

Mast Cells in Developing Subepidermal Bullous Diseases: Emphasis on Tryptase, Chymase and Protease Inhibitors

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The possible involvement of mast cell tryptase and chymase in subepidermal bullous diseases was studied enzyme-histochemically in specimens from erythematous and vesicular skin and from non-involved skin of patients with dermatitis herpetiformis, bullous pemphigoid, erythema multiforme, infective bullous eruption and linear IgA dermatosis. Patients with pemphigus were biopsied for comparison. The immunoreactivity of chymase inhibitors, α_1 -proteinase inhibitor (α_1 -PI) and α_1 -antichymotrypsin (α_1 -AC), in mast cells was demonstrated using the sequential double staining method. Tryptase-positive mast cells were unchanged or only slightly increased in number in erythematous lesions and slightly decreased in blistering skin compared with healthy-looking skin. Only occasionally were mast cells seen in apparent contact with the basement membrane zone. Chymase-positive mast cells and the chymase/tryptase ratio steadily decreased during the development of the lesions in each subepidermal bullous disease. The percentage of α_1 -PI⁺ and/or α_1 -AC⁺ mast cells increased simultaneously, which could explain the disappearance of chymase activity. Similar results were obtained regardless of the bullous disease. The results were also similar in pemphigus, which is an intraepidermal bullous disease. In conclusion, these results show significant alterations in mast cell chymase and protease inhibitors in a range of different bullous diseases, suggesting mast cell involvement. The apparent inactivation of chymase could be due to the action of chymase inhibitors detected in numerous mast cells. However, these alterations probably reflect general inflammation rather than a specific reaction in a certain bullous disease. **Key words:** blister formation; skin inflammation; serine proteinase.

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Skin mast cells contain large amounts of different proteolytic enzymes that can participate in cutaneous inflammation (1). Human mast cells are divided into 3 types based on their protease content in secretory granules: MC_T cells contain only tryptase; MC_{TC} cells produce tryptase, chymase, carboxypeptidase and a cathepsin G-like proteinase; and MC_C cells contain chymase and carboxypeptidase, but not tryptase. MC_{TC} cells are the major cell type in human skin (2–5).

Involvement of mast cells in inflammation and blister formation in subepidermal bullous diseases has been suggested, since in bullous pemphigoid (BP), the early skin lesions show urticarial appearance, the mast cells are hypogranulated, their granules are spread extracellularly (6)

and blister fluid contains increased histamine and tryptase levels (7, 8). In addition, injection of the mast cell degranulator, compound 48/80, into the skin of patients with dermatitis herpetiformis (DH) causes a DH-like bullous lesion in some cases (9). In oral erythema multiforme, the number of mast cells is lower than in normal mucosa, but the number increases between attacks (10). The mechanisms leading to mast cell activation and release of proteases in these diseases are poorly understood, but anaphylatoxins C3a and C5a, neuropeptides, cytokines and numerous other proteins or peptides could be involved (11–13).

Proteases secreted by mast cells could mediate the development of subepidermal blisters, either directly or indirectly by first activating metalloproteinases. Tryptase is able to cleave fibronectin and it probably activates type IV collagenase (14). This enzyme can activate prostromelysin to stromelysin, which in turn activates latent collagenase (15). Human skin chymotrypsin-like proteinases, chymase and cathepsin G, have been reported to cause dermal-epidermal separation at the level of lamina lucida (16). In addition, chymase is known to activate procollagenase (17).

Mast cells could also be involved in bullous diseases characterized by intraepidermal blister formation, since tryptase can activate single-chain urinary-type plasminogen activator (pro-urokinase), which, in turn, could activate plasminogen to plasmin, resulting in acantholysis (18, 19). Furthermore, increased numbers of mast cells have been reported in the lesional skin of pemphigus (20). Alterations in mast cell serine proteinases and apparent inactivation of chymase have also been recorded during the development of skin lesions caused by *Herpes zoster* (21). The inactivation of chymase could be a result of the action of protease inhibitors (21, 22).

Both tryptase and chymase could potentially participate in basement membrane destruction. Therefore, alterations in the activity of these enzymes could have marked consequences in blister development. Since no previous studies are available to show changes in mast cell proteases during the development of bullous diseases, mast cell serine proteinases and protease inhibitors in different bullous skin diseases were studied using enzyme- and immunohistochemical methods.

MATERIALS AND METHODS

Chemicals

Carbobenzoxy-Gly-Pro-Arg-4-methoxy-2-naphthylamide (Z-Gly-Pro-Arg-MNA) and Succinyl (Suc)-Val-Pro-Phe-MNA were purchased from Bachem (Bubendorf, Switzerland). Fast black K salt, Fast Garnet GBC, Tween 20, 3,3'-diaminobenzidine, purified goat IgG, α_1 -proteinase inhibitor (α_1 -PI), bovine serum albumin (BSA) and aprotinin were obtained from Sigma (St Louis, MO, USA). Rabbit polyclonal antibodies against α_1 -PI and α_1 -antichymotrypsin (α_1 -AC)

Table I. Quantification of mast cells showing tryptase and chymase activity in different bullous skin diseases

Number of patients	Protease	Healthy-looking skin		Erythematous skin		Vesicular skin	
		Cells/mm ²	C/T ratio %	Cells/mm ²	C/T ratio %	Cells/mm ²	C/T ratio %
Dermatitis herpetiformis							
28	Tryptase	94 ± 36	63 ± 20	123 ± 55	30 ± 22**	88 ± 32	23 ± 15*
	Chymase	60 ± 31	(n=20)	38 ± 39	(n=18)	19 ± 11*	(n=3)
Bullous pemphigoid							
11	Tryptase	112 ± 49	76 ± 9	132 ± 59	40 ± 12**	117 ± 101	32 ± 9**
	Chymase	82 ± 29	(n=4)	54 ± 25	(n=10)	37 ± 34	(n=5)
Infective bullous eruption							
3	Tryptase	146 ± 80	55 ± 12	163 ± 69	36 ± 10	98 ± 27	24 ± 15*
	Chymase	84 ± 58	(n=3)	55 ± 10	(n=3)	22 ± 12	(n=3)
Erythema multiforme							
2	Tryptase	89–144	59–81	120–124	27–75	86–97	26–39
	Chymase	72–85	(n=2)	32–93	(n=2)	25–33	(n=2)
Linear IgA dermatosis							
4	Tryptase	116 ± 25	77 ± 7	122 ± 27	41 ± 12*	91	21
	Chymase	89 ± 18	(n=3)	52 ± 24	(n=5)	19	(n=1)
Pemphigus							
7	Tryptase	125 ± 37	40 ± 15	107 ± 41	34 ± 12	83 ± 30	25 ± 10
	Chymase	49 ± 19	(n=5)	38 ± 24	(n=5)	20 ± 10*	(n=3)

Tryptase-positive cells reflect the total mast cell number (MC_T and MC_{TC}). The values are expressed as mean ± SD except in the case of EM. C/T ratio = chymase/tryptase ratio expressed as percentage.

* $p < 0.05$; ** $p < 0.0005$. The statistical comparison is based on the corresponding value in healthy-looking skin.

n = number of patients biopsied for healthy-looking, erythematous and vesicular skin specimen, respectively.

were purchased from Dako (Glostrup, Denmark). Reagents for immunohistochemical staining were purchased from Vector Laboratories (Burlingame, CA, USA).

Patients and skin samples

The study included 28 subjects with DH (16 females and 12 males, age range 13–71 years, mean age 55.8 years), 11 with BP (7 females and 4 males, age range 58–89 years, mean age 76.2 years), 7 with pemphigus (4 with pemphigus vulgaris, 1 male and 3 females; 3 with pemphigus foliaceus, 2 males and 1 female; age range 41–78 years, mean age 59.9 years), 4 with linear IgA dermatosis (LIGAD) (2 females and 2 males, age range 36–82 years, mean age 56.8 years), 3 female subjects with infective bullous eruption (age range 67–93 years, mean age 80.7 years), 2 with erythema multiforme (EM) (1 female, 1 male, aged 78 and 72 years, respectively), 1 male subject with Darier's disease (age 55 years) and 1 male subject with subcorneal pustular dermatosis (Sneddon-Wilkinson disease) (age 43 years). At the time of biopsy, the patients were not under effective systemic or local treatment. The diagnosis of each autoimmune blistering disease was confirmed by direct positive immunofluorescence examination. Infective bullous eruption was confirmed by positive bacterial cultivation and negative direct immunofluorescence. Each biopsy was also examined by a pathologist. The biopsies were taken from healthy-looking, erythematous and/or vesicular skin.

Skin biopsies were taken after local anaesthesia (1% lidocaine with adrenaline). Following removal, the specimens were immediately embedded in OCT compound (Miles Scientific, Naperville, IL, USA) and frozen in isopentane cooled with a mixture of absolute ethanol and dry ice.

The protocol of this study was approved by the Ethics Committee of Kuopio University Hospital, Kuopio, Finland.

Enzyme-histochemical staining methods for tryptase and chymase

Cryosections of 5 µm thickness were fixed in 0.6% formaldehyde and 0.5% acetic acid, pH 7.2, for 10 min. Tryptase was demonstrated with 1 mM Z-Gly-Pro-Arg-MNA as the substrate, 0.5 mg/ml Fast black K salt or Fast Garnet GBC as the chromogen, at pH 7.5 (21, 23).

Chymase was stained with 1 mM Suc-Val-Pro-Phe-MNA as the substrate, using 0.5 mg/ml Fast black K salt as the chromogen, at pH 7.5 (21, 23).

Sequential double staining method

For immunohistochemistry, the skin sections were fixed in cold acetone for 15 min. Non-specific binding sites were blocked with 100 µg/ml purified goat IgG dissolved in 1% BSA and phosphate buffered saline. After incubation with the primary antibodies (0.28 µg/ml rabbit anti- α_1 -PI and 0.61 µg/ml anti- α_1 -AC), the avidin-biotin-peroxidase technique (Vectastain Elite ABC kit, Vector) was used (21).

The immunoreactivity of protease inhibitors in mast cells was shown with the sequential double-staining method described previously (21, 23). First, mast cell tryptase was stained with Z-Gly-Pro-Arg-MNA and Fast Garnet GBC (but omitting α_1 -PI from the substrate solution). Thereafter, at least 6 adjacent photographs lining the epidermis and extending approximately 0.4 mm down the dermis were taken. Subsequently, the red azo dye was dissolved away by an overnight incubation in 15% Tween 20. The same sections were then fixed in acetone and stained immunohistochemically with anti- α_1 -PI and anti- α_1 -AC antibodies and re-photographed at exactly the same site as the previous pictures. The control skin sections were processed identically, but stained with unrelated rabbit antibodies.

Mast cell counting and statistics

Mast cells stained for tryptase and chymase were counted separately in adjacent skin sections under high magnification ($\times 400$) with the Olympus BH-2 microscope equipped with an 0.2×0.2 mm ocular grid as described previously (21, 23). The cells were counted at every dermal level (0.2 mm each) in an area of 1.6 mm (width) \times 0.4 mm (depth) immediately beneath the papillary dermis. The number and percentage of α_1 -PI⁺ and α_1 -AC⁺ mast cells were counted by comparing the photographs from sequential double staining as described previously (21). The Student's t -test was used for statistical analysis.

RESULTS

Tryptase and chymase in mast cells of bullous diseases

In all diseases with subepidermal blisters (BP, DH, infective bullous eruption, EM and LIgAD), mast cells with tryptase activity were unchanged or insignificantly increased in number in erythematous skin lesions compared with healthy-looking skin, but rather slightly decreased in number in blistering skin lesions, especially when compared with erythematous skin (Table I). These alterations, however, were not statistically significant. Occasionally, tryptase-positive mast cells appeared as disrupted and fragmented cells in the vesicular skin, probably due to aggressive inflammation. In the epidermis or in apparent contact with the basement membrane tryptase-positive cells were observed on rare occasions in some specimens of erythematous, vesicular or even healthy-looking skin.

Mast cells with chymase activity were decreased in number in erythematous lesions compared with non-involved skin and even more so in bullous skin specimens (Table I). Chymase-positive mast cells were never detected in the epidermis. The chymase/tryptase ratio was significantly decreased in erythematous skin lesions in DH, BP and LIgAD, and even more reduced in bullous skin compared with healthy-looking skin (Table I). However, biopsies from non-involved skin were available for analysis from only 4 BP patients out of 11.

In pemphigus, which served as the control (intraepidermal bullous disease), tryptase- and chymase-positive mast cells were reduced in number in erythematous and even more reduced in number in bullous skin specimens (Table I). Similar changes were seen in 1 subject with Darier's disease and one with Sneddon-Wilkinson disease (data not shown).

Protease inhibitors in mast cells of bullous diseases

The presence of α_1 -PI and α_1 -AC immunoreactivity in mast cells was quantitated by using the sequential double-staining method. The clear-cut localization of α_1 -AC in tryptase-positive mast cells is illustrated in Fig. 1. In addition, the presence of these inhibitors in mast cells was confirmed by staining in reverse order: α_1 -PI and α_1 -AC were first stained immunohistochemically, followed by tryptase staining (not shown). The results are summarized in Table II. Numerous cells other than mast cells were also positive for α_1 -PI and α_1 -AC in some specimens, as found previously (21, 23). These cells are probably macrophages that are known to display these inhibitors in immunohistochemistry. The percentage of α_1 -PI⁺ mast cells was significantly increased in erythematous lesions of DH and LIgAD compared with healthy-looking skin. However, in vesicular lesions, α_1 -PI⁺ mast cells were present in lower percentages than in erythematous lesions in DH, infective bullous eruption, BP and LIgAD (Table II).

The percentage of α_1 -AC⁺ mast cells was increased in erythematous lesions compared with lesion-free skin in each subepidermal bullous disease, although statistical significance was reached only in DH (Table II). In 2 cases of EM, α_1 -AC⁺ and α_1 -PI⁺ mast cells steadily increased in the progress of skin lesions (Table II).

In pemphigus, the percentage of α_1 -PI⁺ mast cells was significantly increased in erythematous skin specimens compared with healthy-looking skin, but the percentage of α_1 -AC⁺ mast cells did not show significant alterations (Table II).

DISCUSSION

Previous studies have suggested that skin mast cells may be involved in blister formation of different bullous diseases.

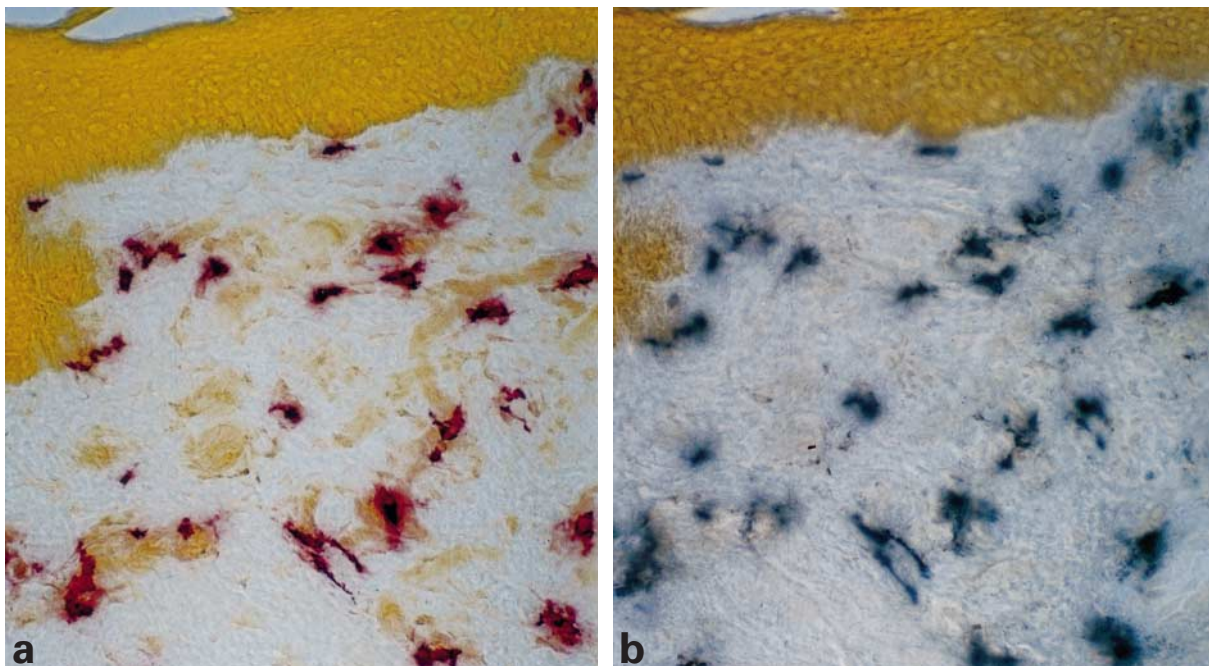


Fig. 1. Section of erythematous skin of dermatitis herpetiformis, stained first for (a) active tryptase, using Z-Gly-Pro-Arg-MNA as the substrate and Fast Garnet GBC as the chromogen. Thereafter, the red azo dye was removed with 15% Tween 20, followed by staining with (b) polyclonal anti- α_1 -antichymotrypsin antibody. All tryptase-positive cells show immunoreactivity for α_1 -antichymotrypsin. Magnification $\times 200$.

Table II. Percentage of tryptase-positive mast cells staining positively for protease inhibitors α_1 -proteinase (α_1 -PI) inhibitor and α_1 -antichymotrypsin (α_1 -AC) in different bullous skin diseases.

Protease inhibitor	Healthy-looking skin % (of total cells)	Erythematous skin % (of total cells)	Vesicular skin % (of total cells)
Dermatitis herpetiformis			
α_1 -PI	36 ± 25	66 ± 20**	43 ± 26
α_1 -AC	89 ± 18	98 ± 3*	100 ± 0
Bullous pemphigoid			
α_1 -PI	54 ± 38	56 ± 30	34 ± 36
α_1 -AC	85 ± 21	98 ± 5	100 ± 0
Infective bullous eruption			
α_1 -PI	37 ± 33	79 ± 19	30 ± 20
α_1 -AC	82 ± 11	98 ± 1	88 ± 20
Erythema multiforme			
α_1 -PI	47–88	52–90	80–95
α_1 -AC	78–97	89–92	93–100
Linear IgA dermatosis			
α_1 -PI	53 ± 5	88 ± 6*	36
α_1 -AC	94 ± 5	95 ± 2	75
Pemphigus			
α_1 -PI	59 ± 34	97 ± 4*	94 ± 7
α_1 -AC	91 ± 8	99 ± 3	98 ± 1

The same number of biopsies was analysed as in Table I. The values are expressed as mean ± SD except in the case of EM.

$p^* < 0.05$; $**p < 0.0005$. The statistical comparison is based on the corresponding value in healthy-looking skin.

Degranulation of mast cells has been observed in the developing lesions of bullous pemphigoid, and was seen together with progressive eosinophil infiltration (6). Furthermore, mast cell derived interleukin-5 (24) could be one potent mediator in attracting and stimulating eosinophils in the blister site (25). Despite the finding that mast cells were only occasionally found in direct morphological contact with the basement membrane zone, released granules could reach this zone, resulting in cleavage of the basement membrane components by tryptase and chymase (14–17).

In the erythematous skin of subepidermal bullous diseases (BP, DH, LIgAD, EM and infective bullous eruption), tryptase-positive mast cells were rather increased in number compared with healthy-looking skin, but both the count of chymase-positive mast cells and the chymase/tryptase ratio were clearly decreased (Table I). This suggests either inactivation of chymase in MC_T cells or new appearance of MC_T cells. The possibility that the decreased number of chymase-positive cells is solely due to extensive degranulation, appearance of phantom cells and clearance of chymase from the extracellular matrix is not likely, since no significant decrease in tryptase-positive cells could be seen. Moreover, chymase-heparin proteoglycan complexes are larger than tryptase-heparin proteoglycan complexes, suggesting their slower diffusion and clearance (26). In parallel with the decreased number of chymase-positive cells, the percentage of α_1 -PI⁺ and/or α_1 -AC⁺ mast cells was increased in these erythematous lesions, which could explain the disappearance of chymase positivity

in mast cells. α_1 -AC showed higher percentages than α_1 -PI in healthy-looking skin, which is in agreement with a previous finding that α_1 -AC is more effective in inhibiting chymase than is α_1 -PI (22). Conversely, chymase is able to cleave these inhibitors efficiently (22). Further support for this inactivation of chymase is given by our previous studies on psoriasis and *Herpes zoster*, which show the presence of chymase protein as well as chymase inhibitors without any apparent chymase activity in mast cells (21, 23).

In the progress from erythematous to vesicular skin, tryptase-positive mast cells tended to decrease in number in each subepidermal bullous disease (Table I). This may indicate degranulation of mast cells, release of tryptase enzyme, appearance of phantom cells, or inactivation of tryptase by an unknown mechanism. Also chymase-positive mast cells and the chymase/tryptase ratio were further decreased in the vesicular lesions compared with the erythematous skin of each disease. Therefore, the early and steady disappearance of chymase activity argues against the assumption that chymase is markedly involved in the dermis-epidermis separation in bullous diseases (16). However, we have detected aprotinin-resistant chymotryptic activity in blister fluids from BP and *Herpes zoster* lesions (27), suggesting that sufficient amounts of enzymatically active chymase can still survive from the action of protease inhibitors in more inflamed lesions. The enzyme is probably present in the lesion site and ready to exert its activity against the basement membrane. This finding is supported by the study revealing decreased antitryptic activity in blister fluids of pemphigoid and pemphigus (28), and a balance between chymase inactivation and cleavage of protease inhibitors may have been reached (22). Since increased tryptase concentrations have previously been detected in blister fluids of BP and contact dermatitis (8) and increased tryptic activity has been measured in blister fluids of pemphigoid (27, 28), the slightly decreased number of tryptase-positive cells in vesicular skin could reflect extensive degranulation and release of tryptase to the blister fluid. After being released from mast cells, tryptase could affect the extracellular matrix (14, 15) and stimulate fibroblasts (29) continuously, since no physiological inhibitors are known for tryptase (1).

In pemphigus, which is characterized by acantholysis, elevated tryptic activity has been detected in blister fluids (28). Tryptase- and chymase-positive mast cells were decreased in number in the erythematous skin and even more decreased in bullous skin specimens (Table I). These results are opposite to those of a previous study that showed increased mast cell numbers in pemphigus vulgaris (20). However, the staining methods are different and the observed alterations in mast cells are similar to those in other bullous diseases as well as in *Herpes zoster* (21) and psoriasis (23). Thus, this behaviour of mast cells is associated with general inflammation, not with blister formation directly.

We can conclude from this study that similar alterations occur in mast cell serine proteinases and protease inhibitors during the development of different bullous diseases. The results suggest that mast cell proteases participate in the inflammatory reaction; however, their direct role in blister formation is not clear. It is possible that tryptase induces and maintains the blisters by continuously activating collagenolytic metalloproteinases and degrading fibronectin (14, 15). Decreased chymase activity in mast cells could allow prolonged survival of pro-inflammatory peptides before

their degradation by chymase (1). Conversely, chymase may degrade extracellular matrix in circumstances of impaired anti-proteolytic activity.

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