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Interaction of murine intestinal mast cell proteinase with inhibitors (serpins) in blood; analysis by SDS–PAGE and Western blotting

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SUMMARY

The interaction of mouse intestinal mast cell proteinase (IMCP) with serine proteinase inhibitors (serpins) in blood was analysed: (i) by examining the capacity of the inhibitors in blood to block the binding of the irreversible serine esterase inhibitor [^{3}H]diisopropyl fluorophosphate (DFP); (ii) by Western blotting. The binding of [^{3}H]DFP to IMCP was blocked very rapidly by inhibitors in mouse serum and, by Western blotting, this inhibition was associated with the appearance of a 73,000 MW proteinase/inhibitor complex together with a series of higher (> 100,000) MW complexes. IMCP was not dissociated from these complexes when electrophoresed under reducing conditions, although prior heat treatment of mouse serum (60° for 30–160 min) abolished the formation of all proteinase/inhibitor complexes. Similarly, the activity of a 48,000 MW inhibitor of chymotrypsin was abolished by heat treatment. A titration experiment established that between 0·5 and 5 mg IMCP were inhibited per ml of serum. The properties and MW of the IMCP inhibitor complexes are typical of serpins and suggest that IMCP secreted during intestinal immunological reactions would be rapidly and irreversibly inactivated by plasma-derived inhibitors.

INTRODUCTION

The concentrations of a highly soluble murine chymotrypsin-like proteinase derived from mast cells of the intestinal mucosa are greatly increase in mice infected with *Trichinella spiralis* (Newlands *et al.*, 1987; Huntley *et al.*, 1989). Isolated serosal mast cells (SMC) apparently lack this intestinal mast cell proteinase (IMCP) (Miller *et al.*, 1988, 1989) and on the basis of its solubility and abundance in parasitized intestinal mucosa (Miller *et al.*, 1989) murine IMCP was considered analogous to the mucosal mast cell (MMC)-derived granule protease of the rat, rat mast cell protease II (RMCPII) (Newlands *et al.*, 1987; Miller *et al.*, 1988), with which it shares 75% amino acid sequence homology (Le Trong *et al.*, 1989).

The development of an enzyme-linked immunosorbent assay (ELISA) facilitated detection of IMCP in the blood of *Trichinella*-infected mice (Huntley *et al.*, 1989), thus further confirming its similarity to RMCPII which is also released systemically during parasite infection. However, the concentrations of IMCP in the blood of parasitized mice were substantially greater (Huntley *et al.*, 1989) than the concentrations of RMCPII in peripheral blood of parasitized rats (Woodbury *et al.*, 1984).

Serine proteinases in contact with blood are normally rapidly and irreversibly inhibited by a group of plasma proteins synthesized in the liver and collectively known as the serpins (Travis & Salvesen, 1983). These highly polymorphic proteins include α_1-proteinase inhibitor and α_1-anti-chymotrypsin. Concentrations of IMCP in blood ranged from 0·2 μg/ml in normal mice to 25 μg/ml in *Trichinella*-infected mice (Huntley *et al.*, 1989), and, because this proteinase is potentially damaging to the circulatory system, the interaction of serpins with IMCP was examined. The results show that, when added to serum, IMCP is rapidly bound to a group of inhibitory proteins demonstrable by SDS–PAGE and Western blotting. Typically, in view of the inhibitory properties of serpins, IMCP was both inhibited and irreversibly bound.

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MATERIALS AND METHODS

Animals

Inbred NIH mice (6–8 weeks old) were purchased from OLAC and were maintained in positive-pressure isolators. Randomly bred 8–10-week-old Swiss white mice were raised at Moredun Research Institute. All mice were fed and watered *ad libitum*.

Parasite infection

NIH mice were infected orally with 300 *Trichinella spiralis* muscle larvae (Alizadeh & Wakelin, 1982). The muscle larvae were obtained by pepsin digestion from NIH mice infected several months previously (Alizadeh & Wakelin, 1982). Serum was collected 10–14 days after infection.

Collection of serum
To obtain serum, anaesthetized mice were killed by cervical dislocation and exsanguinated. Serum samples were stored at −20° prior to assay.

Protein estimations
Protein concentrations were estimated with BCA protein assay reagent (Catalogue No. 23225, Pierce and Warriner U.K. Ltd, Chester, as described by the manufacturer using an IL Multistat III FCS Microcentrifugal analyser and bovine serum albumin (grade V Sigma, Poole, Dorset) as a protein standard.

Purification of IMCP
Mouse intestinal mast cell proteinase was recovered from intestines of *T. spiralis*-infected mice and purified by cation exchange chromatography as described previously (Newlands *et al.*, 1987).

Antibody and antibody-peroxidase conjugates
Antibodies to IMCP were raised in rabbits and isolated by affinity chromatography on IMCP–Sepharose 4B as described previously (Newlands *et al.*, 1987). Rabbit anti-IMCP was coupled to peroxidase according to the method of Nakane & Kawaoi (1974).

Addition of IMCP to serum
Serum samples were incubated with IMCP (50 μg–5 mg/ml) for varying periods at 37°, in a shaking water bath.

SDS–PAGE
Discontinuous SDS–PAGE was as described by Laemmli (1970) using 15% gels with electrophoresis for 1 hr at a constant 200 volts in the BioRad Mini Protean II system (Biorad Laboratories, Watford, Herts). The following pre-stained markers were employed for visualization of molecular weights on Western blots: phosphorylase B (130,000), bovine serum albumin (BSA) (75,000) ovalbumin (50,000), carbonic anhydrase (39,000), soybean trypsin inhibitor (27,000), lysozyme (17,000). Moreover, the following [^{14}C]methylated protein standards were used for SDS–PAGE of samples bound to [^{3}H]DFP, myosin (200,000), phosphorylase B (92,500), BSA (69,000), ovalbumin (46,000), carbonic anhydrase (30,000) and lysozyme (14,300) (Amersham International plc, Amersham, Bucks).

Western blots
Western blotting was with the Khys-Anderson (1984) semi-dry method and each blot was washed in buffer containing PBS (2l), Tween-20 (10 ml), EDTA (0·74 g) and NaCl (40·8 g), before probing with rabbit IgG anti-IMCP HRPO antibody conjugate (25 μg/ml) for 1 hr at room temperature. Peroxidase activity was revealed with diaminobenzidene and hydrogen peroxidase.

Binding of [^{3}H]diisopropyl fluorophosphate (DFP)
Samples (10 μl) were incubated with 1·5 μCi [^{3}H]DFP (91% pure; 5·8 Ci/mmole), (Amersham International plc) and then subjected to SDS–PAGE.Gels were prepared for autoradiography by the method of Laskey & Mills (1975). Radiographs were usually exposed at −70° and developed after 4–14 days.

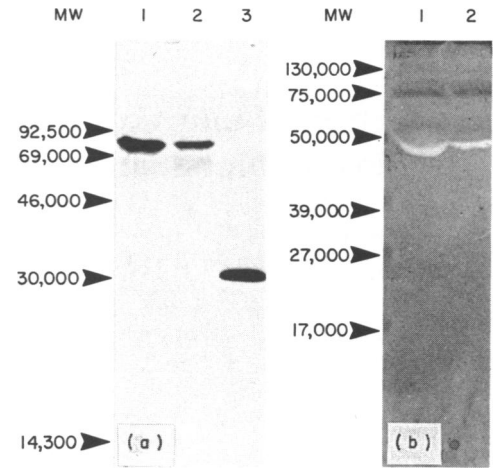

Figure 1. Characterization of proteinases or their inhibitors in serum from normal (lane 1) and *Trinchinella*-infected (lane 2) mice showing: (a) autoradiograph of samples preincubated with 0·5 μCi [^{3}H]DFP (lane 3 contains 80 ng IMCP) and electrophoresed under reducing conditions; (b) the inhibitory profiles of normal and *T. spiralis*-infected (Day 10) sera against chymotrypsin. Each lane contains 0·25 μl serum. SDS–PAGE run under non-reducing conditions.

Localization of proteinase inhibitors by SDS–PAGE
The method of Uriel & Berges (1968) for the detection of natural inhibitors in biological fluids after electrophoresis on acrylamide agarose gels was adapted to SDS–PAGE. The SDS was removed after each run by incubating the gel in 2·5% Triton X-100 for 1 hr at room temperature. The gel was then transferred into a solution of chymotrypsin (20 μg/ml) (Sigma) for 10 min at room temperature. The excess enzyme solution was drained and the gel covered and left at 37° for 30 min to allow the enzyme to bind to inhibitors separated by electrophoresis. Chymotryptic activity was detected by a colour reaction produced by adding acetyl-DL-phenylalanine-B napthyl ester (Sigma). Inhibitors appear as clear bands against a dark background.

RESULTS

Characterization of sera from normal and *Trichinella*-infected mice

Samples of serum from mice infected 10 days previously with *T. spiralis* and from controls were tested (i) for their capacity to bind [^{3}H]DFP, after which the proteins were separated by SDS–PAGE, and (ii) for the presence of inhibitors of chymotrypsin in an 'inhibitor gel'. Similar [^{3}H]DFP binding proteins (75,000–80,000 MW) were present in both control and immune serum (Fig. 1a). There was no evidence of a [^{3}H]DFP binding protein unique to serum from infected mice. The inhibitory profiles of control and immune sera (Fig. 1b) suggest that there is one major inhibitor of chymotrypsin (48,000 MW) in both samples.

Time–course of IMCP binding to proteinase inhibitors in mouse serum

IMCP was added to normal mouse serum (800 μg/ml) and incubated for varying time periods from 0 to 60 min. The reaction was stopped by adding either [^{3}H]DFP or an equal volume of Laemmli's SDS sample buffer (either with or without

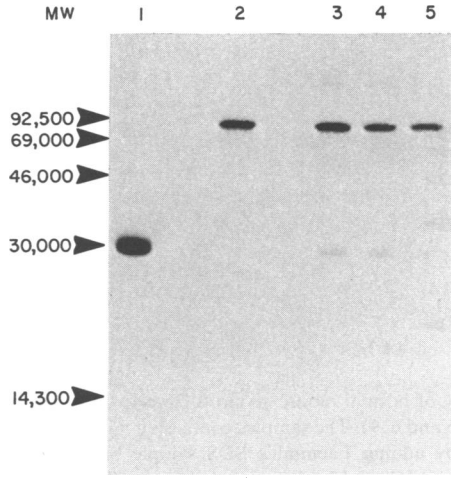

Figure 2. SDS–PAGE under reducing conditions and autoradiography of normal mouse serum. Samples were incubated with IMCP (800 μg/ml) at 37° for 0, 5, and 10 min (lanes 3–5). The reaction was stopped by adding 0·15 μl (0·75 μCi) [^{3}H]DFP. IMCP (80 ng) in lane 1 and normal mouse serum in lane 2 were also incubated with [^{3}H]DFP. Lanes 2–5 each contained 0·25 μl of serum.

2-mercaptoethanol). Samples were then electrophoresed and examined by autoradiography for [^{3}H]DFP-binding proteins (Fig. 2) or by Western blotting (Fig. 3).

When IMCP was added to mouse serum there was virtually instantaneous inhibition of the binding of [^{3}H]DFP to IMCP (Fig. 2). Comparison of lane 1, which contains 80 ng of enzyme, with lanes 3–5, each containing 100 ng IMCP mixed with 0·25 μl serum, demonstrates very little free enzyme in lanes 3–5 (Fig. 2) and, with time, there was a further reduction in labelling intensity of free IMCP and the appearance of an additional faintly labelled band of greater than 100,000 MW. (Fig. 2, lanes 3–5).

A comparable experiment under reducing conditions omitting [^{3}H]DFP but employing Western blotting and developed with rabbit anti-IMCP peroxidase conjugate gave a similar result (Fig. 3a). Native IMCP was present at time 0 but was only barely visible at 15 and 60 min. Under reducing conditions the Western blot was unsatisfactory but very faint bands (> 100,000 MW) at 0 and 15 min and a more prominent band of about 73,000 MW were just visible at 0–60 min (Fig. 3a). Under non-reducing conditions the pattern was both more readily visualized and more complex (Fig. 3b). Again, native IMCP was detected at time 0 but was virtually absent after 15 and 60 min of incubation with serum. However, in addition to a prominent band of 73,000 MW, there was a series of additional bands of greater than 100,000 MW, which were specifically labelled on the blot. In the non-reduced gel, a doublet of > 100,000 MW was present in lane 2 containing normal serum (Fig. 3b). A similar doublet was present in a blot of immune (Day 10) serum, which was identical to that of normal serum with no other visible bands (data not shown).

Titration of IMCP in serum

The proteinase binding capacity of inhibitors in normal mouse serum was titrated. Serum and IMCP (50 μg–5 mg/ml serum) were incubated together for 1 hr at 37°. Western blot (Fig. 4a) and [^{3}H]DFP analysis (Fig. 4b) of this titration experiment show that 50 and 500 μg of enzyme can be fully bound by inhibitory proteins in 1 ml serum within 60 min. When 5 mg IMCP/ml serum were blotted, large amounts of enzyme remained unbound (Fig. 4a) and retained [^{3}H]DFP binding capacity (Fig. 4b).

Heat-inactivation of proteinase inhibitors

Serpins are known to be relatively heat-labile in their native (i.e. unbound) form and are denatured on incubation at 60° (Carrell & Owen, 1985). To determine whether the inhibitors identified in Fig. 3b were also heat-labile, serum was heated at 60° for 30, 60 or 120 min. The treated samples were then incubated with IMCP (2 mg/ml) and electro-blotted (Fig. 5). Heat treatment abolished the binding of IMCP to all serum proteins. The 73,000

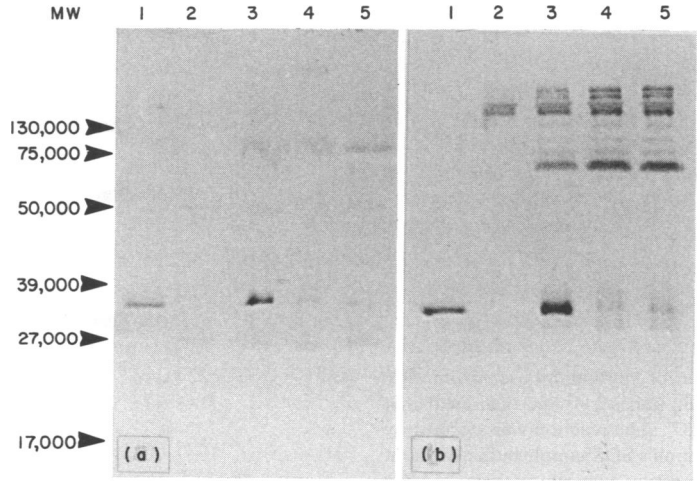

Figure 3. Western blot of normal mouse serum electrophoresed under (a) reducing and (b) non-reducing conditions and probed with rabbit IgG anti- IMCP–horseradish peroxidase conjugate. Serum was incubated with IMCP (800 μg/ml) at 37° for 0, 15 and 60 min (lanes 3–5). The reaction was stopped by adding Laemmli's SDS sample buffer (with 2-mercaptoethanol for reduced samples in a). IMCP (80 ng) was loaded in lane 1 and normal mouse serum in lane 2. Lanes 2–5 each contained 0·25 μl of serum.

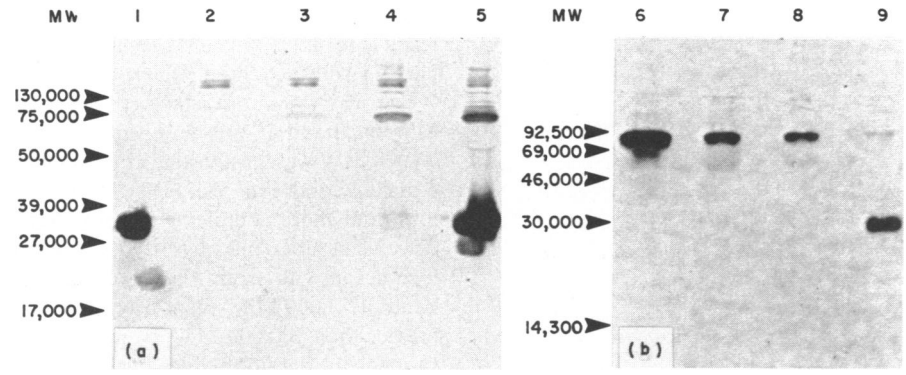

Figure 4. SDS–PAGE followed by (a) Western blotting or (b) autoradiography of normal mouse serum (0·25 μl) which had been incubated at 37° for 60 min with 0, 0·05, 0·5 and 5 μg of IMCP per μl (lanes 2–5 and 6–9). The samples containing 5 μg IMCP were diluted 1:4 before application (lanes 5 and 9). The reaction was stopped by adding Laemmli's SDS sample buffer (without 2-mercaptoethanol for Western blots) or [^{3}H]DFP (0·75 μCi) for autoradiography. IMCP (0·55 μg) was run in lane 1. (The gross excess of IMCP present in lanes 1 and 5 caused smearing artefacts on the blot in the 20,000–30–000 MW range).

and higher MW IMCP/inhibitor complexes were no longer visible but a peroxidase labelled band of approximately 30,000 MW was detected (Fig. 5).

Serum that was heat treated (60°) for 60 min and control serum were electrophoresed and the gels were examined for the presence of inhibitors of chymotrypsin. The 48,000 MW proteinase inhibitor was inactivated by this treatment (Fig. 6).

DISCUSSION

The systemic release of mast cell granule proteinases into blood or lymph has been described in mouse, rat and sheep and is most readily demonstrable during parasitic infection or anaphylactic shock (Miller *et al.*, 1983; Huntley *et al.*, 1987; Miller *et al.*, 1989). Concentrations as high as 2 mg RMCPII/ml have been recorded in rat peripheral blood during anaphylactic shock (King & Miller, 1984) and, during primary infection of mice with *T. spiralis*, up to 25 μg IMCP/ml of serum were detected (Huntley *et al.*, 1989). Such high concentrations would have detrimental systemic effects if the proteinases were not immediately inhibited. Interestingly, the interaction of IMCP with inhibitors in blood only partially interferes with quantification of this proteinase by ELISA (Huntley *et al.*, 1989) whereas the assay for sheep mast cell proteinase is severely affected (Huntley *et al.*, 1987).

The results of Western blotting suggest that a 73,000 MW

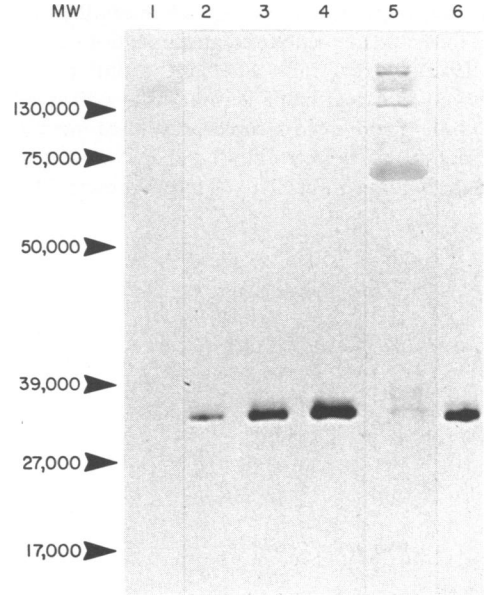

Figure 5. SDS–PAGE/Western blot of normal mouse serum pre-incubated at 60° for 30, 60 or 120 min (lanes 2–4) and then incubated with IMCP (500 μg/ml) for 1 hr at 37°. The reaction was stopped by adding an equivalent volume of Laemmli's SDS sample buffer (without 2-mercaptoethanol). Normal mouse serum was run in lane 1, while IMCP (500 μg/ml) in serum which had no 60° pretreatment was run in lane 5. Lane 6 contained 80 ng IMCP. The blot was probed with rabbit IgG anti-IMCP–HRPO conjugate. Lanes 1–5 contained 0·25 μl of serum.

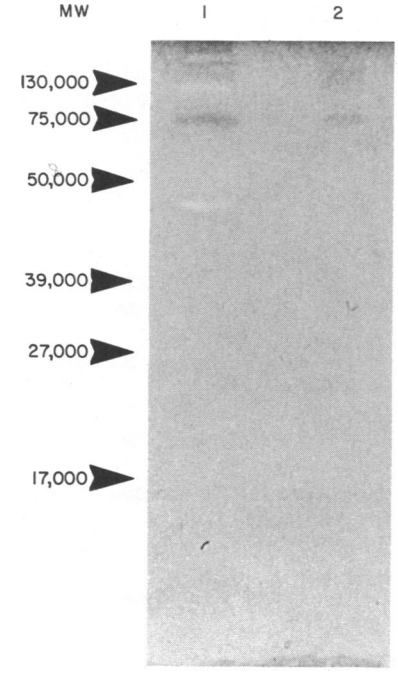

Figure 6. Characterization by SDS–PAGE (non-reducing) of chymotrypsin inhibitors in normal mouse serum (lane 1) and in serum preincubated at 60° for 120 min (lane 2). (0·25 μl serum/lane.)

inhibitor/IMCP complex is formed after adding IMCP to normal serum. This complex, which was resistant to reduction with 2-mercaptoethanol, probably results from the covalent bonding of IMCP to a 48,000 MW serpin. The molecular weights of the serpin or of the serpin/proteinase complex have not been measured accurately in the rather crude systems employed here because of the abundance of serum proteins which interfere with gel migration and because the serpins comprise a relatively small percentage of this total protein (Travis & Salvesen, 1983). Nevertheless, a single molecule or proteinase is known to bind to one molecule of serpin resulting in the cleavage of a peptide of about 4000 MW (Hercz, 1973; Johnson & Travis, 1976; Hunt & Dayhoff, 1980), therefore the serpin/IMCP complex would have an expected MW of 73,000–75,000.

The inhibitor gel profiles suggested that the major chymotrypsin inhibitor was approximately 48,000 MW (Fig. 1b) and that it was very heat-liable (Figs 5 and 6). Heat-lability is a well-known characteristic of the serpin family (Carrell & Owens, 1985), and the present results demonstrated that heat-inactivation of serum proteins prevented the formation of the 73,000 MW proteinase/inhibitor complex. This would again suggest that the 48,000 MW inhibitor preferentially bound IMCP. Titration experiments indicated that up to 500 μg of IMCP were bound per ml of serum but that there was inhibitor saturation at 5 mg enzyme/ml with a substantial excess of free IMCP (Fig. 4).

In addition to the 73,000 MW enzyme/inhibitor complex, an array of serum proteins of > 100,000 MW bound IMCP and were also apparently heat-labile. The nature of these binding proteins is not clear. Using the irreversible serine esterase inhibitor [^{3}H]DFP some residual enzyme activity (Fig. 2) was associated with one band of > 100,000 MW and it is possible that at least some of these proteins bind to IMCP through ionic interaction rather than through direct proteinase inhibition. The significance of a > 100,000 MW doublet on Western blots of normal and immune serum which contain, respectively, 0·2 and 20–25μg IMCP/ml (Huntley *et al.*, 1989) has yet to be determined. The intensity of staining of the doublet was increased when IMCP was added to serum and it was apparently heat-labile. It is possible that these are novel inhibitory molecules when high affinity for IMCP that are present at rather low concentrations. Studies with radiolabelled IMCP might resolve this question.

The 73,000 MW complex, readily visualized at concentrations of 500 μg IMCP/ml serum, was only just visible on Western blots where concentrations of IMCP were less than 50 μg/ml of serum. The relative abundance of serum proteins within the 73,000 MW weight range may, by competing for binding sites on the nitrocellulose, reduce the sensitivity of detection. This possibility is suggested by ELISA analysis of the distribution of IMCP in immune mouse serum (20–25 μg IMCP/ml), where IMCP was maximally detected in the 70–80,000 MW range with an additional 30,000 MW peak, possibly of free enzyme (J. F. Huntley *et al.*, unpublished data). A less plausible alternative is that other more potent inhibitors are available and that they do not enter the gel. For example, α_2-macroglobulin has at least three binding mechanisms: steric trapping (Barrett & Starkey, 1973), covalent linking (Barrett, 1981) and a non-covalent, non-steric adherence reaction with a number of macromolecules which is unrelated to proteolytic activity (Salvesen, 1981). Whatever the mechanism of its binding to α_2-macroglobulin, the active site of any serine proteinase would still be available to [^{3}H]DFP (Barrett, 1981). It is, however, unlikely that the labelled polypeptides in the higher molecular weight (> 100,000 MW) range revealed by Western blotting are related to α_2-macroglobulin since they were detected under non-reducing conditions and α_2-macroglobulin with a MW of 700,000 would not enter the gel under these conditions.

Whilst the techniques used in this study establish that serpin-like inhibitors in blood rapidly inactivate IMCP, the relative importance of each of the inhibitors will only be determined when its rate of association with IMCP has been measured. It is, however, clear that the inhibitory capacity of serum is substantial and is presumably sufficient to counteract the potentially damaging effects of systemic release of mast cell proteinases. Since, in the rat, systemic secretion of RMCPII begins during the neonatal period and continues throughout life (Cummins *et al.*, 1988), there is an early requirement to minimize the potential damage induced by secreted mast cell proteases. The present results establish a useful baseline for determining the fate of secreted IMCP. Further progress will entail molecular definition of the range of polypeptides which bind IMCP.

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