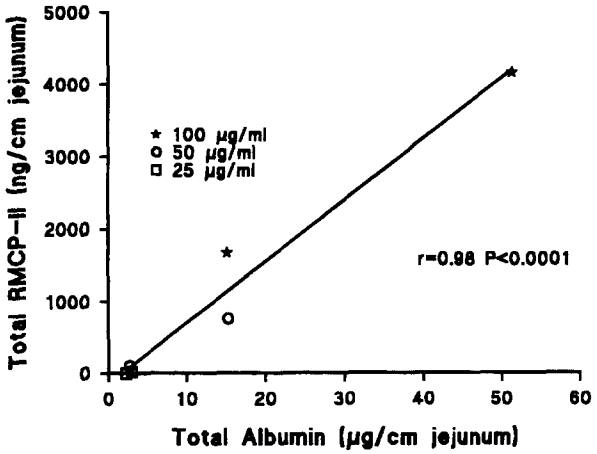

Figure 9. Correlation between the total cumulative RMCP-II and EB-HSA (corrected for length of gut perfused) translocated into the gut lumen of individual rats during the 10-min period of RMCP-II infusion. ($y = 84.8x - 154.5$; $r = 0.98$; $P < 0.0001$).

separation from the villi to suggest that immunological release of RMCP-II was associated with significant epithelial damage (Table 1).

Direct infusion of RMCP-II at concentrations in excess of those observed in ex vivo challenged rats but at levels seen in sera of rats in vivo 5–15 min after the induction of anaphylaxis (20) also failed to produce significant changes in mucosal histology (Table 1 and Fig. 10).

Discussion

The functional significance of the translocation of the mast cell granule chymases into the gut lumen during intestinal hypersensitivity reactions is not known. Therefore, the present experiments, using ex vivo perfused rat jejunum, were designed to address the following questions: (*a*) what is the temporal relationship between the intravascular and transmucosal release of the MMC granule chymase RMCP-II? and (*b*) is the translocation of RMCP-II into the gut lumen associated with increased permeability to other macromolecules and histologically detectable mucosal damage? In addition, does infusion of RMCP-II directly into the cranial mesenteric artery alter mucosal permeability or cause histological damage? Answers to these questions would provide an insight into the function of the soluble granule chymases and the role of mast cells at mucosal surfaces.

Ex vivo perfusion removes the plasma proteins and formed elements of the blood so that some of the potential effectors of systemic shock such as IgG and complement, and erythrocytes, which aggregate within the postcapillary venules of the gut, are absent or greatly reduced in concentration. Nevertheless, intravascular challenge of sensitized rats with soluble worm antigen resulted in a significant release of RMCP-II into the vascular perfusate within 30 s (Figs. 2 and 3) with maximum values of 6–10 µg/ml 1 min later. The rapid passage of this 30-kD macromolecule across the endothelium is most likely mediated by histamine or platelet-activating factor released from activated mast cells (30). By contrast, translocation of RMCP-II into the jejunal lumen was delayed for at least a further 30–60 s. The dissociation between the vascular and epithelial events suggest that distinct mechanisms may be operating in each compartment or that it reflects the difference in diffusion path lengths. When EB-HSA was added to the vascular perfusate, concentrations of RMCP-II and EB-HSA in the intestinal perfusate displayed a highly significant positive correlation; indicating that EB-HSA leak could be contingent on permeability changes brought about by the proteolytic activity of RMCP-II. Analysis of the intestinal perfusates by SDS-PAGE and Western blotting confirmed the ELISA and Evan's blue data, showing that both macromolecules are translocated concomitantly. The relationship between RMCP-II and mucosal permeability to macromolecules was investigated futher by infusing purified RMCP-II into the vasculature. In both series of experiments, the infusion of RMCP-II into normal jejunum resulted in the

Table 1. *Scores Obtained for Histological Features of Sections Taken from Perfused Jejunum Median (Range)*

	Treatment group	*n*	Epithelial shedding at tips of villi	Blebbing of epithelia at sides of villi
Experiment 1	Naive	5	0.5 (0–1)	0 (0–1)
	Immune	5	1.5 (0–3)	0 (0–1)
	Immune/control	5	0.5 (0–3)	1 (0–2.5)
Experiment 2	Naive	5	0 (0–1)	0 (0–0.5)
	Immune	4	1.25 (0–2)	0.75 (0–2)
	Immune/control	3	0 (0–2.5)	0
Infusion of RMCP-II	No albumin	4	1 (0–2)	0
	Albumin	6	1 (0–3)	0 (0–3)

0, no lesion; 5, extensive lesions. No significant differences were noted in lesion scores between treatment groups.

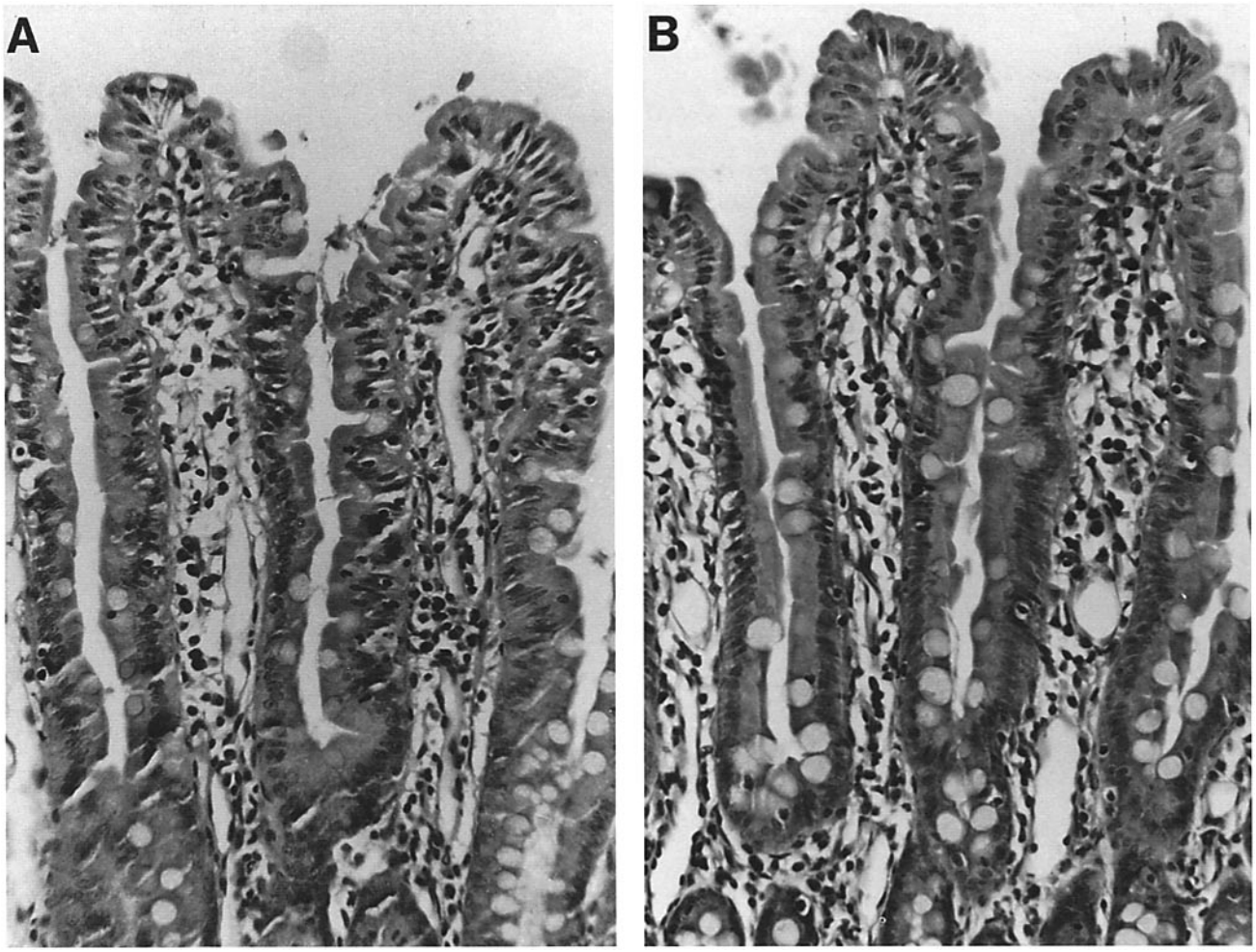

Figure 10. Light micrographs of sections of jejunum taken after ex vivo perfusion, stained with hematoxylin and eosin. Sections from naive rat jejunum (*A*) after challenge with *N. brasiliensis* worm antigen and (*B*) after infusion of the maximum dose of RMCP-II. Note the absence of gross morphological changes after perfusion. (× 350.)

appearance of the protease in the lumen of the jejunum 2–6 min later. Importantly, the translocation of EB-HSA was significantly correlated with the concentration of RMCP-II in the gut lumen, over the period of infusion (Fig. 9). These results were again supported by SDS-PAGE showing increased amounts of EB-HSA correlating with the appearance of RMCP-II in the gel and on the Western blot. Thus, in both active anaphylaxis and in passive infusion of RMCP-II, the development of mucosal permeability to EB-HSA was invariably associated with the concomitant appearance of RMCP-II in the gut lumen.

These findings again suggest that RMCP-II could have a direct role in causing epithelial permeability. However, the relationship may not be a simple one. Two aspects will require further investigation: (*i*) what part, if any, does RMCP-II have in promoting endothelial permeability? and (*ii*) could native inhibitors of RMCP-II be active in the interstitium of the lamina propria? The extent to which the luminal surface of the endothelium might be a substrate for RMCP-II when it has been infused intravascularly is not known. Furthermore, variable hemodynamics in individual ex vivo perfusions could affect the rates at which the infused RMCP-II reaches the mucosal interstitium. The relationship between the amount of RMCP-II infused and mucosal permeability could, similarly, be complicated by the presence of inhibitors. Two distinct α_1-protease inhibitors (α_1-PI) have been identified in rat plasma with an association rate constant (K_{ass}) for RMCP-II of $1–2 \times 10^5$ M^{-1} s^{-1} (31) and there are preliminary data to suggest that other α_1-PI–like serpins are present in the gut mucosa (32). It may be significant, therefore, that the earliest detection of RMCP-II immunoreactivity in the gut lumen, after infusion, is a high mol wt (70-kD) species possibly complexed to α_1-PI (Fig. 8 *B*). The presence of a second, weakly labeled band of slightly lower mol wt (59 kD) at later times presumably reflects the degradation of the complex by free RMCP-II (Fig. 8 *B*).

In vivo, RMCP-II released locally should, theoretically, be inactivated by plasma-derived serpins flooding through the endothelial gaps opened by histamine and other inflammatory mediators. However, our preliminary attempts to mimic the in vivo role of serpins by infusing either native

or synthetic inhibitors ex vivo were not successful because of the very high concentrations that were required (data not shown). When these same inhibitors were tested (at inhibitor/enzyme ratios of up to 10:1) against purified RMCP-II at the molarities used in the ex vivo infusions, a residual enzyme activity was consistently observed. It was not, therefore, possible to use inhibitors to investigate the proteolytic activity of RMCP-II in the ex vivo perfusion model. Nor is it likely that native inhibitors (the concentration of α_1-PI in plasma is ~3 mg/ml) are sufficiently abundant to nullify the activity of the abundant RMCP-II (4–6 mg/g wet wt of mucosa) released into the mucosa during systemic anaphylaxis in vivo (32).

Absence of significant histological change, despite substantial leak of EB-HSA, was a consistent feature in this study. Others have also noted increased mucosal permeability in the absence of detectable lesions during immediate hypersensitivity reactions, even when ultrastructural analysis was carried out (33). There are, however, several reports suggesting that mast cell activation is associated with the separation of epithelium from basal lamina (4). A few lesions of this type were noted in both control and experimental rats but were not specifically related to permeability changes nor to the release of RMCP-II. One of the proposed targets for RMCP-II is the epithelial basement membrane (20) but, with laminin or type IV collagen as potential targets, proteolysis would probably take several hours (34) and is more likely to be mediated by metalloproteases, activated by RMCP-II (35), than by the direct action of RMCP-II itself. It is interesting to note, therefore, that ultrastructural lesions of the basement membrane associated with IgE-mediated secretion of RMCP-II, were observed 4 h after challenge (4).

The very rapid development of macromolecular leak, within 5 min of challenge or of infusion of RMCP-II, suggests that proteolysis is directed against an immediately accessible substrate. Therefore, the most likely target is a paracellular, junctional complex protein which normally maintains functional epithelial integrity. One possible target could be the rat equivalent of the recently described chicken zonula occludens protein, occludin, which, from its predicted amino acid sequence, has several chymotrypsin-sensitive sites in its extracellular domains (36). In vitro studies using a pulmonary epithelial cell line have already shown that nanomolar concentrations of RMCP-II can promote rapid and reversible permeability to macromolecules (37) by a mechanism independent of active ion secretion (2). Similarly, a bacterial serine protease has recently been shown to cause paracellular permeability in a cultured epithelial cell line in association with loss of expression of the tight junction protein, ZO-1 (38). There is, therefore, the intriguing possibility that the mucosal mast cell chymases have a restricted substrate specificity so that, when released, they can alter the permeability of the mucosa, but not its structural integrity.

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