

©2004, Acta Pharmacologica Sinica
Chinese Pharmacological Society
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
<http://www.ChinaPhar.com>

Inhibition of tryptase and chymase induced nucleated cell infiltration by proteinase inhibitors¹

Shao-heng HE^{2,3}, Han-qiu CHEN, Jian ZHENG

Allergy & Inflammation Research Institute, Shantou University Medical College, Shantou 515031, China;

²Immunopharmacology Group, University of Southampton, UK

KEY WORDS tryptase; chymase; nucleated cells; proteinase inhibitor

ABSTRACT

AIM: To investigate the ability of proteinase inhibitors to modulate nucleated cell infiltration into the peritoneum of mice induced by tryptase and chymase. **METHODS:** Human lung tryptase and skin chymase were purified by a similar procedure involving high salt extraction, heparin agarose affinity chromatography followed by S-200 Sephacryl gel filtration chromatography. The actions of proteinase inhibitors on tryptase and chymase induced nucleated cell accumulation were examined with a mouse peritoneum model. **RESULTS:** A selective chymase inhibitor Z-Ile-Glu-Pro-Phe-CO₂Me (ZIGPPF) was able to inhibit approximately 90 % neutrophil, 73 % eosinophil, 87 % lymphocyte and 60 % macrophage accumulation induced by chymase at 16 h following injection. Soy bean trypsin inhibitor (SBTI), chymostatin, and α_1 -antitrypsin showed slightly less potency than ZIGPPF in inhibition of the actions of chymase. While all tryptase inhibitors tested were able to inhibit neutrophil, eosinophil, and macrophage accumulation provoked by tryptase at 16 h following injection, only leupeptin, APC366, and aprotinin were capable of inhibiting tryptase induced lymphocyte accumulation. The inhibitors of tryptase tested were also able to inhibit tryptase induced neutrophil and eosinophil accumulation at 6 h following injection. When being injected alone, all inhibitors of chymase and tryptase at the concentrations tested by themselves had no significant effect on the accumulation of nucleated cells in the peritoneum of mice at both 6 h and 16 h. **CONCLUSION:** Proteinase inhibitors significantly inhibited tryptase and chymase-induced nucleated cell accumulation *in vivo*, and therefore they are likely to be developed as a novel class of anti-inflammatory drugs.

INTRODUCTION

Tryptase and chymase are serine proteases that are almost exclusively located to the secretory granules of mast cells. They are the most abundant protein products in mast cell granules, which consist of approxi-

mately 50 % total protein in the granules^[1]. Upon degranulation, tryptase and chymase are released from mast cells along with histamine, heparin, and other mast cell granule products^[2]. Large quantities of active form tryptase and chymase (up to 40 pg per mast cell) in mast cells^[1] implicate that these mast cell unique mediators are likely to play a role in mast cell related diseases.

Tryptase has been found to be able to profoundly alter the behavior of certain cell types, such as provocation of release of IL-8 from epithelial and endothelial cells^[3,4], activation of mast cells^[5,6], stimulation of pro-

¹ Project supported by the Planned Science and Technology Project of Guangdong Province, China (No 2003B31502) and the Li Ka Shing Foundation, Hong Kong, China (No C0200001).

³ Correspondence to Dr Shao-heng HE. Phn 86-754 890-0405.

Fax 86-754-890-0192. E-mail shoahenghe@hotmail.com

Received 2004-04-19

Accepted 2004-08-12

liferation of epithelial cells^[3] and fibroblasts^[7], induction of cytokine production and release from human peripheral blood eosinophils^[8], stimulation of TNF- α , IL-6 and IL-1 β synthesis and release from peripheral blood mononuclear cells^[9]. Chymase was reported to be able to provoke microvascular leakage in the skin of guinea pig^[10], activate IL-1 β ^[11], and participate in the activation-secretion process of mast cells^[12]. Of particular importance is that tryptase and chymase are able to induce infiltration and activation of eosinophils and neutrophils^[13-15], which represents a novel and pivotal communication mechanism between mast cells, the primary effector cells of allergic reactions and eosinophils and neutrophils, the secondary effector cells of allergic reactions.

In recent years, searching to develop tryptase and chymase inhibitor drugs has become one of the major targets for the development of anti-inflammatory drugs. Certain inhibitors of tryptase and chymase have been found to possess anti-inflammatory activity including that a specific tryptase inhibitor APC 366 was able to attenuate allergen-induced late-phase airway obstruction when being inhaled into asthmatic airways^[16]; a potent tryptase inhibitor nafamostat mesilate dramatically suppressed pulmonary dysfunction induced in rats by a radiographic contrast medium^[17]; a novel chymase inhibitor, 4-[1-([*bis*-(4-methyl-phenyl)-methyl]-carbonyl)3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yl]oxy]-benzoic acid (BCEAB), suppressed cardiac fibrosis in cardiomyopathic hamsters^[18,19] and a chymase inhibitor TEI-E548 improved survival in hamsters with myocardial infarction^[20]. However, little is known of the effects of proteinase inhibitors on tryptase and chymase induced nucleated cell infiltration *in vivo*. In the current study, we investigated the potential abilities of inhibitors of tryptase and chymase, which were used in our previous study with enzyme assay^[21], to inhibit tryptase and chymase induced nucleated cell infiltration in the peritoneum of mice.

MATERIALS AND METHODS

Preparation of tryptase The procedures for isolation of human tryptase were reported previously^[21]. In brief, macroscopically normal lung tissue dissected from the patient with lung cancer at lobectomy was collected from the Pathology Department, Medical College, Shantou University and was minced into small pieces. After being extracted with high salt buffer con-

taining MES (Sigma) 10 mmol/L (pH 6.1) and NaCl 2 mol/L, the extract was applied to heparin agarose (Sigma) in an equilibration buffer containing MES 10 mmol/L (pH 6.1) and NaCl 0.4 mol/L, and followed by a S-200 Sephacryl agarose (Sigma) chromatography procedure in a buffer containing MES 100 mmol/L (pH 6.1) and NaCl 2 mol/L. The fractions containing high tryptase activity were collected and stored at -80 °C.

Tryptase activity and purity determination As described previously^[5], tryptase activity was determined by its ability to cleave a synthetic substrate *N*-benzoyl-*D,L*-arginine-*p*-nitroanilide (BAPNA, Sigma) 20 mmol/L in Tris-HCl 0.1 mol/L (pH 8.0) and glycerol 1 mol/L. The reaction was spectrophotometrically monitored at 410 nm. Protein concentration was determined by Coomassie Brilliant Blue G (Pierce, USA) method. The specific activity of tryptase was expressed as mU tryptase activity per mg protein, where one unit (U) of enzyme was taken as the amount that catalyzed the cleavage of 1 μ mol of BAPNA per minute at 25 °C. The purity of tryptase was estimated with the numbers of diffuse bands on 10 % sodium dodecyl sulphate-polyacrylamine gel electrophoresis (SDS-PAGE). The identity as tryptase was confirmed by Western blotting with monoclonal antibody AA₅ (a present from Dr Andrew F WALLS, University of Southampton, UK) against human tryptase. The tryptase prepared for this study had a single diffuse band on SDS-PAGE and their specific activities were 2.1 kU/g protein.

Preparation of chymase The procedures for isolation of human chymase were reported previously^[21]. In brief, macroscopically normal skin tissue dissected from amputation legs was collected from the Pathology Department, Medical College, Shantou University, and chymase was purified from the human skin tissue by high salt extraction, heparin agarose and S-200 Sephacryl gelfiltration chromatography procedures. The purified chymase was then concentrated in C-10 Centricon centrifugal concentrators (Amicon, UK) and stored at -80 °C until use. Enzymatic activity was determined spectrophotometrically (410 nm) by the rate of hydrolysis of 0.7 mmol/L *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPP, Sigma) in NaCl 1.5 mol/L, Tris 0.3 mol/L, pH 8.0. The specific activity of chymase was expressed as mU activity per mg protein, where 1 U of enzyme represents that required to hydrolyse 1 μ mol of SAAPP per min at 25 °C. Purity was evaluated using 10 % SDS-PAGE, and the identity of the protein band confirmed by Western blotting with monoclonal

antibody CC₁ (a present from Dr Andrew F WALLS, University of Southampton, UK) against human chymase. The specific activity was 4.9 kU/g.

Preparation of compounds As trypsin and chymase are enzymatically unstable in physiological solutions, considerable care was taken in its preparation. Purified trypsin and chymase stored in high salt buffer were diluted immediately prior to its injection, first with sterile distilled water, the NaCl concentration was adjusted to 0.15 mol/L, and then with normal saline to obtain the required enzyme concentration. BSA in saline was used as a foreign protein control. Where added, proteinase inhibitors and trypsin or chymase were incubated for 20-30 min on ice before injection. The concentrations of inhibitors applied in the current study were selected according to our previous experimental results^[13,14,21].

Immunodepletion of trypsin and chymase activities An excess quantity of purified AA₅ anti-trypsin antibody (100 µg in a volume of 20 µL) was incubated with 25 µg trypsin at 4 °C for 24 h. The trypsin-antibody complexes were then immunoprecipitated with protein A Sepharose beads. After centrifugation, the supernatant was assayed for residual trypsin activity using BAPNA as substrate. The supernatant was then diluted to 550 µL and injected into the peritoneum of five mice with 100 µL per mouse. Similarly, 25 µg chymase was treated with purified CC₁ anti-chymase antibody (100 µg in a volume of 25 µL) following the procedure above.

Mouse peritoneal injection and cell count Trypsin, chymase, bovine serum albumin (BSA), normal saline, or inhibitors were injected in 0.5 mL volumes into the peritoneum of male BALB/c mice (18-22 g, obtained from Guangdong Experimental Animal Centre, China, Grade II, Certificate No 2001A049), whose abdominal skin was swabbed with 70 % ethanol. This model was adapted from that described by Thomas *et al*^[22], which complied with the European Community guidelines for use of experimental animals and was approved by Ethics Committee of Shantou University Medical College. At 6 h or 16 h following injection, animals were killed by inhalation of carbon dioxide, and peritoneal lavage was performed in a standardized manner with 5 mL normal saline. The fluid was collected into heparinized tubes and centrifuged at 2000 r/min for 10 min at 4 °C. Cells were resuspended in 2.0 mL MEM, stained with 0.1 % Trypan blue, and enumerated using an Improved Neubauer haemocytometer (for to-

tal cell numbers). Cyto-centrifuge preparations were made, air dried and stained with modified Wright's stain. Differential cell counts were performed for a minimum of 500 cells. The results were expressed as absolute numbers of each cell type per mouse peritoneum. For certain experiments, the peripheral blood of mice was taken from tail vein before peritoneal lavage being collected and blood smear slides were prepared. After staining with modified Wright's stain, a minimum of 300 nucleated cells was counted for differential cell counts and the result was expressed as percentage of total cells.

Statistics Statistical analysis were performed by using SPSS software. Data were expressed as mean±SEM. Where analysis of variance indicated significant differences between groups with ANOVA, for the preplanned comparisons of interest, *t*-test was applied. For all analysis, *P*<0.05 was taken as significant.

RESULTS

Induction of nucleated cell accumulation by chymase and trypsin Chymase at 5 µg was able to potently induce nucleated cell infiltration in the peritoneum of mice at 6 h and 16 h following injection. Among these cells, the numbers of neutrophils were dramatically increased by up to approximately 15 fold at 16 h, and they were the predominant cell type in the peritoneum of mice at 6 h following injection with the absolute numbers reaching as many as 2.21×10⁶ per mouse peritoneum. The numbers of lymphocytes were also greatly elevated at 6 h and 16 h following injection of chymase with up to approximately 6 fold increase in the numbers of lymphocytes being achieved at 16 h. Similarly, the numbers of eosinophils and macrophages were largely increased in the peritoneum of mice, but this was only observed at 16 h following injection (Tab 1).

Trypsin showed a similar potency to chymase in induction of nucleated cell infiltration in the peritoneum of mice. It provoked up to some 44 fold increase in neutrophil numbers and approximately 3 fold increase in eosinophil numbers. The numbers of lymphocytes and macrophages were also greatly elevated by trypsin in the peritoneum of mice (Tab 2). In the peripheral blood of mice, both trypsin and chymase were able to induce significant increase in the numbers of neutrophils at 16 h following injection (Tab 3).

The effect of inhibitors of chymase on chymase induced nucleated cell accumulation All inhibitors

Tab 1. Induction of nucleated cell infiltration in the peritoneum of mice by mast cell chymase. *n*=6-8. Mean±SEM. Compounds were injected into the peritoneum of mice for 0 h, 6 h, and 16 h. ^b*P*<0.05 vs saline control.

Nucleated cells	10 ⁶ ×Cell numbers in the peritoneum of mice								
	0 h	Saline			BSA (50 μg)			Chymase (5 μg)	
		6 h	16 h	0 h	6 h	16 h	0 h	6 h	16 h
Total	1.67±0.19	1.40±0.23	1.26±0.26	2.20±0.61	1.34±0.24	1.62±0.31	2.48±0.43	3.81±0.38 ^b	3.47±0.41 ^b
Neutrophils	0.03±0.01	0.17±0.06	0.05±0.01	0.05±0.02	0.14±0.08	0.17±0.07	0.04±0.02	2.21±0.22 ^b	0.76±0.16 ^b
Eosinophils	0±0.01	0.04±0.01	0.02±0.00	0±0.01	0.03±0.01	0.04±0.01	0±0.01	0.06±0.01	0.16±0.03 ^b
Lymphocytes	0.12±0.07	0.09±0.02	0.06±0.01	0.15±0.05	0.06±0.01	0.09±0.02	0.16±0.01	0.23±0.05 ^b	0.36±0.07 ^b
Macrophages	1.5±0.22	1.10±0.11	1.13±0.24	1.99±0.54	1.11±0.18	1.47±0.14	2.28±0.33	1.39±0.19	2.19±0.23 ^b

Tab 2. Induction of nucleated cell infiltration in the peritoneum of mice by mast cell trypsinase. *n*=6-8. Mean±SEM. Compounds were injected into the peritoneum of mice for 0 h, 6 h, and 16 h. ^b*P*<0.05 vs saline control.

Nucleated cells	10 ⁶ ×Cell numbers in the peritoneum of mice								
	0 h	Saline			BSA (50 μg)			Trypsinase (5 μg)	
		6 h	16 h	0 h	6 h	16 h	0 h	6 h	16 h
Total	1.67±0.19	1.61±0.23	1.21±0.24	2.20±0.61	1.37±0.25	1.67±0.33	2.43±0.24	4.46±0.62 ^b	3.24±0.59 ^b
Neutrophils	0.03±0.01	0.24±0.06	0.02±0.01	0.05±0.02	0.15±0.08	0.07±0.02	0.1±0.04	2.40±0.50 ^b	0.88±0.15 ^b
Eosinophils	0±0.01	0.03±0.01	0.03±0.00	0±0.01	0.03±0.01	0.04±0.01	0.02±0.01	0.10±0.02 ^b	0.12±0.03 ^b
Lymphocytes	0.12±0.07	0.09±0.02	0.06±0.02	0.15±0.05	0.06±0.01	0.09±0.02	0.18±0.04	0.16±0.02	0.24±0.03 ^b
Macrophages	1.5±0.22	1.24±0.17	1.10±0.21	1.99±0.54	1.12±0.20	1.47±0.17	2.13±0.21	1.83±0.34	2.00±0.40 ^b

Tab 3. The effect of trypsinase (Try) and chymase (Chy) on nucleated cell count in mouse peripheral blood at 16 h following injection. Mean±SEM. *n*=5. ^b*P*<0.05 vs saline control. ^c*P*<0.05 vs corresponding antibody-treated group.

Compound injected (μg)	% of the total nucleated cells			
	Neutrophils	Eosinophils	Lymphocytes	Monocytes
Try 5.0	35.6±3.8 ^{bc}	0	63±3.7	0.7±0.1
Chy 5.0	32±3.9 ^{bc}	0	66.3±4.1	1.1±0.2
dTry 5.0	23.2±2.8	0	72.7±2.9	2.3±1.0
dChy 5.0	20.3±3.4	0	77.9±3.5	1.7±0.4
AA ₅ 20	21.3±2.0	0.4±0.1	76.9±2.8	1.3±0.2
CC ₁ 20	21.5±1.8	0.3±0.1	77.1±2.9	1.2±0.3
Normal saline	22.4±2.0	0.4±0.1	76.2±2.0	1.0±0.2

dTry=trypsinase was immunodepleted with AA₅ before being injected into the peritoneum of mice. dChy=chymase was immunodepleted with CC₁ before being injected into the peritoneum of mice.

of chymase at the concentrations tested were able to inhibit nucleated cell accumulation induced by chymase at both 6 h and 16 h following injection. Among these inhibitors, a selective chymase inhibitor ZIGPPF was able to inhibit up to some 90 % neutrophil, 73 % eosinophil, 87 % lymphocyte, and 60 % macrophage accumulation induced by chymase, respectively. Although SBTI and α₁-antitrypsin were relatively less potent than ZIGPPF in inhibition of the actions of chymase, they showed a similar pattern of inhibitory actions to ZIGPPF in the peritoneum of mice. In comparison with the other three inhibitors, chymostatin appeared to be a relatively weak inhibitor on chymase induced lymphocyte accumulation, and a much potent inhibitor on chymase induced macrophage accumulation (Fig 1). When being injected alone, all inhibitors of chymase at the concentrations tested by themselves had no significant effect on the accumulation of nucleated cells in the peritoneum of mice at both 6 and 16 h (data not shown).

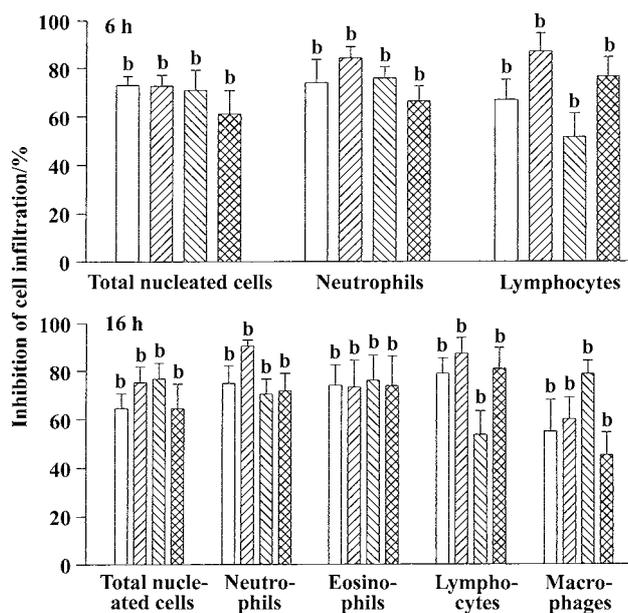


Fig 1. Percentage inhibition of chymase-induced nucleated cell infiltration in the peritoneum of mice by inhibitors of chymase at 6 h and 16 h following injection. Chymase (5 μ g) was incubated with SBTI (50 μ g, open bars), ZIGPPM (0.5 μ g, left hatched bars), chymostatin (5 μ g, right hatched bars), or α_1 -antitrypsin (50 μ g, crossed bars), respectively on ice for 20 min before being co-injected into the peritoneum of mice. ^b $P < 0.05$ vs the uninhibited control.

The effect of inhibitors of trypsin on trypsin induced nucleated cell accumulation As for inhibitors of chymase, all five inhibitors of trypsin at the concentrations tested were capable of reducing the numbers of nucleated cells accumulated in the peritoneum of mice in response to trypsin. While a broad spectrum inhibitor of trypsin leupeptin appeared to be able to inhibit up to some 94 % neutrophil, 95 % eosinophil, 93 % lymphocyte, and 57 % macrophage accumulation provoked by trypsin, a selective inhibitor of trypsin APC366 inhibited up to approximately 95 % neutrophil, 68 % eosinophil, 87 % lymphocyte, and 64 % macrophage infiltration induced by trypsin at 16 h following injection. However, the other two broad spectrum inhibitors of trypsin tested benzamidine and antipain (a small microbial peptide) showed less potency than leupeptin and APC366 in inhibition of trypsin-induced neutrophil, eosinophil and lymphocyte accumulation at 16 h. Aprotinin, an inhibitor of tissue kallikrein, possesses some trypsin inhibition activity. It was able to potently inhibit trypsin-induced neutrophil, eosinophil, lymphocyte and macrophage accumulation. It was noted that benzamidine and antipain failed to inhibit trypsin-induced lymphocyte accumulation at 16 h fol-

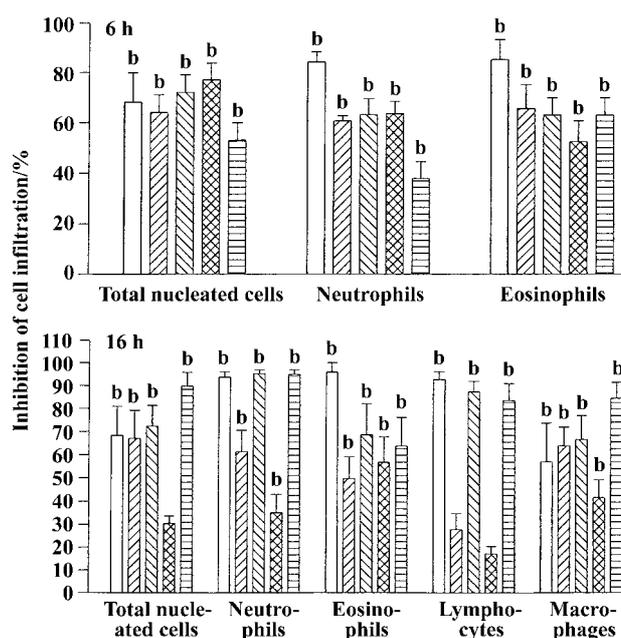


Fig 2. Percentage inhibition of trypsin-induced nucleated cell infiltration in the peritoneum of mice by inhibitors of trypsin at 6 h and 16 h following injection. Trypsin (5 μ g) was incubated with leupeptin (50 μ g, open bars), benzamidine (50 μ g, left hatched bars), APC366 (50 μ g, right hatched bars), antipain (50 μ g, crossed bars) or aprotinin (50 μ g, rectangular bars), respectively on ice for 20 min before being co-injected into the peritoneum of mice. ^b $P < 0.05$ vs the uninhibited control.

lowing injection (Fig 2). The inhibitors of trypsin tested were also able to inhibit trypsin induced neutrophil and eosinophil accumulation at 6 h following injection (Fig 2). All inhibitors of trypsin at the concentrations tested by themselves had no significant effect on the accumulation of nucleated cells in the peritoneum of mice at both 6 and 16 h (data not shown).

Effect of immunodepletion on trypsin and chymase-induced nucleated cell accumulation The immunodepletion procedure employed was able to reduce approximately 96 % trypsin and 94 % chymase activities. However, it only reduced approximately 70 % neutrophil, 80 % eosinophil, 64 % lymphocyte, and 55 % macrophage accumulation induced by trypsin 16 h following injection. The immunodepletion procedure was also able to diminish approximately 67 % neutrophil, 90 % eosinophil, 56 % lymphocyte, and 91 % macrophage accumulation induced by chymase. AA5 and CC1 had no effect on nucleated cell accumulation when they were injected into mouse peritoneum alone (Tab 4).

Tab 4. The effect of immunodepletion on tryptase (Try) and chymase (Chy) induced nucleated cell accumulation in mouse peritoneum at 16 h following injection. Mean±SEM. n=5. ^bP<0.05 compared with saline control. ^fP<0.01 vs corresponding antibody-treated group.

Compound injected (µg)	10 ⁻⁶ × Cell numbers in the peritoneum of mice			
	Neutrophils	Eosinophils	Lymphocytes	Macrophages
Try 5.0	3.2±0.7 ^{bf}	0.13±0.03 ^{bf}	3.1±0.4 ^{bf}	7.4±1.2 ^{bf}
Chy 5.0	3.5±1.0 ^{bf}	0.13±0.03 ^{bf}	2.6±0.5 ^{bf}	8.9±1.1 ^{bf}
dTry 5.0	1.0±0.3	0.05±0.01	1.2±0.1	4.7±1.0
dChy 5.0	1.2±0.3	0.04±0.01	1.2±0.4	3.1±0.9
AA ₅ 20	0.08±0.02	0.03±0.01	0.15±0.03	2.9±0.4
CC ₁ 20	0.09±0.02	0.03±0.01	0.14±0.02	2.8±0.4
Normal saline	0.06±0.01	0.03±0.01	0.12±0.02	2.5±0.5

dTry=tryptase was immunodepleted with AA₅ before being injected into the peritoneum of mice. dChy=chymase was immunodepleted with CC₁ before being injected into the peritoneum of mice.

DISCUSSION

It is important to learn that inhibitors, the selective inhibitors in particular, of tryptase or chymase possess potent inhibitory actions on tryptase or chymase induced nucleated cell accumulation *in vivo*. Up to approximately 95 %, 95 %, 93 %, and 64 % inhibition of neutrophil, eosinophil, lymphocyte and macrophage infiltration respectively by tryptase, and up to approximately 90 %, 73 %, 87 %, and 60 % inhibition of neutrophil, eosinophil, lymphocyte and macrophage infiltration respectively by chymase indicates that the actions of tryptase and chymase in provocation of infiltration of nucleated cells are dependent on the intact catalytic sites of the enzymes. The immunodepletion of tryptase and chymase activities with their specific monoclonal antibodies further confirmed that the actions of tryptase and chymase on nucleated cell accumulation were dependent on their enzymatic activities. This catalytic site-dependent inhibition mechanism was observed previously with other experimental systems including proliferation of epithelial cells^[2], endothelial cells^[3], smooth muscle cells^[23] and induction of microvascular leakage by tryptase^[5], and by chymase^[10].

The standard quantity of tryptase and chymase (5 µg), which was applied throughout the study, was chosen based on the doses of tryptase^[13] and chymase^[14] used in the previous studies. The doses of inhibitors

used were able to completely abolish the ability of tryptase and chymase to cleave the chromogenic substrate BAPNA *in vitro* under the conditions described^[21], and did not provoke a significant change of nucleated cell numbers in the peritoneum of mice.

Leupeptin and APC366 showed similar pattern and potency in inhibition of infiltration of each cell type at 16 h and 6 h indicating they are likely to share the same inhibitory mechanism, which is directly block tryptase enzymatic activity. Benzamidine appeared a relative weaker inhibitor than leupeptin in inhibition of neutrophil, eosinophil and particularly lymphocyte infiltration at 16 h following injection suggesting that it is either a short-lived inhibitor *in vivo* or using a different inhibitory mechanism from leupeptin. It seems likely that antipain is a rather different inhibitor from leupeptin *in vivo* as it had no effect on tryptase-induced lymphocyte infiltration and relatively small inhibitory actions in tryptase-induced neutrophil, eosinophil and macrophage infiltration at 16 h. Aprotinin, a weak inhibitor of tryptase, and potent inhibitor of tissue kallikrein^[24] showed a strong inhibitory actions in tryptase-induced nucleated cell infiltration, indicating that a kallikrein related mechanism may be involved in the process. The degree of inhibition achieved with aprotinin at 16 h was greater than that achieved at 6 h suggested that the actions of aprotinin were a relatively slow process. All four inhibitors of chymase tested showed a similar pattern in inhibition of chymase-induced neutrophil and eosinophil infiltration, indicating that the enzymatic activity of chymase was required to provoke these two cell type to accumulate in the peritoneum of mice. However, chymostatin appeared less effective in inhibition of chymase-induced lymphocyte infiltration, but more effective in inhibition of chymase-induced macrophage infiltration than the other three inhibitors tested. This may suggest that chymase adopted different mechanism to accumulate lymphocytes and macrophages.

In recent years, mast cells were further found to be associated with asthmatics^[25,26], acute appendicitis^[27], blood clotting^[28], hay fever^[29], and skin allergic reactions^[30], which suggests that this cell type is undoubtedly involved in the pathogenesis of a range of diseases. Therefore, as the most abundant and unique secretory mediators of mast cells tryptase and chymase are very likely to play a key role in mast-cell associated diseases. The ability of tryptase inhibitor APC366 to block allergen-induced late-phase airway obstruction in asthmatic airways^[16] and MOL 6131^[31] to abolish airway inflam-

mation in a mouse asthma model implicates that tryptase inhibitors could be an important target for therapeutic intervention in inflammation. Lack of assay for measuring the levels of chymase in biological fluids makes direct evaluation of the roles of chymase in diseases difficult. However, it is still worthwhile to develop anti-chymase drugs for the treatment of mast cell-associated diseases as chymase possesses a number of proinflammatory functions in man.

In conclusion, the current study proved that the proteinase inhibitors tested were highly effective in inhibition of tryptase and chymase-induced nucleated cell infiltration *in vivo*, and therefore development of anti-tryptase and anti-chymase drugs will be beneficial to the treatment of inflammatory diseases.

REFERENCES

- Schwartz LB. Tryptase from human mast cells: biochemistry, biology and clinical utility: In: Schwartz LB, editor. Neutral proteases of mast cells. Basel, Karger: Monogr Allergy; 1990. p 90-113.
- Walls AF, He S, Buckley MG, McEuen AR. Roles of the mast cell and basophil in asthma. *Clin Exp Allergy Rev* 2001; 1: 68-72.
- Cairns JA, Walls AF. Mast cell tryptase is a mitogen for epithelial cells: stimulation of IL-8 production and intercellular adhesion molecule-1 expression. *J Immunol* 1996; 156: 275-83.
- Compton SJ, Cairns JA, Holgate ST, Walls AF. The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 and IL-8 and stimulates the selective release of IL-8 from HUVEC. *J Immunol* 1998; 161: 1939-47.
- He S, Walls AF. Human mast cell tryptase: a potent stimulus of microvascular leakage and mast cell activation. *Eur J Pharmacol* 1997; 328: 89-97.
- He S, Gaça MDA, Walls AF. A role for tryptase in the activation of human mast cells: modulation of histamine release by tryptase and inhibitors of tryptase. *J Pharmacol Exp Ther* 1998; 286: 289-97.
- Cairns JA, Walls AF. Mast cell tryptase stimulates the synthesis of type I collagen in human lung fibroblasts. *J Clin Invest* 1997; 99: 1313-22.
- Temkin V, Kantor B, Weg V, Hartman ML, Levi-Schaffer F. Tryptase activates the mitogen-activated protein kinase/activator protein-1 pathway in human peripheral blood eosinophils, causing cytokine production and release. *J Immunol* 2002; 169: 2662-9.
- Malamud V, Vaaknin A, Abramsky O, Mor M, Burgess LE, Ben-Yehudah A, *et al*. Tryptase activates peripheral blood mononuclear cells causing the synthesis and release of TNF-alpha, IL-6 and IL-1beta: possible relevance to multiple sclerosis. *J Neuroimmunol* 2003; 138: 115-22.
- He S, Walls AF. The induction of a prolonged increase in microvascular permeability by human mast cell chymase. *Eur J Pharmacol* 1998; 352: 91-8.
- Mizutani H, Schechter NM, Lazarus GS, Black RA, Kupper TS. Rapid and specific conversion of precursor interleukin 1 β (IL-1 β) to an active IL-1 species by human mast cell chymase. *J Exp Med* 1991; 174: 821-5.
- He S, Gaca MDA, McEuen AR, Walls AF. Inhibitors of chymase as mast cell stabilizing agents: the contribution of chymase in the activation of human mast cells. *J Pharmacol Exp Ther* 1999; 291: 517-23.
- He S, Peng Q, Walls AF. Potent induction of neutrophil- and eosinophil-rich infiltrate *in vivo* by human mast cell tryptase: selective enhancement of eosinophil recruitment by histamine. *J Immunol* 1997; 159: 6216-25.
- He S, Walls AF. Human mast cell chymase induces the accumulation of neutrophils, eosinophils and other inflammatory cells *in vivo*. *Br J Pharmacol* 1998; 125: 1491-500.
- Tomimori Y, Muto T, Fukami H, Saito K, Horikawa C, Tsuruoka N, *et al*. Chymase participates in chronic dermatitis by inducing eosinophil infiltration. *Lab Invest* 2002; 82: 789-94.
- Krishna MT, Chauhan A, Little L, Sampson K, Hawksworth R, Mant T, *et al*. Inhibition of mast cell tryptase by inhaled APC366 attenuates allergen-induced late-phase airway obstruction in asthma. *J Allergy Clin Immunol* 2001; 107: 1039-45.
- Sendo T, Itoh Y, Goromaru T, Sumimura T, Saito M, Aki K, *et al*. A potent tryptase inhibitor nafamostat mesilate dramatically suppressed pulmonary dysfunction induced in rats by a radiographic contrast medium. *Br J Pharmacol* 2003; 138: 959-67.
- Nakajima M, Naya N. Development of a chymase inhibitor: pharmacological characterization of a chymase inhibitor in inflamed tissue remodeling and fibrosis. *Jpn J Pharmacol* 2002; 90: 206-9.
- Takai S, Jin D, Sakaguchi M, Katayama S, Muramatsu M, Sakaguchi M, *et al*. A novel chymase inhibitor, 4-[1-([bis-(4-methyl-phenyl)-methyl]-carbamoyl)3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yloxy]-benzoic acid (BCEAB), suppressed cardiac fibrosis in cardiomyopathic hamsters. *J Pharmacol Exp Ther* 2003; 305: 17-23.
- Hoshino F, Urata H, Inoue Y, Saito Y, Yahiro E, Ideishi M, *et al*. Chymase inhibitor improves survival in hamsters with myocardial infarction. *J Cardiovasc Pharmacol* 2003; 41 Suppl 1: S11-8.
- He S, Chen P, Chen HQ. Modulation of enzymatic activity of human mast cell tryptase and chymase by proteinase inhibitors. *Acta Pharmacol Sin* 2003; 24: 923-9.
- Thomas CA, Yost Jr FJ, Snyderman R. Cellular serine proteinase induces chemotaxis by complement activation. *Nature* 1977; 269: 521-2.
- Berger P, Perng DW, Thabrew H, Compton SJ, Cairns JA, McEuen AR, *et al*. Tryptase and agonists of PAR-2 induce the proliferation of human airway smooth muscle cells. *J Appl Physiol* 2001; 91: 1372-9.
- Azougagh Oualane F, Toulemonde F, Doutremepuich F,

- Doutremepuich C. Effects of aprotinin on heparin activities and heparin neutralization with protamine. *Thromb Res* 1992; 68: 185-93.
- 25 Bettiol J, Radermecker M, Sele J, Henquet M, Cataldo D, Louis R. Airway mast-cell activation in asthmatics is associated with selective sputum eosinophilia. *Allergy* 1999; 54: 1188-93.
- 26 Carroll NG, Mutavdzic S, James AL. Increased mast cells and neutrophils in submucosal mucous glands and mucus plugging in patients with asthma. *Thorax* 2002; 57: 677-82.
- 27 Coskun N, Sindel M, Elpek GO. Mast cell density, neuronal hypertrophy and nerve growth factor expression in patients with acute appendicitis. *Folia Morphol (Warsz)* 2002; 61: 237-43.
- 28 Samoszuk M, Corwin M, Hazen SL. Effects of human mast cell tryptase and eosinophil granule proteins on the kinetics of blood clotting. *Am J Hematol* 2003; 73: 18-25.
- 29 KleinJan A, McEuen AR, Dijkstra MD, Buckley MG, Walls AF, Fokkens WJ. Basophil and eosinophil accumulation and mast cell degranulation in the nasal mucosa of patients with hay fever after local allergen provocation. *J Allergy Clin Immunol* 2000; 106: 677-86.
- 30 Macfarlane AJ, Kon OM, Smith SJ, Zeibecoglou K, Khan LN, Barata LT, *et al*. Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. *J Allergy Clin Immunol* 2000; 105: 99-107.
- 31 Oh SW, Pae CI, Lee DK, Jones F, Chiang GK, Kim HO, *et al*. Tryptase inhibition blocks airway inflammation in a mouse asthma model. *J Immunol* 2002; 168: 1992-2000.