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Tryptase and chymase: comparison of extraction and release in two dog mastocytoma lines

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SUMMARY

Mast cell secretory granules contain unique tryptic and chymotryptic serine proteases that differ between species and tissues. Direct comparison of these proteases in single-cell types has been hindered by the difficulty of obtaining adequate numbers of pure mast cells. In this study, we were able to compare tryptic and chymotryptic enzyme activity in cells of presumed monoclonal origin, using two stable lines ('BR' and 'G') of dog mastocytomas The gel-filtration profiles, inhibitor susceptibilities and substrate preferences of tryptic and chymotryptic mastocytoma protease activities established their close resemblance to the tryptases and chymases of human and rodent mast cells. Striking heterogeneity was observed in the amounts and solubilities of the tryptic and chymotryptic activity in the two different mastocytoma cell lines. Incubation of cells from both lines with calcium ionophore A23187 caused non-cytotoxic release of protease activity. In contrast to chymase from rat connective tissue mast cells, protease activity that was insoluble after extraction at low ionic strength became soluble following ionophore-stimulated release. Neither tryptic nor chymotryptic activity was activated during degranulation, suggesting the absence of inactive precursors. Cells of the 'BR' line released both tryptic and chymotryptic activity in parallel with the granule marker histamine; cells of the 'G' line released a much smaller proportion of tryptic activity than of either chymotryptic activity or histamine. These differences in release of granule constituents from cells of common origin could be explained by developmental variations in the production of performed mediators or by differential regulation of preformed mediator release. We conclude that the differences in protease content, solubility and release in these mastocytoma lines are useful in evaluating the potential pathophysiological significance of the contribution of proteases to mast cell heterogeneity.

INTRODUCTION

Granule-associated tryptic and chymotryptic proteases (tryptases and chymases) are released from mast cells during degranulation (Schwartz *et al.*, 1981b,c; Miller *et al.*, 1983). Enzyme and immunohistochemical studies suggest that these proteases are present in high intracellular concentrations and are exclusive to mast cells (Schechter *et al.*, 1986; Irani *et al.*, 1986), and that mast cells differ between tissues and between species in the specificity of their proteases (Glenner & Cohen, 1960; Chiu & Lagunoff, 1972). Thus, in humans, 90% of lung mast cells appear to contain only tryptase, whereas in the skin, 88% contain chymase as well as tryptase (Irani *et al.*, 1986; Schwartz, Lewis & Austen 1981a). In the rat, two different chymases segregate between connective tissue and mucosal mast cells (Woodbury & Neurath, 1980). No unique functions have been demonstrated clearly for tryptases and chymases. However, *in vitro* experiments have shown that human lung tryptase releases C3a from C3 (Schwartz *et al.*, 1983) and degrades high molecular weight kininogen (Maier, Spragg & Schwartz, 1983), and that rat chymase cleaves type IV collagen (Sage, Woodbury & Bornstein, 1979) and converts angiotensin I to angiotensin II (Reilly *et al.*, 1982). Thus, differences in protease content and release invite speculation that the proteases define subsets of mast cells that differ in function.

In previous studies, release of chymase and tryptase from mast cells has not been compared directly. The two proteases have been examined separately in pooled cell populations, rather than in single-cell types. For example, tryptase release has

Abbreviations: bzRpNA, *N*-benzoyl-DL-arginine-*p*-nitroanilide; bzVGRpNA, *N*-benzoyl-L-val-gly-arg-*p*-nitroanilide; DFP, diisopropyl fluorophosphate; EDTA, ethylenediamine tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; HSE, high-salt extract; LSE, low-salt extract; Me_2SO, dimethylsulphoxide; PI, $alpha_1$-protease inhibitor; SBTI; soybean trypsin inhibitor; sucFPFpNA, *N*-succinyl-L-phe-pro-phe-*p*-nitroanilide; sucFpNA, *N*-succinyl-L-phe-*p*-nitroanilide; TLCK, tosyl-L-lysine chloromethylketone; TPCK, L-1-tosylamide-2-phenylethyl chloromethylketone.

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been studied in human lung mast cells, which are heterogeneous in size, histamine release and protease phenotype (Schulman *et al.*, 1983; Irani *et al.*, 1986), and may contain mast cells of differing origin. Because of our interest in the roles of tryptic and chymotryptic mast cell enzymes, we wanted to examine proteases from well-defined, specific types of mast cells. Recently, several dog mastocytoma lines were adapted to serial passage in athymic mice (Lazarus *et al.*, 1986), and this allowed us to compare protease activities in pure, stable clones of differentiated mastocytoma cells. Like the 'TC' mast cells in human lung and skin that have been defined histochemically (Irani *et al.*, 1986), these mastocytoma cells contain both tryptic and chymotryptic proteases (Caughey *et al.*, 1986). In the present study we show that protease activities of canine mastocytoma cells and those of rodent and human mast cells are similar. We also show substantial differences in content, solubility and release of tryptic and chymotryptic activity in a comparison of the two mastocytoma lines.

MATERIALS AND METHODS

Protease extractions

We passaged dog mastocytomas (UCSF Animal Care Facility) serially as subcutaneous nodules in BALB/c athymic mice (Lazarus *et al.*, 1986). When the cells were not studied immediately after harvest, tumours were stored at −70° prior to extraction of proteases. According to a modification of the method of Smith, Hougland & Johnson (1984), tissue was rinsed in cold isotonic saline, minced and homogenized in 10 ml/g cold 10 mM 2-(*N*-morpholino)ethane sulphonic acid (MES), 0·02% Na azide, pH 6·1 (low-salt buffer). Organelles and debris were sedimented from the homogenate at 20,000 *g* for 30 min at 4°, yielding a low-salt extract (LSE1). The pellet was rehomogenized in the same volume of low-salt buffer, and centrifuged as above. The supernatant (LSE2) was decanted and saved; the pellet was extracted twice with the same volume of cold buffer containing 2 M NaCl (high-salt buffer) followed by sedimentation as above, yielding supernatant high-salt extracts (HSE1 and 2, respectively). The low- and high-salt extracts were subjected to studies as described below, and were stored at −70°.

Enzyme assays

Tryptic activity was measured with either *N*-benzoyl-DL-arg-*p*-ntiroanilide (bzRpNA; Sigma, St Louis, MO) or *N*-benzoyl-L-val-gly-arg-*p*-nitroanilide (bzVGRpNA; Vega, Tucson, AZ) depending on the sensitivity required. Reactions were carried out in 1 ml of 0·06 M Tris-HCl (Sigma) (pH 7·8) with 0·4% dimethylsulphoxide (Me_2SO) (Sigma), at either ambient temperature or 37°, at a substrate concentration of 80 μg/ml. Chymotryptic activity was measured with either *N*-succinyl-L-phe-*p*-nitroanilide (sucFpNA; Sigma) or *N*-succinyl-L-phe-L-pro-L-phe-*p*-nitroanilide (sucFPFpNA; Bachem, Torrance, CA) in 0·03 M Tris-HCl, 1 M NaCl (pH 8·0) with 0·4% Me_2SO at a substrate concentration of 80 μg/ml. For the investigation of reaction velocity as a function of substrate concentration, cleavage was carried out in the above buffers plus 10% Me_2SO to enhance the solubility to substrates at higher concentrations. There was no significant effect of 10% Me_2SO on the rate of enzymatic cleavage. All substrates were stored as stock solutions of 20 mg/ml in Me_2SO at −20°. Reactions were initiated by addition of enzyme. Blanks contained substrates in assay buffer without enzyme. Change in absorption with substrate cleavage was followed spectrophotometrically at 394 nm. Under the conditions of their respective assays, the changes in molar absorbtivity at 394 nm for bzVGRpNA and sucFPFpNA were calculated to be 11,300 and 11,000 M/cm, respectively.

For inhibition studies, tryptic and chymotryptic enzyme assays were performed in duplicate as above in 10% Me_2SO. BR HSE proteases were preincubated for 10 min with inhibitor prior to the initiation of the assay by addition of substrate. Assay solutions contained 50 μg/ml of bovine lung heparin (Sigma) to stabilize mastocytoma tryptic activity during preincubation (Schwartz & Bradford, 1986). Controls were HSE-assay solutions preincubated in the absence of inhibitor. DFP (Sigma) was diluted from 0·2 M stock in propylene glycol. Other inhibitors were diluted from freshly prepared stocks in assay buffer or Me_2SO.

Mastocytoma tryptic and chymotryptic activity was compared with that of trypsin and chymotrypsin by measuring the specific activity of the latter enzymes versus bzVGRpNA and sucFPFpNA, respectively, under the same conditions used for the corresponding mastocytoma enzyme activities. To determine the amount of active trypsin and chymotrypsin for this measurement, TPCK-trypsin and TLCK-chymotrypsin were active-site titrated with *p*-nitrophenyl guanidinobenzoate (Chase & Shaw, 1969).

Other assays

Protein assays were performed by the method of Bradford (1976), with bovine serum albumin as the standard. To measure microgram amounts of protein in high-salt buffer, the assay was modified as follows: 0·1 ml of sample or standard in high-salt buffer was diluted with 1·2 ml of water and vortexed with 0·2 ml concentrated dye reagent. This resulted in a linear standard curve over a range of 1–10 μg of albumin. Histamine was measured by automated *o*-phthalaldehyde fluorometry (Siraganian, 1974). Heparin was assayed via Azure A metachromasia (Sigma) according to Jaques & Wollin (1967), with 50 mM Tris-HCl, pH 7·6, substituting for barbital buffer. With this modification, the assay was linear in the range of 1–8 units, using bovine lung heparin as a standard.

Granule release studies

Fragments of freshly excised tumours were incubated for 90 min at 37° in Joklik-modified minimal essential medium (UCSF Cell Culture Facility) containing 25 mM HEPES, pH 7·4, 2 g/100 ml of bovine serum albumin, and 80 U/ml of collagenase. Disaggregated cells were pelleted, washed with Ca- and Mg-free Tyrode's buffer, and resuspended in Dulbecco's modified Eagle's-H16, 1 g/l glucose, 25 mM HEPES (pH 7·4) and 10% fetal calf serum, supplemented to 250 mg/l histidine and 2 mM glutamine, for 24–48 hr of further incubation prior to use. Cells were identified by staining with toluidine blue and viability was assessed by exclusion of erythrosin B.

To compare the release of proteases and histamine in response to a stimulus to degranulation, cells were washed three times in Ca- and Mg-free Tyrode's buffer and resuspended in complete Tyrode's buffer containing 25 mM HEPES and 0·1% bovine serum albumin. Prewarmed cells, 10^5–10^6/ml, were incubated at 37° in polypropylene tubes for 30 min, in qudruplicate, with varying concentrations (0·01–5 μM) of ionophore A23187 (Calbiochem-Behring, San Diego, CA). The time dependence of release was investigated in 5 μM ionophore over a

range of incubation times (1–30 min). To establish spontaneous release of granule constituents, parallel incubations of cells were carried out in the absence of ionophore. Incubation was terminated by cooling in an ice-water slurry for 10 min, and the cells pelleted. Two tubes were used for the quantification of histamine and two for the assay of protease activity. Prior to assay of histamine, perchloric acid was added to separated cell and supernatant fractions to a concentration of 0·2 M, and samples stored at −70°. Aliquots of supernatant were removed from the remaining tubes for the assay of tryptic and chymotryptic activity. Cells in the remaining pellet were resuspended and sonicated on ice for 30 seconds, with the microtip attachment of a Model 350 Sonifier (Branson, Danbury, CT) at its lowest output. Aliquots were assayed as above for protease activity. Net percentage release of histamine and protease activity was calculated according to Schwartz *et al.* (1981b). For each of the lines, cytotoxicity was evaluated via an assay for lactic dehydrogenase release, employing LD-L reagent (Sigma) following 30 min of incubation with 5 μM of A23187. In a separate experiment to test for release of granule-bound protease, supernatant obtained from the degranulation of BR cells with 3 μM A23187 was recentrifuged at 20,000 *g* for 30 min to sediment any intact granules. Percentage release of tryptic and chymotryptic activity into the resulting 20,000 *g* supernatant was compared with that into the original 400 *g* supernatant. To test for protease bound to the cell surface after release, G cells pelleted after 30 min incubation with 5 μM A23187 in complete Tyrode's were washed with the same buffer containing 1 M NaCl (Schwartz *et al.*, 1981c). The release of protease activity into Tyrode's buffer at physiological ionic strength was compared with the total obtained after inclusion of the additional activity obtained after extraction in 1 M NaCl.

Gel filtration

A 5 × 46 cm column of Sephacryl S200 (Pharmacia, Uppsala, Sweden) was poured for preparative separation of tryptic and chymotryptic activity, calibrated with globular protein standards provided by the manufacturer, and equilibrated at 4° with high-salt buffer. Thirty millilitres of mastocytoma extract were applied to the column. G LSE was made up to 2 M NaCl before application to the column; BR HSE was applied directly. Aliquots of 10-ml fractions were assayed for tryptic and chymotryptic activity.

RESULTS

Extraction of protease activity from cell homogenates

The two mastocytomas showed striking differences in content and extraction of tryptic and chymotryptic activities at low and high ionic strength, as shown in Fig. 1. The G line contained the highest concentration of tryptic activity, and most of this activity was extracted at low ionic strength. In contrast, BR cells contained slightly less tryptic activity, but much more chymotryptic activity, and the majority of both activities were extracted at high ionic strength. Within a given cell line, the protease activity and extraction pattern was constant in different tumour nodules.

Heparin-like activity, as assayed by Azure A metachromasia, also varied between the two mastocytoma lines (Table 1). G cells, which released the most protease activity into low-salt buffer, also released substantially more heparin into the low ionic strength extracts. In BR cells, metachromatic activity, like protease activity, was more evenly partitioned between LSE and HSE.

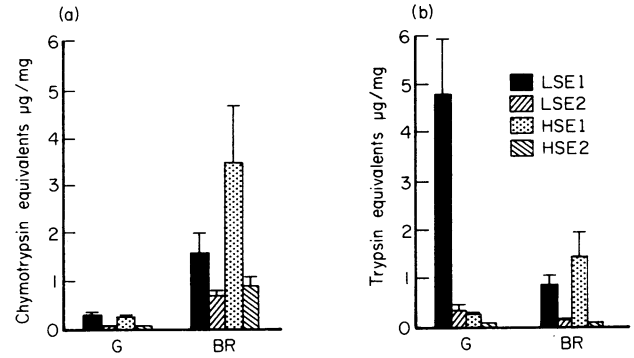

Figure 1. Sequential extraction of protease activity from two mastocytoma cell lines, BR and G. (a) Extraction of sucFPFpNA-cleaving activity in chymotrypsin equivalents and (b) extraction of bzVGR-pNA-cleaving activity in trypsin equivalents, both normalized per sum (mg) of protein extracted in low and high salt; LSE1, first low-salt extract; LSE2, second low-salt extract; HSE1, first high-salt extract; HSE2, second high-salt extract.

Table 1. Heparin-like Azure A metachromasia in mastocytoma extracts

	Units of heparin equivalents in extracts*	
Mastocytoma line	LSE1	HSE1
G	7·92 ± 0·67	4·22 ± 0·18
BR	8·18 ± 1·24	7·06 ± 1·96

* Units of heparin-like activity ml/mg protein (sum of protein concentration among LSE and HSE) with bovine lung heparin as standard; values = mean ± SE; $n = 3$ tumours per line.

Granule release with calcium ionophore (A23187)

Incubation of either BR or G cells with ionophore resulted in time- and dose-dependent release of proteases and histamine (Fig. 2). However, the two cell lines differed markedly. BR cells released 60–80% of tryptic and chymotryptic activity, as well as histamine, in parallel both in respect of dose and time (Fig. 2a and b). G cells responded differently: the overall release was lower and did not reach a plateau with time (Fig. 2c and d). Furthermore, although G cells contain substantial tryptic activity (Fig. 1), little release occurred during stimulation with ionophore. Total tryptic or chymotryptic activity (pellet + supernatant) did not differ between stimulated and unstimulated cells of either line (Fig. 3), suggesting that no inactive zymogen forms in the cell were activated during degranulation. Viability of BR and G cells prior to ionophore exposure was 90 ± 4 and 90 ± 3% (mean ± SE), respectively. No increase in release of lactic dehydrogenase activity occurred with ionophore incubation.

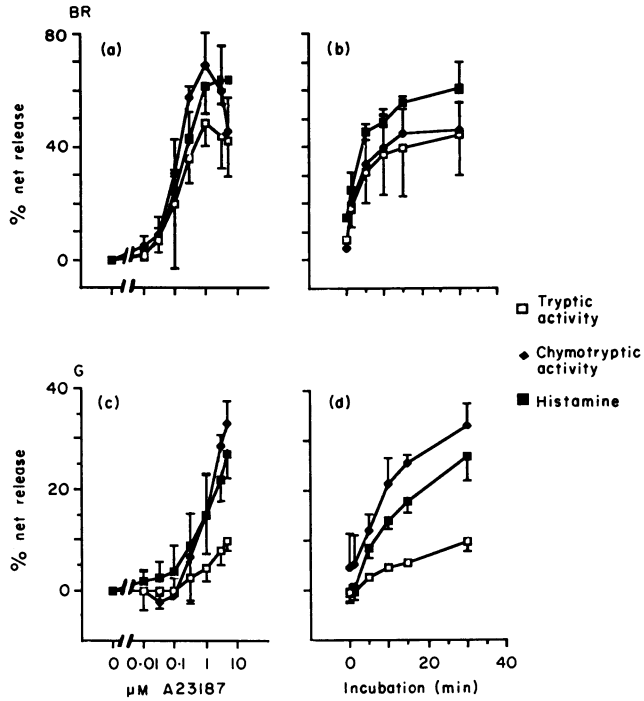

Figure 2. Ionophore (A23187) dose- and time-dependent release of granule constituents from disaggregated BR (a, b) and G (c, d) mastocytoma cells. Tryptic substrate, bzVGRpNA; chymotryptic substrate, sucFPFpNA; values, mean net percentage release ± SE; $n = 3$ experiments for each line, a different nodule for each experiment. Incubation time = 30 min in the dose-response study; ionophore = 5 μM in the time-dependence study. Mean spontaneous release values are < 10% for each constituent in each experiment.

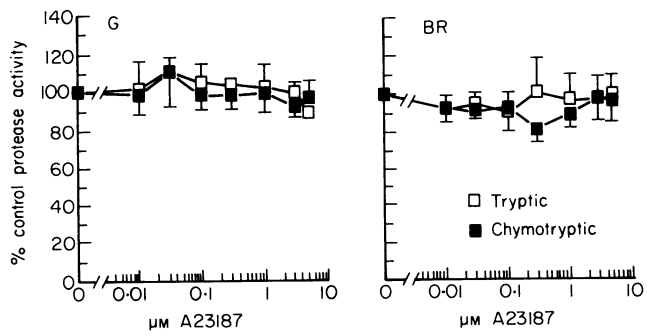

Figure 3. Total protease activity (sum of activity in pellet and degranulation supernatant) in ionophore-stimulated mastocytoma cells. Values = percentage of total activity in cells not exposed to ionophore A23187 ± SE; G and BR = 'G' and 'BR' mastocytoma lines, respectively; each incubation = 30 min; $n = 3$ experiments for each line, a different nodule for each experiment.

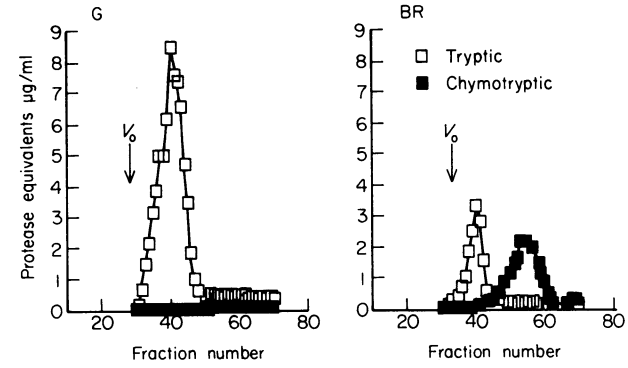

Figure 4. S200 gel filtration of protease activity from G mastocytoma LSE (left panel) and BR mastocytoma HSE (right panel). Tryptic activity = micrograms of trypsin equivalents/millilitres of effluent; chymotryptic activity = micrograms of chymotrypsin equivalents/millilitres effluent; V_o = void volume.

Following recentrifugation of the BR 400 *g* supernatant at 20,000 *g*, tryptic and chymotryptic activity in the new supernatant were 99% and 87%, respectively, of the corresponding activities in the parent supernatant. Thus protease activity did not remain attached to exocytosed granules in the 400 *g* supernatant. Indeed, we have no evidence to suggest that granules are released intact from the mastocytoma cells. In cells of the G line, washing of 5 μM ionophore-degranulated cells with 1 M NaCl resulted in only 5·0% and 8·0% increase in release of tryptic and chymotryptic activity, respectively, indicating that attachment to the cell surface following release was unlikely to account for the low percentage or release of tryptic relative to chymotryptic activity in this line.

Gel filtration of mastocytoma proteases

Despite the differences in the ionic strength at which the proteases were extracted, the gel-permeation profiles of G 1 LSE and BR HSE were indistinguishable, except for the differences in ratio of tryptic to chymotryptic activity (Fig. 4). In extracts of both mastocytomas, tryptic activity eluted at an apparent MW of 140,000 and chymotryptic activity at 27,000.

Substrate and inhibitor specificities

The marked preferences of BR proteases for extended peptide substrates are demonstrated in Fig. 5. For each enzyme activity, subsite interactions appeared to boost specificity constants by more than an order of magnitude. For bzVGRpNA and sucFPFpNA, double reciprocal replots (1/mM substrate versus 1/velocity) of the data depicted in Fig. 5 gave Michaelis constants (K_m) of 0·7 and 0·9 mM, respectively. The linear correlation coefficient was 0·99 in each instance. K_m was much greater for bzRpNA and sucFpNA. The exact value could not be determined because solubility limits for both substrates were exceeded before K_m was achieved. Both proteases exhibited a broad range of optimum activity from pH 7 to 9 when the tripeptide nitroanilides were used as substrates. Activity declined sharply below pH 7 and was minimal under the conditions of extraction, at pH 6·1.

Tryptic activity was inhibited by aprotinin, benzamidine, leupeptin and TLCK at the concentrations indicated (Fig. 6),

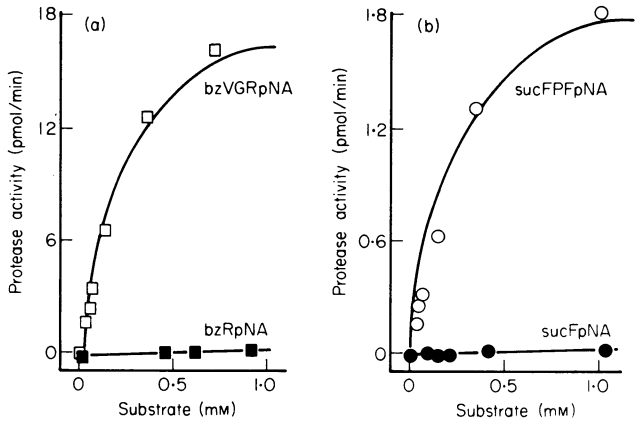

Figure 5. Substrate preferences of BR mastocytoma proteases: (a) depicts cleavage of tryptic substrates and (b) cleavage of chymotryptic substrates. All measurements represent initial rates of cleavage at 37°.

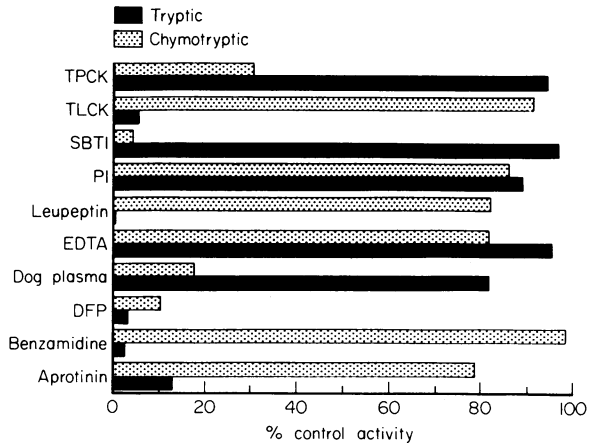

Figure 6. Inhibition of BR mastocytoma protease activity; black bars, tryptic activity; grey bars, chymotryptic activity, expressed as percentage of activity compared to controls preincubated in the absence of inhibitor; 10-min duplicate preincubations and assays performed at 37°; inhibitor concentrations used were 10% dog plasma, 0·01 mg/ml PI and aprotinin, 0·1 mg/ml SBTI, 2 mM TLCK, DFP and EDTA, 0·2 mM TPCK and leupeptin.

but was virtually unaffected by dog plasma. Chymotryptic activity was inhibited by SBTI (Sigma), and partially by TPCK and dog plasma. Both types of activity could be abolished by DFP, though for chymotryptic activity this required 30 or more min of incubation (data not shown). Neither activity was inhibited by α_1-protease inhibition (PI, human; Sigma).

DISCUSSION

The characteristics that separate mast cell tryptases and chymases from other serine proteases are shared by the mastocytoma protease activities studied here. Among these features are release from the cell during degranulation, preference for extended peptide substrates, and the lack of demonstrable activation from inactive forms in the cell. The susceptibility to inhibitors is also characteristic: tryptase resistance to SBTI and plasma, chymase resistance to aprotinin, and the resistance of both to α_1-protease inhibition distinguish the mast cell proteases from neutrophil and clotting proteases (Smith *et al.*, 1984; Schwartz *et al.*, 1981a; Tanaka *et al.*, 1983; Fraki *et al.*, 1985; Seppa & Jarvinen, 1978; Schechter *et al.*, 1983; Powers *et al.*, 1985).

G mastocytoma cells release a greater proportion of chymase activity than tryptase activity. These data suggest that non-parallel release is the response to degranulation in mast cells that contain both types of protease activity. The significance of this finding is that histochemical or biochemical estimates of tryptase and chymase activity within mast cells may fail to predict how much of one protease relative to the other will appear outside of the cells following degranulation.

The reason for the differences in release of G mastocytoma tryptase and chymase is not yet clear. One possibility is that release of the two activities from mature granules is differentially regulated. At present, however, there is no evidence in these or other cells for packaging of proteases in different granules, nor of separate stimulus-secretion pathways for any preformed granule constituent. A more likely explanation is the presence of greater amounts of tryptase activity in an unreleasable form in immature granules or organelles involved in protein synthesis. Higher expression of tryptase in young cells with immature granules could account for the low percentage release of tryptase compared to chymase in cells of common origin but of varying age.

An additional finding of this study is a lack of positive correlation between solubility of protease activity extracted into low-salt buffer and either percentage release or solubility following release. This contrasts with inferences drawn from studies of rat chymases. Rat mucosal mast cell chymase is soluble at low ionic strength and can be detected in the circulation following intestinal anaphylaxis (Miller *et al.*, 1983). However, rat connective tissue mast cell chymase is freed from ionic association with macromolecular heparin proteoglycan in tissue extracts only at high concentrations of salt (Seppa & Jarvinen, 1978), and remains granule associated and insoluble following degranulation into solutions of low ionic strength (Schwartz *et al.*, 1981c). These differences are potentially important, for the actions of a protease which remains attached to an exocytosed granule are likely to be local, whereas those of a soluble enzyme are potentially regional or systemic. In BR mastocytoma cells, even though only about one-third of extracted chymase and tryptase activity was soluble at low ionic strength (Fig. 1), approximately two-thirds of each protease activity could be released in soluble form into low ionic strength buffer by degranulation (Fig. 2). The probable explanation for this is that the macromolecular proteoglycans (i.e. heparins and/or chondroitins) to which mast cell proteases are attached are degraded to smaller fragments during degranulation, resulting in granule dissolution and release of proteases into solution. The very high percentage of G mastocytoma tryptase activity extracted in low-salt buffer (Fig. 1) may be due to association with soluble fragments rather than low-salt insoluble (macromolecular) forms of proteoglycan. The distribution of metachromatic activity shown in Table 1 supports this speculation: in G cells, nearly twice as much heparin-like activity is extracted into low compared to high ionic strength buffer, whereas BR cell protease and heparin-like activity are both more evenly distributed.

In summary, this study compared tryptase- and chymase-like protease activity in two dog mastocytoma lines. Like human mast cell populations, the lines contained both protease activities in different amounts. Release of the two proteases during degranulation was parallel in one line and non-parallel in the other. In contrast to chymase of rat mast cells, protease that was insoluble in low ionic strength extracts was soluble following ionophore-stimulated release. We conclude that the differences in protease content, solubility and release in these cell lines provide useful insights into the bases of mast cell heterogeneity.

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