Anaphylactic Release of Mucosal Mast Cell Granule Proteases: Role of Serpins in the Differential Clearance of Mouse Mast Cell Proteases-1 and -2[1]

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The granule-derived mouse mast cell proteases-1 and -2 (mMCP-1 and -2) colocalize in similar quantities in mucosal mast cells but micrograms of mMCP-1 compared with nanograms of mMCP-2 are detected in peripheral blood during intestinal nematode infection. This differential systemic response was investigated both in vitro and in vivo. Bone marrow-derived mucosal mast cell homologs released similar quantities of mMCP-1 and-2 concomitantly with β-hexosaminidase in response to calcium ionophore (~60% release) or IgE/DNP (25% release). In contrast, serum from mice sensitized by infection with *Nippostrongylus brasiliensis* 10 days earlier contained >1500-fold more mMCP-1 (10,130 ± 1,609 ng/ml) than mMCP-2 (6.4 ± 1 ng/ml), but, in gut lumen, the difference was ~8-fold. After OVA sensitization, >600-fold more mMCP-1 (7,861 ± 2,209 ng/ml) than mMCP-2 (12.8 ± 4.7 ng/ml) was present in blood 1 h after challenge, but, in gut lumen, there were relatively comparable levels of mMCP-1 and -2. To estimate the rates of systemic accumulation and clearance, 10 μg of mMCP-1 or -2 was injected i.p. Plasma levels of injected mMCP-2 peaked (1%) at 15 min then declined, whereas levels of mMCP-1 were maximal (~25%) at 3 h. Inactivation of mMCP-1 with PMSF before injection resulted in mMCP-2-like kinetics, but inhibition of mMCP-1 by serum gave kinetics similar to that of native mMCP-1. mMCP-1 isolated from serum is complexed with serpins and we conclude that both the accumulation and the longevity of mMCP-1 in blood is due to complex formation, protecting it from a pathway that rapidly clears mMCP-2, which is unable to form complexes with serpins. *The Journal of Immunology,* **2006, 176: 899–904.**

The accumulation, in peripheral blood, of mast cell granule neutral proteases is used widely as a specific marker of mast cell activation after an allergic response, a drug-induced anaphylactoid reaction, or as a consequence of a pathological condition involving mast cells (1, 2). The β-chymase rat mast cell protease-2 (rMCP-2)[3] was the first mast cell protease to be quantified in peripheral blood (3), and several milligrams of rMCP-2 per milliliter of blood were recorded in rats subjected to systemic anaphylaxis (4). Subsequent studies in mice again showed that microgram quantities the β-chymase mouse mast cell protease-1 (mMCP-1), which is expressed by mucosal mast cells (MMC), are released systemically and can be detected in peripheral blood during intestinal nematode infection (1).

The systemic concentrations of β-chymases rMCP-2 and mMCP-1 in rodents are much higher in response to allergic challenges than those of mast cell tryptase in human blood where basal concentrations of ~5 ng of tryptase per milliliter increase to 15–90 ng of tryptase per milliliter (5). The tryptase assay does not detect all patients with allergic responses, but it is thought to distinguish mast cell involvement from a predominantly basophil-mediated allergic reaction. Studies on timing of release of the mast cell mediators, histamine and tryptase, show much earlier systemic accumulation of histamine with a half-life of 2 min, whereas tryptase levels in the blood are maximal at 1–2 h and then decline thereafter, with a half-life of ~2 h (6, 7). Similarly, the early kinetics of the systemic release of rMCP-2 are well described (3, 4) and both rMCP-2 and mMCP-1 enter the gut lumen (4, 8), but little is known of the half-life of rodent β-chymases in the systemic circulation.

Jejunal MMC from nematode-infected mice, as well as bone marrow-derived MMC homologs, cultured in the presence of TGF-β_1, contain comparable levels of mMCP-1 and -2 (9). However, mMCP-2 is detected in the circulation at concentrations that are similar (<20 ng/ml; Ref. 9) to those of tryptase in allergic patients, which is ~100-fold lower than systemic concentrations of mMCP-1 in the same animals (9). mMCP-2 is a serine esterase that binds tritiated diisopropyl fluorophosphate (9, 10), but, unlike mMCP-1, has no apparent chymotryptic or other substrate activity (9).

The purpose of the present study was to determine whether the very different systemic levels of mMCP-1 and -2 in parasitized mice were due to differential release and/or differential rates of clearance of each chymase. The in vitro and in vivo results suggest that there is constitutive differential release, but that equivalent levels of mMCP-1 and -2 are released under anaphylactic challenge. Furthermore, binding to plasma serpins that do not bind to mMCP-2, appears to substantially prolong the half-life of mMCP-1 in the systemic circulation.

Materials and Methods

Materials

Mast cell proteases mMCP-1 and mMCP-2 were purified from bone marrow-derived mast cell (BMMC) cultures as previously described (9). Abs

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[3] Abbreviations used in this paper: rMCP-2, rat mast cell protease-2; AAT, α_1-antitrypsin; β-hex, β-hexosaminidase; BMMC, bone marrow-derived mast cell; MMC, mucosal mast cell; mMCP, mouse mast cell protease.

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used in mMCP-1- and mMCP-2-specific ELISAs were purified as described (9, 11). Electrophoresis was performed using the miniProtean system (Bio-Rad). Unless otherwise stated, chemicals and reagents were obtained from Sigma-Aldrich. All experimental procedures involving laboratory animals were approved by the Biological Services ethical review committee at the University of Edinburgh and were performed under license, as required by the United Kingdom's Animals (Scientific Procedures) Act 1986.

Bone marrow mast cell culture

Bone marrow was removed from BALB/c mice and cultures were set up in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (Serotec), penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), recombinant human TGF-β_1 (1 ng/ml), recombinant mouse IL-3 (1 ng/ml), IL-9 (5 ng/ml), and stem cell factor (50 ng/ml), as described previously (9). Day 15 cultured mast cells were incubated overnight with or without the addition of mouse monoclonal anti-DNP IgE. Cells were washed in Tyrode's buffer (125 mM NaCl, 4.7 mM KCl, 1.4 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 10 mM D-glucose, (pH 7.4)) and re-suspended in DNP-human serum albumin (10 ng/ml) or calcium ionophore A23187 (10^{-6} M) diluted in Tyrode's buffer. Samples were incubated at 37°C, and cells were placed on ice at 0- and 30-min time points. Samples were centrifuged, and the supernatant was removed and stored on ice. The cell pellet was dissolved in 0.5% Triton X-100. All samples were tested for β-hexosaminidase (β-hex; Ref. 12) and mMCP-1 and -2 release, and the percentage of degranulation was calculated.

Nippostrongylus brasiliensis *infections*

BALB/c mice were infected s.c. with 500 *N. brasiliensis* larvae. Fecal egg counts on day 7 confirmed infection (16,000 eggs per gram ± 4,500). Mice were bled out on day 10 and serum samples were stored for analysis. Ten centimeters of jejunum was excised and the was lumen flushed with 0.5 ml of 10 mM Tris-HCl (pH 7.5); 0.15 M NaCl containing "complete Mini" EDTA-free protease inhibitors (Roche; 1 tablet per 7 ml of buffer). Flushings were placed in a tube containing EDTA on ice. The flushings were then centrifuged, and the supernatant was frozen in aliquots along with the residual pellet. Flushed jejunum was homogenized and centrifuged, and aliquots were frozen. Uninfected male BALB/c mice were used as controls.

OVA-induced gut anaphylaxis and release of MMC granule proteases

BALB/c mice were sensitized twice by i.p. injection, 2 wk apart, with 50 μg of OVA and alum as described (13). Four weeks after the first sensitizing injection of OVA, mice were gavaged every 2–3 days with 50 mg of OVA in 250 μl of saline or, for control purposes, with saline. Mice were deprived of food for 3 h before gavage. One hour after the fifth gavage, mice were killed and bled out, and samples of jejunum and of jejunal luminal flushings were collected and stored as described above.

Inhibition of mMCP-1 with PMSF

PMSF (200 μl of 12.5 mM in DMSO) was incubated with 400 μg of mMCP-1 in PBS (784 μl) at room temperature for 40 min. After centrifugation to remove precipitate, inhibited mMCP-1 was buffer exchanged into PBS using a Hi-Trap desalting column (5 ml, G.E. Healthcare). The product was concentrated to 700 μl (YM-10 concentrator; Millipore), to give a protein concentration of 0.43 mg/ml (yield, 75%). The inhibited mMCP-1 preparation had a residual activity of 0.006% when compared with uninhibited mMCP-1 using the substrate succinyl-Phe-Leu-Phe thiobenzyl ester (9).

Intraperitoneal injection of mast cell proteases

BALB/c mice were injected i.p. with 10 μg each of mMCP-1 and -2, 10 μg each of mMCP-1 and -2 pretreated with 20 μl of BALB/c mouse serum for 5 min, or 10 μg of PMSF-inhibited mMCP-1. Mice were bled out at 15-, 60-, 90-, and 180-min time points, and their peritoneal cavity was washed with 500 μl of PBS containing 0.1% BSA (four mice per time point). Peritoneal wash was centrifuged and the supernatant and pellet frozen in aliquots. ELISAs were performed on peritoneal wash fluid and serum for mMCP-1 (11) and mMCP-2 (9) as appropriate. The total amount of protease present in peritoneal wash was calculated based on a total volume of 500 μl, and that value was expressed as a percentage of the original amount (10 μg) injected. The percentage of original amount injected present in serum was calculated assuming a standard 1 ml of serum per mouse.

Ability of mMCP-1 and mMCP-2 to form complexes with human α_1-antitrypsin (AAT) or other serum serpins

Human AAT (1.0 μg) was incubated at 21°C for 30 min with mMCP-1 (0.5 μg), mMCP-2 (0.5 μg), or buffer (50 mM Tris-HCl (pH 7.5); 0.15 M NaCl) in a total of 10 μl of buffer. The product was analyzed on an SDS-gel (12% acrylamide) and stained with Coomassie brilliant blue. Similarly, native mMCP-1 (0.5 μg) was compared with PMSF-inhibited mMCP-1 (0.5 μg) when incubated with excess human AAT (10 μg). Additionally, mMCP-1 (0.25 μg), mMCP-2 (0.25 μg), and buffer (PBS) were incubated with normal mouse serum (0.5 μl) and heat-inactivated FCS (0.5 μl) in a total reaction volume of 10 μl, at 21°C for 15 min.

Purification and analysis of mMCP-1:serpin complexes from mouse serum

An anti-mMCP-1 affinity column was prepared using 1.25 mg of affinity purified sheep anti-mMCP-1 Ig (11) conjugated to a Hi-Trap NHS-activated cartridge (1 ml; G.E. Healthcare) according to the manufacturer's instructions. BALB/c mouse serum (1.8 ml), pooled from mice killed at day 10–12 of *N. brasiliensis* infection, was applied to the affinity column in PBS. The column was eluted with 2 ml of 0.1 M citric acid, and eluate was neutralized with 1 M Tris base, before concentration to 0.5 ml (YM-10; Millipore). A sample (~1 μg) of the product was separated by SDS-PAGE and the main component band was cut from the Coomassie blue-stained gel for tryptic peptide mass fingerprinting (Moredun Research Institute).

Results

Expression and release of mMCP-1 and mMCP-2 from BMMC

BMMC cultured in the presence of TGF-β_1 were >95% pure from day 7 onwards, and expressed similar amounts of mMCP-1 and mMCP-2, as assessed by ELISA (Fig. 1). However, from day 9 of culture onwards, a significantly higher concentration of mMCP-1 than mMCP-2 was detected in the culture supernatant. Calcium ionophore-induced degranulation of BMMC resulted in equal proportions of total β-hex, mMCP-1, and mMCP-2 being released. IgE-mediated degranulation resulted in ~25% release of β-hex, and also of mMCP-1 and mMCP-2. Therefore, there was no significant difference in chymase release during degranulation, but constitutive release of mMCP-1 appeared to be greater than that of

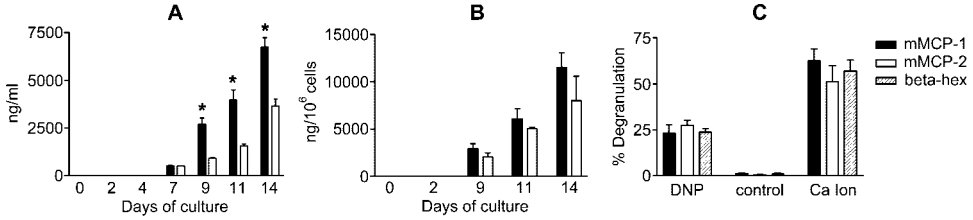

FIGURE 1. Expression and release of mMCP-1 and mMCP-2 from cultured BMMC. Cells were grown in the presence of TGF-β_1 as described in *Materials and Methods*. The amount of mMCP-1 present in supernatant was significantly higher ($p < 0.05$) than that of mMCP-2 from day 9 onward (*A*), whereas the total amounts of mMCP-1 and mMCP-2 in cell pellets (*B*) were not significantly different. *C*, The percent release of mMCP-1, mMCP-2, and β-hex from cultured BMMC (day 15) when stimulated by calcium ionophore (Ca ion) or IgE crosslinking (DNP, see *Materials and Methods*). In all cases, equivalent amounts of each analyte were released.

mMCP-2. The data shown are representative of three independent experiments.

Differential release of mMCP-1 and mMCP-2 during N. brasiliensis *infection*

ELISA analysis of jejunal extracts from day 10 of *N. brasiliensis* infection showed, as previously described (9), that the level of mMCP-1 detected by the ELISA in serum (10,130 ± 1,609 ng/ml) was very much higher than that of mMCP-2 (6.4 ± 1 ng/ml) (Fig. 2*A*). The concentration of mMCP-1 present in jejunal luminal flushings (523 ± 125 ng/ml) was ~8-fold higher than that of mMCP-2 (66 ± 21 ng/ml) (Fig. 2*B*; $p < 0.05$). However, mMCP-1 and mMCP-2 were both present at ~500 ng per mg of whole intestinal tissue (Fig. 2*C*). These results indicate a selective release of mMCP-1 into the jejunal lumen, and that much lower levels of mMCP-2 persist in serum compared with mMCP-1. The results of a repeat experiment were consistent with these results.

Anaphylactic release of mMCP-1 and -2 in OVA-sensitized mice

After OVA sensitization and challenge of BALB/c mice, jejunal MMC hyperplasia was evident in OVA-challenged mice compared with saline-challenged controls (6.8 ± 0.9 vs 0.8 ± 0.2, respectively, toluidine blue-positive mast cells per villus-crypt unit; $n = 6$). In OVA-challenged mice, >600-fold more mMCP-1 (7,861 ± 2,209 ng/ml) than mMCP-2 (12.8 ± 4.7 ng/ml) was present in blood 1 h after challenge (Fig. 3*A*), but in jejunal luminal flushings, there was 3.5-fold less mMCP-1 (13.8 ± 9 ng/ml) than mMCP-2 (49 ± 26 ng/ml) (Fig. 3*B*). The concentration of mMCP-1 (352 ± 133 ng/mg) was ~3-fold higher than that of mMCP-2 (106 ± 23 ng/mg) in jejunal homogenate. Neither mMCP-1 nor mMCP-2 was detected in any sample from any of the saline-treated controls. These results show that within 1 h of gavaging with OVA, the levels of mMCP-1 and mMCP-2 in serum are comparable to those seen in mice infected with *N. brasiliensis*, but that differences in the levels of both proteases in jejunal luminal flushings was much less pronounced. The results shown are representative of three independent experiments.

Peritoneal injection of proteases in mice.

A pilot study (n = 1 per data point, data not shown) was conducted to optimize the amount of protease to inject and timing of sampling. This showed that the residual activity of 10 μg of mMCP-1 was reduced to <5% within 15 min of i.p. injection (data not

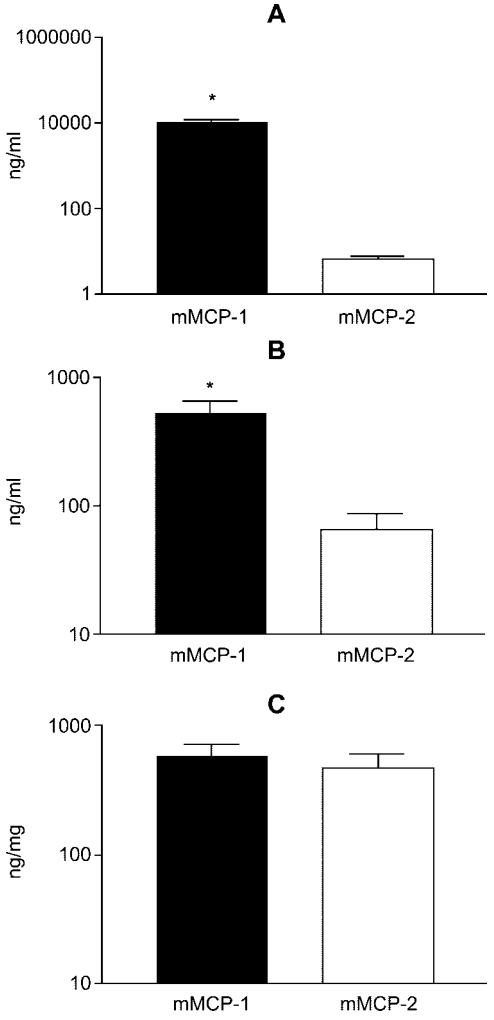

FIGURE 2. Quantification of mMCP-1 and mMCP-2 in serum (*A*), jejunal lumen (*B*), and jejunal homogenate (*C*) of mice infected with *N. brasiliensis* (day 10). Significantly more mMCP-1 than mMCP-2 was detected in serum and jejunal lumen ($p < 0.05$).

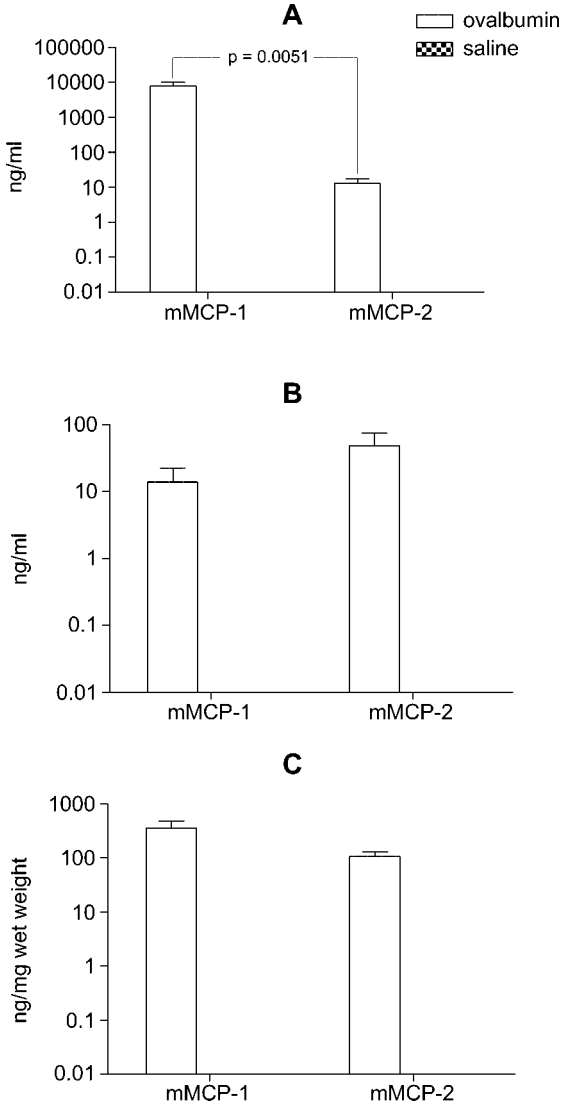

FIGURE 3. Quantification of mMCP-1 and mMCP-2 in serum (*A*), jejunal lumen (*B*), and jejunal homogenate (*C*) 1 h after intragastric challenge with either 50 μg of OVA or with saline. mMCP-1 and -2 were not detected in any of the saline-challenged controls.

shown). The following results ($n = 4$ per data point) are consistent with the pilot data.

After i.p. injection, the proteases appeared in peripheral blood serum (Fig. 4*A*) and were cleared from the peritoneal cavity (Fig. 4*B*). At 3 h postinjection, only native mMCP-1 and serum-treated mMCP-1 were detectable at residual levels of over 2% in the peritoneal wash, whereas the other preparations were apparently cleared more rapidly. The proportion of mMCP-2 present in circulating blood peaked at a level of just 1% at 15 min, and declined to <0.2% by 3 h. However, the proportion of mMCP-1 present in the circulation was rising at 15 min, and reached a relatively steady plateau of 20–25% between 1 and 3 h postinjection. Indeed, after 24 h, there was still 8–16% of mMCP-1 remaining in serum.

The ability of mMCP-1 to form a complex with the human plasma serpin, AAT was demonstrated (Fig. 5*A*), while the inactive mMCP-2 was unable to make such a complex. Treatment of mMCP-1 with PMSF resulted in an inactive chymase, unable to form a complex with human AAT (Fig. 5*B*), but with effectively unaltered m.w. When this was injected into the peritoneum of mice, the proportion remaining in serum at 3 h postinjection was only 0.5%; significantly lower than that of uninhibited mMCP-1, and showing the same kinetics of clearance as mMCP-2.

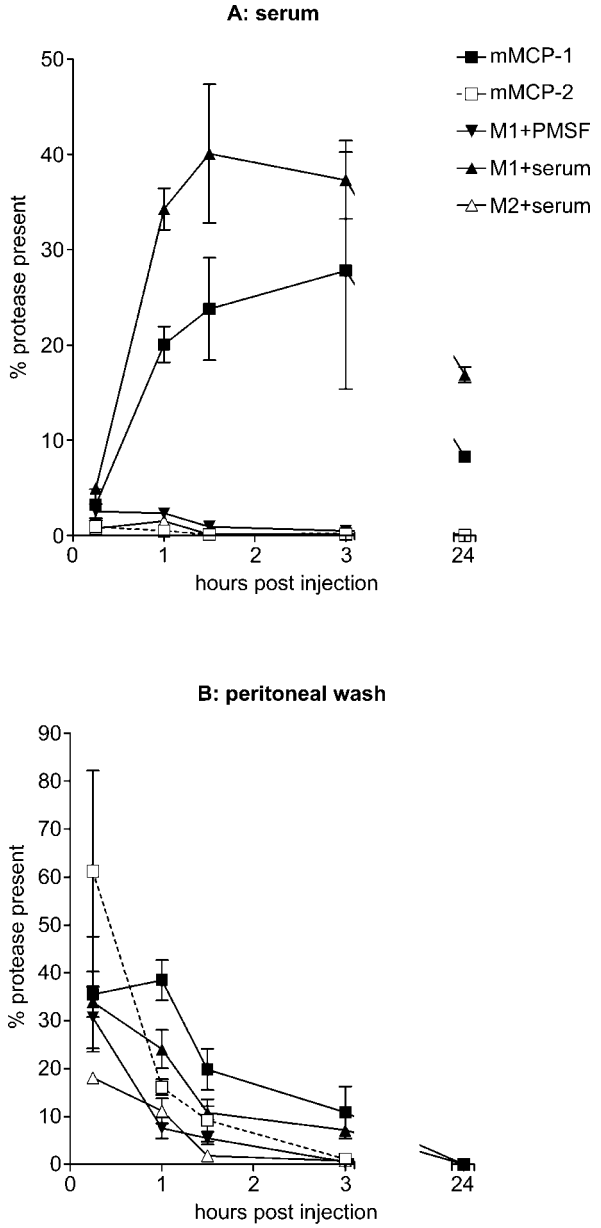

FIGURE 4. Levels of mMCP-1 and mMCP-2 in peritoneal wash and peripheral blood serum after i.p. protease injection. Each mouse was injected with 10 μg of mMCP-1 or mMCP-2, either in native form or inhibited with mouse serum or PMSF. The percent of originally injected protease present in the blood or peritoneal compartments was calculated as described in *Materials and Methods*.

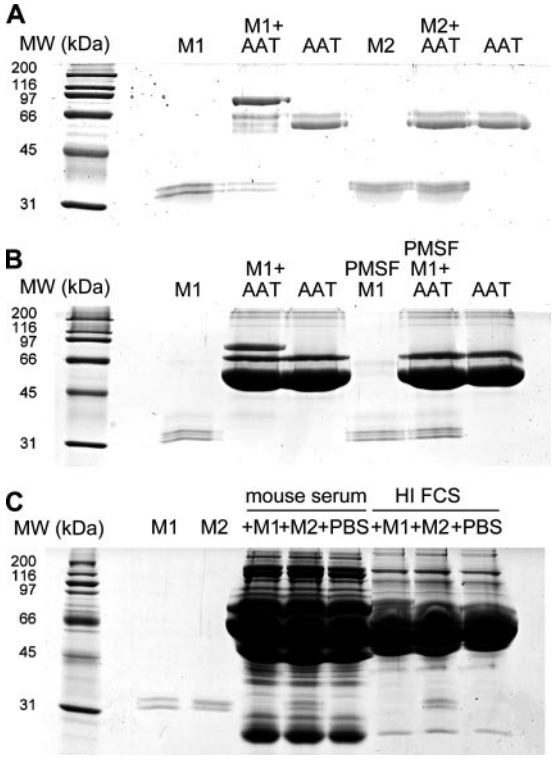

FIGURE 5. Ability of mMCP-1 and mMCP-2 to form complexes with the serpin, α_1-proteinase inhibitor. The reaction of mMCP-1 (M1; 0.5 μg) and mMCP-2 (M2; 0.5 μg) with human AAT (1.0 μg) is shown in *A*. An SDS-stable complex was made in the case of mMCP-1, but not with mMCP-2. *B*, The reaction of mMCP-1 (0.5 μg) and PMSF-inhibited mMCP-1 (0.5 μg, see *Materials and Methods*) with AAT (10 μg). Preinhibition of mMCP-1 with PMSF prevented the formation of a complex with AAT. *C*, The reaction of mMCP-1 (0.25 μg) and mMCP-2 (0.25 μg) with mouse serum (0.5 μl) and heat-inactivated FCS (HI FCS; 0.5 μl). Incubations with serum were conducted for 15 min at room temperature before denaturing SDS-PAGE. The disappearance of mMCP-1 bands in both serum incubations indicates the formation of SDS-stable complexes, whereas mMCP-2 appears unaffected by addition of serum.

If mMCP-2 were able to form complexes with other (non-AAT) serpins in serum, this may affect its clearance and/or detection. Therefore, we compared the addition of mMCP-1 and mMCP-2 to normal mouse serum and to FCS (Fig. 5*C*); the latter also being of relevance to detection of mMCP-2 in tissue culture supernatants. When added to either serum, mMCP-1 bands disappeared due to formation of higher MW complexes. However, mMCP-2 bands were apparently unaffected, consistent with a lack of ability of mMCP-2 to form serpin-enzyme complexes.

Therefore, we conclude that serine protease activity and ability to form the serpin-enzyme complex is necessary for the prolonged presence of mMCP-1 in the circulation.

Analysis of mMCP-1 in circulation

Serum from *N. brasiliensis*-infected mice was applied to a column of immobilized anti-mMCP-1 Abs, and ~40 μg of bound protein

was eluted per milliliter of serum applied. The eluted protein consisted of predominantly a single product, with M_r 77,000 by SDS-PAGE (Fig. 6). Tryptic peptide mass fingerprinting of this protein band was consistent with a complex between mMCP-1 and one or more mouse serpins (Table I). The serpin A1AT1 had the greatest number of matching peptides, but unique matching peptides corresponding to A1AT2 and A1AT4 were also detected, suggesting that mMCP-1 forms complexes with a number of AAT variants in serum, and not just a single form. This confirms that mMCP-1 in serum is present as a serpin-enzyme complex.

Discussion

Detection of the MMC proteases mMCP-1 and rMCP-2 in mouse and rat serum, respectively, has long been used as a measure of MMC activity in rodents (1). Compared with the levels of other mast cell proteases, notably tryptases in human serum (2), these levels seem unfeasibly high, being in the micrograms per milliliter region rather than the nanograms per milliliter range. The more recent discovery that mMCP-2, like tryptase, is present in serum at levels three orders of magnitude lower than mMCP-1 (9) has prompted the present investigation into the reasons behind this dramatic difference.

The difference could arise due to differential expression, release, and/or clearance of mMCP-1 compared with mMCP-2. Therefore we began the investigation by determining the relative expression and release of mMCP-1 and mMCP-2 in cultured BMMC.

In this model, we noted similar levels of mMCP-1 and mMCP-2 in cell pellets, although there was a significantly higher level of mMCP-1 secreted into the culture medium. There was no difference in relative levels of both proteases during ionophore or IgE-mediated degranulation, which suggests that constitutive release of mMCP-1 is more likely than degranulation to contribute to a higher level of secreted mMCP-1 than mMCP-2.

In *N. brasiliensis*-infected mice, levels of mMCP-1 and mMCP-2 were very similar, but in flushings of gut lumen, mMCP-1 was more abundant. It is possible that, as in the cultured mast cells, selective constitutive release of mMCP-1, rather than degranulation, is responsible for this disparity. Constitutive release of mMCP-1 was noted, for example, in *Trichinella*-infected mice deficient in IgE, although levels in plasma were significantly reduced compared with levels wild-type mice (14). In the present study, mMCP-1 levels in the gut lumen were less than one order of magnitude higher than those for mMCP-2, and are unlikely to account for concentrations of mMCP-1 that are three orders of magnitude greater relative to mMCP-2 in serum. These latter observations on systemic levels of mMCP-1 and -2 were very similar to those noted in the OVA sensitized and challenged mice. The main difference in this experimental model being that higher levels of mMCP-2 than mMCP-1 were present in the jejunal lumen, which suggests that, in this case, both proteases are released anaphylactically into the milieu of the gut and that plasma levels are dictated by a different mechanism.

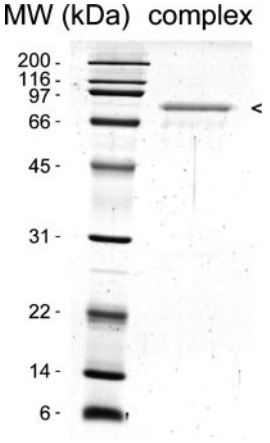

FIGURE 6. Analysis of mMCP-1 in peripheral blood serum of *N. brasiliensis*-infected mice. Serum from mice at day 10–12 of *N. brasiliensis* infection was passed down an affinity column containing immobilized anti-mMCP-1 Ig. Specifically bound material was eluted using a low pH buffer (see *Materials and Methods*). Approximately 1 μg of the eluate was analyzed by SDS-PAGE, showing that it consisted predominantly of a single band (marked "<") at approx. M_r 77,000.

Table I. *Results of tryptic peptide mass fingerprinting of mMCP-1:serpin complex isolated from* N. brasiliensis*-infected mouse serum*

Protein	Swiss-Prot ID	Number of Hits (% coverage)	Unique Matches (matched peptide)
Serpina1a (A1AT1)	P07758	15 (33%)	662.4 (293–297) 981.6 (301–308) 1214.7 (278–287) 1230.7 (278–287; Metox)
Serpina1b (A1AT2)	P22599	12 (30%)	975.6 (301–308) 2032.0 (214–230)
Serpina1d (A1AT4)	Q00897	11 (26%)	1108.6 (309–318)
mMCP-1	P11034	10 (32%)	n/a

[a] The fingerprint was consistent with a mixture of mMCP-1 and one or more closely related serpin variants.

Therefore we investigated the clearance of mMCP-1 and mMCP-2, after injection of 10 μg of each protease into the peritoneal cavity of mice. In some respects, this model has similarities to the natural release of mast cell proteases in the gut, where clearance occurs through the lymphatic system before reaching the circulation. In this study, we noted that mMCP-1 and mMCP-2 behaved very differently. MMCP-2 was cleared relatively rapidly from the peritoneal cavity and was detectable in serum at 15 min, although only ~1% of the injected mMCP-2 was present in the circulation at that time. The amount of mMCP-2 in serum remained low thereafter. In contrast, mMCP-1 cleared notably slower from the peritoneum, and its concentration in serum built up over time, containing between 20 and 28% of injected protease from 1 to 3 h post injection. The fact that 8% of the protease was still present in serum after 24 h indicates that the protease has a long serum half-life (at least 10 h).

The main functional difference between mMCP-1 and mMCP-2 is the apparent lack of proteolytic activity of the latter (9). A deletion in the substrate binding cleft of mMCP-2 is believed to be responsible for this lack of activity (9, 10), preventing access to peptide substrates. However, the active site catalytic center is still fully active, as shown by binding of the low m.w. inhibitor, diisopropyl fluorophosphate (9, 10). Here we have shown that the lack of activity against proteases extends to interactions with serpins; mMCP-1 forming an SDS-stable complex with human AAT, while mMCP-2 cannot.

Therefore, we investigated the effect of preincubating mMCP-1 with sufficient mouse serum to inhibit mMCP-1 activity through interaction with native serum serpins (residual activity <2%; data not shown). When injected i.p. into mice, this preparation showed the same kinetics of clearance as native mMCP-1, and over 15% of the original mMCP-1 was still present in serum after 24 h.

It was noted that peritoneal washes recovered from the earliest time point after active mMCP-1 injection had negligible mMCP-1

activity (data not shown), suggesting that rapid inhibition of mMCP-1 occurs with serpins present in the peritoneal cavity. To interfere with this process, we injected mMCP-1 that had been preinhibited with the low m.w. inhibitor PMSF. This preparation had effectively the same molecular size as native mMCP-1, but was inactive and unable to form complexes with serpins. On injection into the peritoneal cavity of mice, this preparation followed the same clearance kinetics as mMCP-2. Therefore, it appears that the rapid formation of a serpin-enzyme complex is necessary to maintain the presence of mMCP-1 in the circulation. In the absence of this process, proteases are cleared rapidly from the circulation and cannot build up in concentration. Therefore, the large concentrations of mMCP-1 in nematode-infected mice represent the cumulative effect of mMCP-1 release over the hours or days preceding sample collection.

The natural inhibition of mMCP-1 with serpins was verified by affinity purification of mMCP-1 from nematode-infected mouse serum. The product was a single band of serpin-enzyme complex, where the serpin appeared to be a mixture of mouse AAT variants, most notably serpin A1AT1.

The fact that formation of serpin-enzyme complexes protects mMCP-1 from rapid, nonserpin-mediated clearance from tissues and serum is unusual, considering that serpin-enzyme complex formation is more often associated with the rapid clearance of proteases. For example, cathepsin G, human neutrophil elastase, and thrombin, complexed with cognate serpins (α_1-antichymotrypsin, AAT, and heparin cofactor II, respectively), are all cleared from the circulation of mice within minutes (15–17). Clearance of serpin-enzyme complexes is believed to be mediated via low-density lipoprotein receptor-related-protein-1 (LDLR1) in hepatocytes, which recognizes complexed but not uncomplexed serpins (18). Therefore, it is possible that the complex formed between mMCP-1 and AAT adopts a conformation that is not recognized by the receptor.

Serpin-enzyme complexes may perform a signaling role, since for example, the human neutrophil elastase-AAT complex is chemotactic for neutrophils (19). It is interesting to speculate whether the mMCP-1:AAT complex has any biological role, and whether the high circulating concentration during nematode infections is of any relevance. One would expect that a high circulating concentration would negate any chemotactic gradient, and the slow rate of clearance may be indicative of a lack of binding to receptors generally.

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Disclosures

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