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Human mast cell chymase induces the accumulation of neutrophils, eosinophils and other inflammatory cells *in vivo*

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1 The roles of chymase in acute allergic responses are not clear, despite the relative abundance of this serine proteinase in the secretory granules of human mast cells. We have isolated chymase to high purity from human skin tissue by heparin-agarose affinity chromatography and Sephacryl S-200 gel filtration procedures, and have investigated the ability of human mast cell chymase to stimulate cell accumulation following injection into laboratory animals.

2 Injection of chymase provoked marked neutrophilia and eosinophilia in the skin of Dunkin Hartley guinea-pigs. Compared with saline injected control animals, there were some 60 fold more neutrophils and 12 fold more eosinophils present at the injection site.

3 Following injection of chymase into the peritoneum of BALB/c mice, there were up to 700 fold more neutrophils, 21 fold more eosinophils, 19 fold more lymphocytes and 7 fold more macrophages recovered than from saline injected controls at 16 h. Doses of chymase as low as 5 ng (1.7×10^{-13} mole) stimulated an inflammatory infiltrate, and significant neutrophilia was elicited within 3 h.

4 The chymase induced cell accumulation in both the guinea-pig and mouse models was dependent on an intact catalytic site, being reduced by co-injection of proteinase inhibitors or heat inactivation of the enzyme.

5 Co-injection of histamine or heparin significantly reduced the chymase induced neutrophil accumulation, whereas neither histamine nor heparin by themselves had any effect on the accumulation of nucleated cells. No synergistic or antagonist interactions between chymase and tryptase were observed when these two major mast cell proteinases were co-injected into the mouse peritoneum.

6 Our findings suggest that chymase may provide an potent stimulus for inflammatory cell recruitment following mast cell activation.

Keywords: Chymase; neutrophil; eosinophil; lymphocyte; macrophage; guinea-pig skin; mouse peritoneum

Introduction

Chymase is a chymotrypsin-like serine proteinase which is present in the secretory granules of mast cells and released on mast cell degranulation. It is co-stored with other proteases, including tryptase, carboxypeptidase and a cathepsin G-like enzyme and with other preformed mediators such as histamine, heparin and certain cytokines (Walls, 1998). The quantity of chymase in human skin mast cells (4.5 pg cell^{-1}; Schwartz *et al*., 1987) is greater than that of histamine (1–2 pg mast cell^{-1}), but the mediator roles of this major mast cell product have been relatively little investigated.

The involvement of chymase in processes of allergic inflammation has been suggested by the ability of this major mast cell product to increase microvascular permeability in guinea-pig skin (He & Walls, 1998). *In vitro* this proteinase has been demonstrated to activate the potent proinflammatory cytokine IL-1β (Mizutani *et al*., 1991) to degrade IL-4 (Tunon de Lara *et al*., 1994) and to cleave membrane bound stem cell factor provoking its release from cells (Longley *et al*., 1997). In addition, the highly efficient conversion of angiotension I to angiotension II by chymase (Urata *et al*., 1990) may also be of relevance in inflammation given reports that angiotension II may stimulate cell chemotaxis and chemokine release *in vitro* (Wolf *et al*., 1997). Roles in tissue remodelling are suggested by the ability of chymase to activate procollagenase (Saarinen *et al*., 1994) and to cleave type I procollagen to initiate collagen fibril formation *in vitro* (Kofford *et al*., 1997), and it has been found to induce extensive epidermal-dermal separation in skin tissues (Briggaman *et al*., 1984).

Studies with the counterparts of human chymase in other species support the concept that this mast cell proteinase may contribute to allergic inflammation. Thus, dog chymase has been reported to enhance histamine-induced vascular leakage in the skin of dogs (Rubinstein *et al*., 1990). Rat chymase may be able to induce vascular leakage in rat skin (Seppä, 1980), to activate rat mast cells either directly (Schick *et al*., 1984) or indirectly (by forming a histamine-releasing peptide from albumin; Cochrane *et al*., 1993), to degrade thrombin (Pejler *et al*., 1994) and complement C3a (Gervasoni *et al*., 1986) and to generate a chemotactic factor for neutrophils from IgG (Katunuma & Kido, 1988).

Our recent observation that human mast cell tryptase may induce neutrophil and eosinophil recruitment in both the skin of guinea-pigs and the peritoneum of mice (He *et al*., 1997a) has highlighted the potential of human mast cell proteinases to contribute to cell accumulation in allergic disease. It has been reported previously that a chymotryptic protease isolated from human skin which has some similarity to human chymase, may induce neutrophil accumulation following injection into the skin of rabbits (Hatcher *et al*., 1977) or the peritoneum of mice (Thomas *et al*., 1977), but the actions of well characterized preparations of chymase in such models has not been investigated. As the recruitment of granulocytes is one of the hallmarks of allergic inflammation, we have investigated the actions of purified human chymase in guinea-pig and mouse models of inflammatory cell accumulation, and have investigated the potential of this proteinase to interact with other mast cell products *in vivo*.

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Methods

Purification of chymase and tryptase

Chymase was purified from human skin tissue following procedures described previously (He & Walls, 1998). Briefly, about 500 g of skin excised from amputated legs was minced finely by passing three times through a meat mincer, and then washed three times with a low salt buffer [0.01 M 3-(*N*-morpholino)propane-sulphonic acid (MOPS), 0.1 M NaCl, pH 6.8]. Chymase was extracted with a high salt buffer (0.01 M MOPS, 2 M NaCl, pH 6.8) and passed through a heparin agarose affinity column (eluting with 0.01 M MOPS, 0.4 M NaCl, pH 6.8), and then through a Sephacryl S-200 gel filtration column (with 2 M sodium chloride, 0.1 M MOPS, pH 6.8 as running buffer). The purified chymase was concentrated by ultrafiltration and stored at −80°C. Purity was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) with 10% reducing gels, with silver staining. Western blotting was performed with rabbit antiserum to human chymase as described previously (McEuen *et al*., 1995).

Tryptase was isolated using an immunoaffinity purification procedure which has been reported (He *et al*., 1997a). Chopped human lung tissue (500 g) collected *post mortem* was blended and washed with a low salt buffer [0.15 M NaCl, 10 mM 2-(*N*-morpholino)ethane-sulphonic acid (MES), and 0.02% sodium azide; pH 6.1], and the homogenate extracted using a high salt buffer (2 M NaCl, 10 mM MES, and 0.02% sodium azide, pH 6.1). Tryptic activity in the supernatant was filtered, and purified by sequential heparin-agarose and immunoaffinity chromatography procedures with AA5 monoclonal antibody specific for tryptase (Walls *et al*., 1990). Tryptase fractions were concentrated in centrifugal concentrators and stored at −80°C until use. The purity of tryptase was assessed by SDS–PAGE with 10% reducing gels and by Western blotting with monoclonal antibody AA5.

Assays of enzyme activity

Chymotryptic activity was measured spectrophotometrically at 410 nm, 25°C, by monitoring the hydrolysis of 0.7 mM *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide in 1.5 M NaCl, 0.3 M Tris (pH 8.0) and 5% ethanol (Schechter *et al*., 1988). Tryptic activity was measured using 20 mM *N*-benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA) in 0.1 M Tris-HCl, pH 8.0, 1 M glycerol, containing 1 mg ml^{-1} bovine serum albumin (BSA, Smith *et al*., 1984). In order to investigate the possible presence of elastase as a contaminant in purified preparations, the elastolytic activity was assayed using 1.4 mM *N*-succinyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide in the same buffer as used with BAPNA. In all assays, 50 μl of sample was added to a total reaction volume of 1 ml in a 1 cm pathlength cuvette. Multiple readings were taken and reaction rates calculated assuming a molar extinction coefficient of 8800 M^{-1} cm^{-1} for *p*-nitroanilide. Protein concentrations were determined using Coomassie Brilliant blue G (Bradford, 1976) with BSA as the standard.

Guinea-pig skin model

Dunkin Hartley guinea-pigs (600–1000 g, Harlan, Bicester, U.K.) were anaesthetized by injection of pentobarbitone saline (21 mg kg^{-1}, i.p.) and injection of Hypnorm (0.35 ml kg^{-1}, i.m.). The procedures for testing cell accumulation in response to injected compounds were performed as described previously (He *et al*., 1997a). After shaving the back, chymase or other compounds (50 μl volume) were injected i.c. in randomized sites placed 3–5 cm apart. Animals were killed by cervical dislocation 6 or 16 h following injection and the skin removed. An area of skin of 10×5 mm^2 around the injection site was excised, fixed in neutral buffered formalin and processed in paraffin wax for histology. It was investigated if the actions of chymase were dependent on an intact catalytic site, by preincubating the purified enzyme with the chymase inhibitor SBTI for 20 min on ice before injection. In addition, the effect of heating chymase for 2 h at 56°C was studied.

Identification and enumeration of granulocytes in tissue

A rabbit antiserum produced against guinea-pig neutrophils was employed to identify neutrophils in sections of guinea-pig skin by immunocytochemistry (He *et al*., 1997a). Following dewaxing and rehydration, sections were incubated with 3% hydrogen peroxide in methanol for 10 min, and then with 10% normal swine serum for 30 min. Following washing with Tris-buffered saline (pH 7.6), anti-neutrophil serum (1 : 3000) in 5% normal guinea-pig serum was added for 30 min. Biotinylated F $(ab')_2$ fragment of swine anti-rabbit immunoglobulins in 10% normal guinea-pig serum was employed as the secondary antibody and followed by streptavidin-biotin-horse raddish peroxidase. The immunoperoxidase stain was developed with 0.5 mg ml^{-1} 3,3′ diaminobenzidine tetrahydrochloride (DAB, in 0.05 M Tris-HCl with 0.01% hydrogen peroxide). Mayer's haematoxylin was used as the counterstain, and after final washing, sections were dehydrated and mounted with DPX.

The carbol-chromotrope procedure was employed for the staining of eosinophils. After dewaxing, rehydration and trypsinization (0.1% trypsin for 20 min at 37°C), sections were stained first with Mayer's haematoxylin (3 min) and then with carbol-chromotrope solution (containing 5 mg ml^{-1} chromotrope 2R and 10 mg ml^{-1} phenol; 45 min). After dehydration, sections were mounted with DPX. The injection site was identified under the light microscope and placed central to the field of vision. Neutrophils and eosinophils were counted in the field (each field covering an area of 0.19 mm^2) containing the greatest number of positively stained cells, and numbers were expressed per mm^2 of tissue in this field.

Mouse peritoneum model

After swabbing the abdominal skin of male BALB/c mice (18–22 g; Harlan, Bicester, U.K.) with 70% ethanol, chymase and other compounds in a standard volume of 0.5 ml, were injected into the peritoneum. Following a protocol employed previously with purified tryptase (He *et al*., 1997a), at 3, 6 or 16 h after injection, animals were killed by inhalation of carbon dioxide, and peritoneal lavage fluid was collected. After centrifugation ($500 \times g$ for 10 min at 4°C), cells were resuspended in 2.5 ml Eagle's Minimal Essential Medium (MEM), stained with Trypan blue, and enumerated using an Improved Neubauer haemocytometer. Cytocentrifuge preparations were made and stained with a modified Wright's stain. Differential cell counts were performed with a minimum of 500 cells. As studies involved large numbers of mice, experiments were performed in stages with no more than two mice in each treatment group injected on any occasion. Data

from animals which received the same treatment on different occasions were pooled and analysed together.

Materials and drugs

The following compounds were purchased from Sigma (Poole, Dorset, U.K.): α_1-antitrypsin, soybean trypsin inhibitor (SBTI), BAPNA, *N*-succinyl-L-Ala-L-Ala-L-Ala-*P*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, porcine heparin glycosaminoglycan, histamine disphosphate salt, pyrilamine maleate, cimetidine, trypsin, BSA (fraction V), MEM containing 25 mM HEPES, heparin agarose, Trypan blue dye, DAB, hydrogen peroxide, normal guinea-pig serum, goat anti-mouse IgG kit, MES, MOPS and Tris-base. Chromotrope 2R, phenol and all other chemicals were of analytical grade and were purchased from BDH (Poole, Dorset, U.K.); Coomassie protein assay reagent and normal swine serum from Pierce (Rockford, IL, U.S.A.); pentobarbitone sodium from Sanofi Animal Health (Watford, U.K.) and Hypnorm (containing 0.11 mg fentanyl citrate and 3.5 mg fluanisone) from Janssen (Oxford, U.K.); biotinylated F $(ab')_2$ fragment of swine anti-rabbit immunoglobulins and streptavidin-biotin-horse raddish peroxidase from DAKO (Glostrup, Denmark); Sephacryl S-200 from Pharmacia (Milton Keynes, U.K.); Mayer's haematoxylin from Raymond A Lamb (London, U.K.); DPX from Koch-Light (Haverhill, Suffolk, U.K.); modified Wright's stain from Miles (Stoke Poges); and silver staining kits for gels from Bio-Rad (Hemel Hempstead, U.K.).

Statistical analysis

Statistical analyses were performed using StatView software (Version 4.02, Abacus Concepts, Berkeley, CA, U.S.A.). As data were not normally distributed, they are presented as the median and range, for the number animals indicated. Where Kruskal-Wallis analysis indicated significant differences between groups, for the pre-planned comparisons of interest the Mann-Whitney *U*-test was employed. $P<0.05$ was taken as significant.

Results

Purification of mast cell proteinases

The procedures employed allowed 1.0 and 1.5 mg chymase to be obtained from each of two preparations of 500 g chopped human skin tissue. There were yields of 20 and 35%, representing approximately 200 and 300 fold increases in specific activity. The two preparations of chymase were pooled and analysed as a single preparation of chymase in order to avoid any problem of batch to batch variation in this study. The specific activity of the chymase used was 6.8 u mg^{-1}, where 1 u of enzyme represents that required to hydrolyse 1 μmole of *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide per min at 25°C, pH 8.0. SDS–PAGE with silver staining revealed a single diffuse band with an apparent molecular weight of approximately 30 kD, and the identity confirmed as chymase by Western blotting. No tryptic or elastolytic activity was detected using chromogenic substrates.

The tryptase preparation appeared homogenous on SDS–PAGE, bound tryptase-specific antibody on Western blotting, and had a specific activity 1.8 u mg^{-1}, where 1 u represents that required to cleave 1 μmole of BAPNA per min at 25°C, pH 8.0. Chymotryptic and elastolytic activity could not be measured in the preparation with the chromogenic substrates used. Contamination with endotoxin was very low, being less than 38 pg mg^{-1} for both the chymase and tryptase preparations.

Granulocyte accumulation in guinea-pig skin

Examination of the injection sites at 6 and 16 h following intradermal injection of chymase revealed a marked neutro-

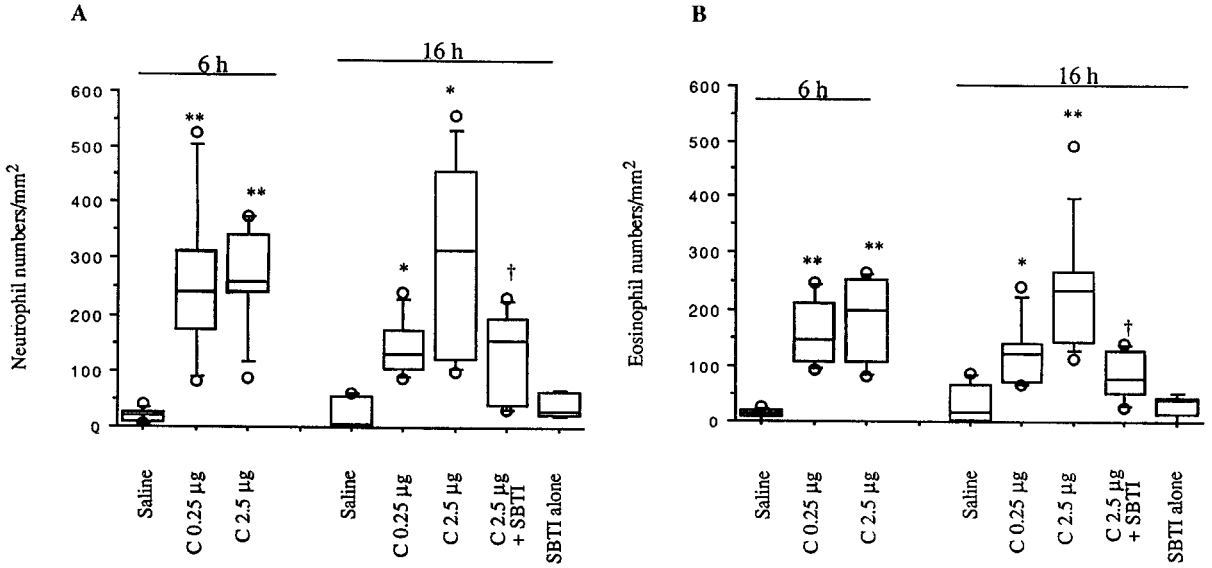

Figure 1 (A) Neutrophil and (B) eosinophil numbers in the skin tissue of guinea-pigs 6 and 16 h following injection of 0.25 μg or 2.5 μg human chymase (C) either in the presence or absence of SBTI (5 μg). Also shown are the responses with the saline diluent alone or with SBTI alone. Data are displayed as a boxplot, which indicates the median, interquartile range, the largest and smallest values other than outliers (whiskers) and the outliers (○) (defined as those which are more than 1.5 box lengths from the median) for 5–7 animals. *$P<0.05$ and **$P<0.005$ compared with the diluent only control (Mann-Whitney *U*-test).

philia and eosinophilia in skin. Very few neutrophils or eosinophils were observed in tissues injected with the saline diluent alone. In chymase-injected tissue, neutrophils and eosinophils were most numerous around the injection site. Counting neutrophils and eosinophils in a standard area centered on the injection site revealed an increase of about 60 fold in the numbers of neutrophils and an increase of some 12 fold in the numbers of eosinophils (Figure 1). Neutrophil numbers at sites where the proteinase inhibitor SBTI had been coinjected with chymase were less than 50% of those at sites injected with chymase alone, and eosinophil numbers were some 60% less (Figure 1).

Accumulation of cells in mouse peritoneum

In the mouse peritoneum, injection of chymase provoked a marked increase in the numbers of nucleated cells in peritoneal lavage fluid at 6 and 16 h (Table 1). Injection of BSA in similar quantities did not result in a significant increase in total numbers of nucleated cells compared with those with the saline diluent. The neutrophil numbers in the peritoneal lavage fluid of mice were found to be dramatically increased following injection with chymase at both 6 and 16 h (Figure 2A). At 3 h following injection, chymase induced significant neutrophilia only at the top dose tested. Mice injected with quantities of chymase as low as 5 ng had greater numbers of neutrophils than those receiving saline alone. The responses were dose-dependent at both 6 and 16 h and most pronounced in the animals which received 50 μg chymase, with apparent increases in median numbers of neutrophils of some 700 fold at 16 h. Eosinophilia was observed in the mouse peritoneum 6 h following injection with 0.5–50 μg chymase, and 16 h following injection with quantities of 0.05 μg and greater (Figure 2B). Median numbers of eosinophils in lavage fluid from the chymase-injected mice were up to 21 fold greater than in the mice injected with diluent alone.

Significant increases in numbers of lymphocytes were observed at both 6 and 16 h following injection (Figure 2C). As little as 5 ng chymase was able to induce significant lymphocyte accumulation at 6 h and the most pronounced lymphocyte response was achieved at 16 h following injection of 50 μg chymase, when there was an increase in the cell numbers of some 19 fold. There was also an increase of more than 7 fold in the numbers of macrophages recovered from the best responding group of mice treated with chymase (Figure 2D). Injection of BSA over the same dose range as for chymase was not associated with significant alterations in the numbers of any of the cell types in lavage fluid at either time point.

Chymase-induced cell accumulation was significantly reduced by preincubating the chymase with chymostatin or heat inactivating the enzyme prior to injection at both 6 h (Table 2) and 16 h following injection (Table 3). Both of these treatments abolished completely the ability of chymase to cleave the chromogenic substrate *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide *in vitro* under the conditions described. At both of the time points, injection of chymostatin alone at the same dose did not result in significant alterations in either total cell numbers or in numbers of any cell type (except lymphocytes at 16 h) in the lavage fluid compared with those injected with saline diluent alone (Tables 2 and 3). Two other proteinase inhibitors SBTI and α_1-antitrypsin were tested for their ability to inhibit the cell accumulation stimulated by chymase. However, at the concentration required to inhibit chymase activity *in vitro* (50 μg), both stimulated an apparent increase in peritoneal cell numbers at both time points when they were injected alone (data not shown).

Table 1 Numbers of nucleated cells recovered in the peritoneal lavage fluid of mice 3, 6 or 16 h following injection of saline, BSA or chymase

Compounds injected (μg)	*Total number of cells* ($\times 10^6$) *3 h*	*6 h*	*16 h*
Saline	2.6 (0.95–3.6)	1.6 (0.7–2.7)	1.0 (0.5–2.7)
BSA 0.005	nd	1.7 (1.2–2.5)	1.4 (1.0–2.2)
0.5	nd	0.7 (0.4–2.9)	2.5 (0.2–3.9)
50	nd	1.4 (0.5–2.1)	2.0 (0.5–4.5)
Chymase 0.005	2.5 (1.4–4.4)	2.4 (1.9–4.4)	2.2 (0.9–3.5)*
0.05	2.3 (1.1–3.1)	2.6 (1.6–4.2)	2.2 (1.0–5.0)*
0.5	3.4 (2.3–4.7)	5.2 (2.0–12)**	6.0 (2.5–7.5)**
5	3.1 (2.3–4.9)	4.1 (2.7–5.8)**	3.5 (1.6–4.9)**
50	nd	4.0 (2.6–4.5)**	15 (6.0–23)**

Median values and the range are shown for 5–9 animals. *$P<0.05$ and **$P<0.005$ compared with saline only control (Mann-Whitney *U*-test). nd = not done.

Table 2 Inhibition of cell accumulation induced by chymase in the mouse peritoneum at 6 h

Compound injected	*Total cells* ($\times 10^6$)	*Neutrophils* ($\times 10^6$)	*Eosinophils* ($\times 10^6$)	*Lymphocytes* ($\times 10^6$)	*Macrophages* ($\times 10^6$)
Chymase	3.9 (2.7–5.8)	1.90 (1.81–3.26)	0.06 (0.03–0.09)	0.30 (0.14–0.37)	1.6 (0.7–2.0)
Chymase + chymostatin	2.4 (1.3–3.8)*	0.62 (0.31–1.32)**	0.06 (0.05–0.11)	0.22 (0.11–0.39)	1.2 (0.6–1.5)
Heat inactivated chymase	2.6 (1.6–2.9)*	0.59 (0.18–1.01)**	0.02 (0.02–0.05)	0.06 (0.03–0.11)*	1.8 (1.2–2.5)
Chymostatin	1.3 (1.2–2.0)	0.10 (0.03–0.12)	0.02 (0.01–0.05)	0.06 (0.03–0.09)	1.1 (1.0–1.8)
Saline	1.6 (0.7–2.7)	0.24 (0–0.79)	0.03 (0–0.08)	0.09 (0.03–0.17)	1.3 (0.5–1.8)

Cells numbers in peritoneal lavage fluid recovered following injection of chymase (5 μg) alone, chymase (5 μg) with chymostatin (5 μg), heat inactivated chymase (5 μg) or the saline diluent. Median values and the range are shown with 6–8 animals (five with chymostatin alone) in each group. *$P<0.05$ and **$P<0.005$ compared with uninhibited chymase (Mann-Whitney *U*-test).

Interactions between preformed mast cell mediators

Co-injection of histamine (5 μg) with various doses of chymase into the mouse peritoneum resulted in a reduced degree of neutrophil influx compared with that induced by injection of chymase alone at both 6 and 16 h (Figure 3). There was no apparent effect of histamine on eosinophil, lymphocyte or macrophage accumulation induced by chymase in the same

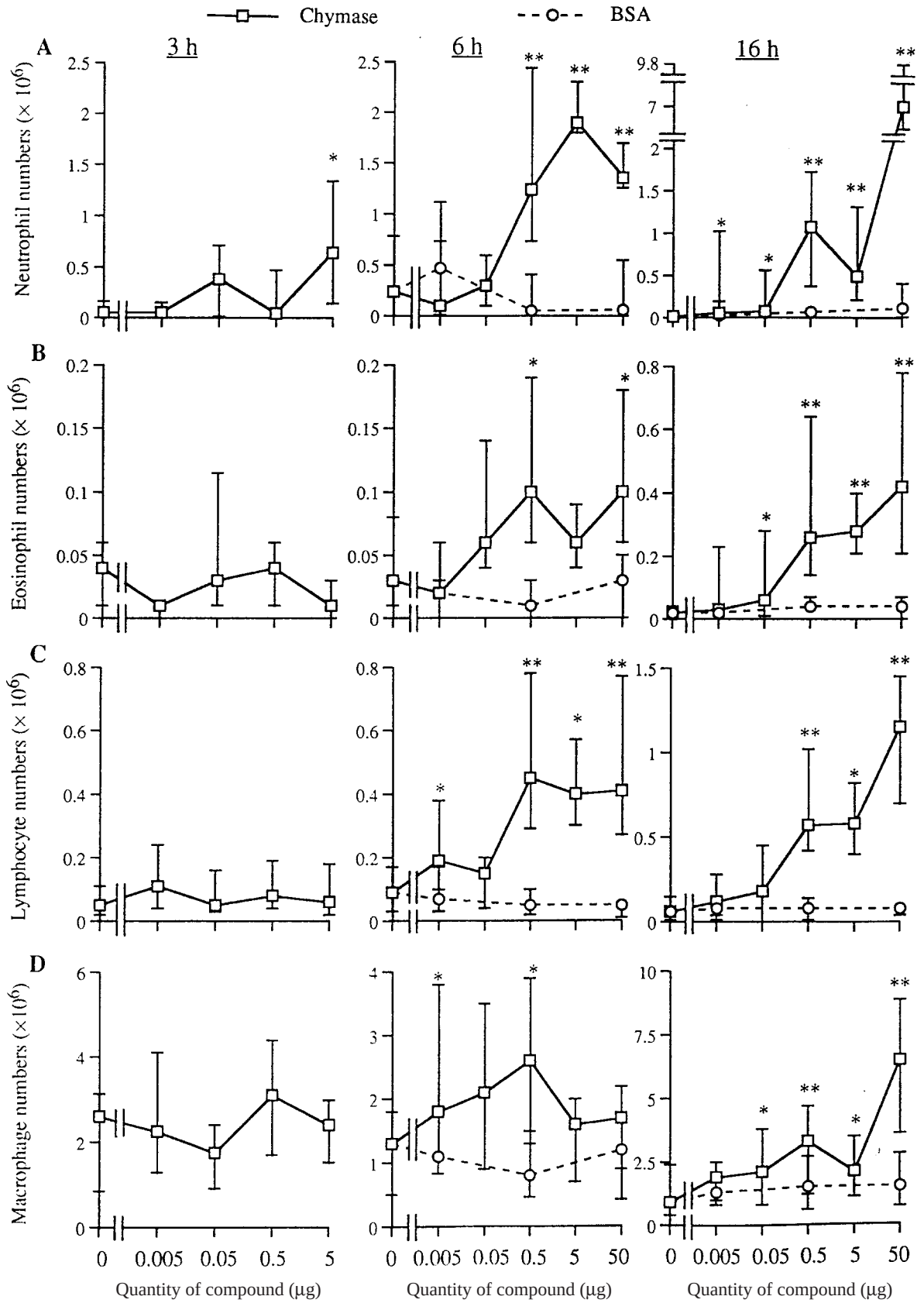

Figure 2 (A) Neutrophil (B) eosinophil (C) lymphocyte and (D) macrophage numbers in peritoneal lavage fluid recovered from mice 3, 6 or 16 h following injection of chymase or BSA. Median values and range are shown for 5–9 mice. $^{*}P<0.05$ and $^{**}P<0.005$ and compared with the response with the saline diluent alone (Mann-Whitney *U*-test).

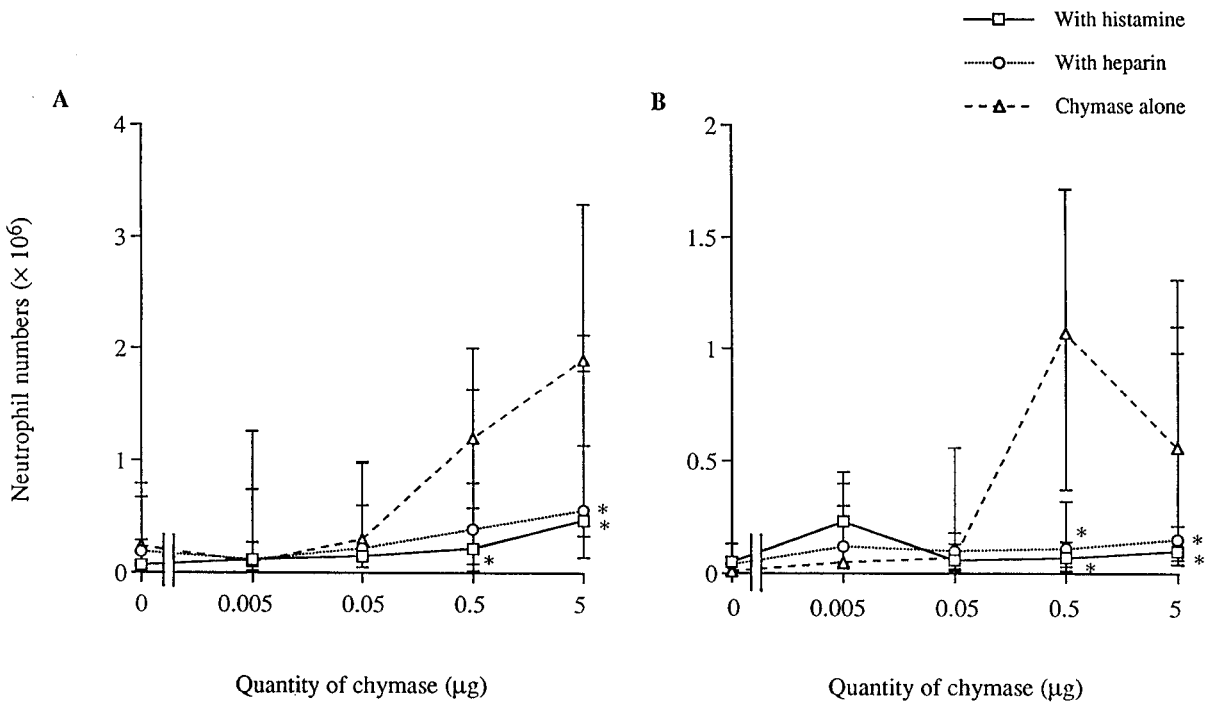

Figure 3 Neutrophil numbers in mouse peritoneal lavage fluid (A) 6 h and (B) 16 h following injection of chymase alone, chymase with 5 μg histamine or chymase with 25 μg heparin. Median values and range are shown for 6–10 animals. *$P<0.05$ for response less than that with chymase alone (Mann-Whitney *U*-test).

Table 3 Inhibition of cell accumulation induced by chymase in the mouse peritoneum at 16 h

Compound injected	*Total cells* ($\times 10^6$)	*Neutrophils* ($\times 10^6$)	*Eosinophils* ($\times 10^6$)	*Lymphocytes* ($\times 10^6$)	*Macrophages* ($\times 10^6$)
Chymase	2.9 (1.6–4.8)	0.56 (0.21–1.3)	0.14 (0.07–0.26)	0.32 (0.29–0.56)	1.9 (0.9–3.3)
Chymase + chymostatin	1.8 (1.0–3.5)*	0.17 (0.04–0.22)*	0.04 (0.01–0.22)*	0.27 (0.16–0.39)	1.2 (0.6–2.5)
Heat inactivated chymase	2.5 (1.8–3.5)	0.09 (0.01–0.21)**	0.05 (0.02–0.16)*	0.07 (0.05–0.13)*	2.1 (1.7–3.2)
Chymostatin	1.3 (1.0–1.9)	0.03 (0.01–0.18)	0.02 (0.01–0.05)	0.15 (0.10–0.32)	1.0 (0.7–1.5)
Saline	1.0 (0.5–2.7)	0.01 (0–0.06)	0.02 (0.01–0.04)	0.06 (0.01–0.17)	0.9 (0.4–2.4)

Cell numbers in peritoneal lavage fluid recovered following injection of chymase (5 μg) alone, chymase (5 μg) with chymostatin (5 μg), heat inactivated chymase (5 μg) or the saline diluent. Median values and the range are shown with 6–8 animals (five with chymostatin alone) in each group. *$P<0.05$ and **$P<0.005$ compared with uninhibited chymase (Mann-Whitney *U*-test).

animals (data not shown). The chymase-induced neutrophil influx also appeared to be inhibited by co-injecting heparin (25 μg) at both of the time points (Figure 3). In addition, heparin seemed to reduce eosinophil recruitment induced by chymase and to abolish completely chymase-induced macrophage accumulation at 16 h (Mann-Whitney *U*-test, data not shown). Injection of neither histamine nor heparin by themselves caused significant alterations in the numbers of nucleated cells or of any cell type in the mouse peritoneum (data not shown). When various quantities of chymase were co-injected with 0.5 μg tryptase into the mouse peritoneum there was no synergistic or inhibitory interaction observed between these two major mast cell products on either total nucleated cell numbers or on differential cell counts at either 6 or 16 h (Kruskal Wallis; data not shown).

Discussion

Our studies provide compelling evidence that human mast cell chymase can induce the accumulation of granulocytes, as well as of lymphocytes and macrophages following injection into laboratory animals. Small quantities of chymase injected either into guinea-pig or mouse models provoked a granulocyte-rich inflammatory cell infiltrate within 6 h. Chymase may thus contribute in an important way to cell recruitment following mast cell activation in allergic disease.

Chymase in a quantity as small as 5 or 50 ng (1.7×10^{-13} or 10^{-12} mole, respectively) was able to induce significant increases in the numbers of neutrophils, eosinophils, lymphocytes and macrophages in the mouse peritoneum. The material employed in the present study was of high purity, and the levels of endotoxin were lower than those permitted for intravenous injection in man (United States Pharmacopeia upper limit: 50 ng ml^{-1}; FDA, 1983). Moreover, heating the enzyme at 56°C for 2 h (a procedure unlikely to alter the biological actions of endotoxin), or inactivating the enzyme with proteinase inhibitors, significantly reduced the ability of chymase to induce cell accumulation. While it is difficult to estimate the concentrations of chymase which will be achieved in tissues or body fluids in allergic disease, it seems likely that those employed in the present study are of physiological relevance. The human skin mast cell has been reported to contain 8.5 mg ml^{-1} chymase (3.5×10^{-3} M; Schechter, 1990). The release of chymase in a macromolecular complex of 400–560 kDa with proteoglycans and carboxypeptidase (Goldstein

et al., 1992), and the tendency to bind to charged components of cells and basement membranes (Briggaman *et al*., 1984) should result in high local concentrations of chymase being present in the immediate vicinity of degranulating mast cells.

There was a massive accumulation of granulocytes following injection of chymase into guinea-pig skin or into the mouse peritoneum. At the top dose tested (5 μg), chymase stimulated an increase in neutrophil numbers in the mouse peritoneum as early as 3 h post injection, but the maximal increase in numbers was observed at 16 h when there were as many as 700 fold more neutrophils than in the saline injected control mice. At the time points employed, the eosinophilia provoked tended to be less marked than the neutrophilia, with an increase in eosinophil numbers of up to 21 fold observed in response to chymase at 16 h. However, in guinea-pig skin the degree of eosinophil infiltration around the injection site of chymase was quite comparable to that of neutrophils. The apparent increase in lymphocyte numbers (up to 19 fold) in the mouse peritoneum was also appreciable; and though the relative increase in numbers of macrophages was smaller (up to 7 fold), the increase in terms of absolute numbers was nevertheless quite considerable for this abundant resident cell of the peritoneum.

The time course of the neutrophilia and eosinophilia observed in response to chymase is quite consistent with that reported following allergen challenge in the skin, lung or other sites of sensitized rodents and other laboratory animals (Walls *et al*., 1991; Wershil *et al*., 1991), as well as in atopic human subjects (Fleekop *et al*., 1987; Montefort *et al*., 1994). An increase in numbers of lymphocytes has also been noted in the bronchoalveolar lavage fluid of asthmatics during allergen-induced late phase reactions (Diaz *et al*., 1989), and increased numbers of mononuclear cells in nasal washings from rhinitic subjects 4–11 h after allergen challenge (Bascom *et al*., 1988). Our observations are thus in keeping with the idea that chymase could be involved in mediating inflammatory cell accumulation following its secretion from mast cells in allergic disease.

The findings with purified chymase have strong parallels with those of earlier studies with a chymotryptic proteinase isolated from human skin, which was reported to induce a neutrophil and mononuclear cell rich infiltration when injected into the skin of rabbits (Fräki, 1977; Hatcher *et al*., 1977), or neutrophilia in the peritoneum of mice (Thomas *et al*., 1977). A number of other proteases have also been demonstrated to have the potential to stimulate the accumulation of inflammatory cells *in vivo*. Tryptase, which is co-released with chymase from mast cells, we have found can induce neutrophilia and eosinophilia following injection into guinea-pig skin and the mouse peritoneum, as well as lymphocyte and macrophage accumulation in the latter model (He *et al*., 1997a). The patterns and time courses of cell accumulation in these models appear to be quite similar, whether provoked by chymase or tryptase, though at least on a weight basis there was a tendency for chymase to be a more potent stimulus. A trypsin-like enzyme (possibly tryptase) derived from human skin has also been effective in inducing neutrophil and lymphocyte infiltration following injection into rabbit skin, and a much smaller degree of leukocyte infiltration has been observed with cathepsin B1 and D-like enzymes purified from the same source (Fräki, 1977). Moreover, human thrombin can induce mild neutrophilia when injected into rabbit skin (Drake *et al*., 1992), and bovine pancreatic trypsin can provoke the sequestration of neutrophils in lung microvessels when infused intravenously into sheep, rats (Tahamont *et al*., 1982) or hamsters (Reichart *et al*., 1996). Proteases thus have the potential to have an important role in processes of cell recruitment, and the range of enzymes demonstrated to induce such a response would suggest that a number of quite different mechanisms may be involved.

The substrates upon which chymase may act *in vivo* to stimulate cell accumulation are not clear. One candidate is IL-1β which may be activated by chymase *in vitro* (Mizutani *et al*., 1991). This cytokine is able to induce the accumulation of eosinophils in the skin of rats (Sanz *et al*., 1995), and neutrophils in the skin of rabbits (Rampart & Williams, 1988), as well as being able to prime human neutrophils *in vitro* for subsequent activation by other stimuli (Sullivan *et al*., 1989; Dularay *et al*., 1990), and to upregulate expression of the cell adhesion molecules E-selectin, ICAM-1 and VCAM-1 on human vascular endothelial cells (Bochner *et al*., 1991; Ebisawa *et al*., 1992). The efficient conversion of angiotensin I to angiotension II by chymase (Urata *et al*., 1990) could be relevant in that angiotensin II has been found to induce expression in rat endothelial cells of the potent eosinophil and monocyte chemoattractant RANTES (Wolf *et al*., 1997); and angiotensin III, a derivative of angiotensin II through the action of aminopeptidase A, has been demonstrated to be a chemoattractant for human neutrophils (Yamamoto *et al*., 1993). Also of importance could be the cleavage of substrates not normally considered in the context of leukocyte recruitment. Chymase could generate chemoattractants through cleavage of α_1-antitrypsin at the Met^{358}-Ser^{359} peptide bond, and α_1-antichymotrypsin at Leu^{358}-Ser^{359} and Leu^{361}-Val^{362} bonds within the reactive-site loop (Schechter *et al*., 1989). The new peptides generated from hydrolysis of Met^{358}-Ser^{359} of α_1-antitrypsin (Banda *et al*., 1988) and from α_1-antichymotrypsin (Potempa *et al*., 1991) have been reported to have chemotactic activity towards neutrophils. The ability of chymase to cleave type I procollagen or more indirectly, to activate human interstitial procollagenase (Saarinen *et al*., 1994) could result in increased production of degradation products of collagen some of which may have neutrophil chemotactic activity *in vitro* (Kofford *et al*., 1997).

A family of protease activating receptors (PAR) has now been identified for tryptic proteases. Three of these, PAR-1 (thrombin receptor 1; Vu *et al*., 1991), PAR-2 (Nystedt *et al*., 1994) and PAR-3 (thrombin receptor 2; Ishihara *et al*., 1997) have been cloned and characterized. Acting on PAR-1 and PAR-3, thrombin can induce profound alterations in cell behaviour some of which may be relevant in inflammation (Vu *et al*., 1991; Ishihara *et al*., 1997). Possibly through the activation of PAR-2 (Molino *et al*., 1997) or other receptors, mast cell tryptase can also provoke cell responses, acting as a growth factor (Walls, 1998), or inducing histamine release from mast cells (He & Walls, 1997; He *et al*., 1998), and chemokine release from epithelial and endothelial cells (Cairns & Walls, 1996; Compton *et al*., 1998). A rat chymase has been reported to activate rat mast cells (Schick *et al*., 1984), canine chymase to stimulate mucus secretion from glandular epithelial cells (Sommerhoff *et al*., 1989) and human cathepsin G to act as a chemoattractant for human neutrophils and monocytes (Chertov *et al*., 1997). However, in contrast to its rat conterpart, human chymase does not appear to stimulate the degranulation of mast cells (He *et al*., 1997b), and the actions of human chymase on cells or the potential presence of a receptor for a chymotryptic protease have been little investigated.

At sites of allergic inflammation chymase will be secreted into the extracellular environment along with a range of other factors which could modulate processes of cell recruitment. In the present studies we found that the pattern of chymase-induced cell accumulation could be altered by co-administra-

tion of other preformed products of mast cells. Histamine itself at the concentrations tested did not provoke a cellular influx when injected into the mouse peritoneum. However, when histamine was co-injected with chymase it was noted that fewer neutrophils were recovered than when chymase was injected alone, and that numbers of each of the other cell types were apparently unaffected. Using the same mouse model we have observed previously that administration of histamine can selectively enhance tryptase induced eosinophil and lymphocyte accumulation in the mouse peritoneum, without any alteration in the degree of neutrophil influx (He *et al*., 1997a). Our studies indicate that histamine may have a role in modulating cellular accumulation induced by mast cell serine proteinases, and the overall effect may be to increase the relative numbers of eosinophils in the inflammatory infiltrate.

The co-injection of heparin with chymase appeared to reduce the neutrophil and macrophage influx in the mouse peritoneum. Anti-inflammatory actions have been observed previously for heparin, and it has been reported that a purified preparation of heparin can reduce the neutrophil recruitment elicited by tryptase in the mouse peritoneum (He *et al*., 1997a), inhibit eosinophil infiltration induced by allergen in the airways (Seeds *et al*., 1993) and skin of guinea-pigs (Teixeira & Hellewell, 1993), and reduce allergen-induced airway and cutaneous responses in allergic sheep (Ahmed *et al*., 1993). On the other hand, we failed to observe either a synergistic or inhibitory interaction when tryptase and chymase were injected together. The simple additive cell accumulation responses observed would be in keeping with these proteinases acting through different pathways.

In conclusion, using two different animal models, we have found that human chymase is a potent stimulus for inflammatory cell accumulation. The relative importance of chymase in mediating a granulocyte rich inflammatory infiltrate in allergic disease will depend on the concentrations of other products released from mast cells and from other cell types, and on the levels of proteinase inhibitors. Reports that the greatest proportion of chymase containing mast cells are present in human skin and in the submucosal tissues of the intestinal and respiratory tracts (Irani & Schwartz, 1990) may indicate a greater contribution for this proteinase in conditions affecting connective tissue sites. However, a recent study employing sensitive immunocytochemical techniques has suggested that at least in the intestine, the great majority of mucosal mast cells may also contain chymase (Beil *et al*., 1997). Chymase could have an important role as a mediator of cell recruitment in inflammatory disease, and may be a useful target for therapeutic intervention.

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