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Biochemical Pharmacology 64 (2002) 1187–1193

Biochemical Pharmacology

Role of mast cell chymase in allergen-induced biphasic skin reaction

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Received 10 October 2001; accepted 7 March 2002

Abstract

Intradermal injection of human chymase (EC 3.4.21.39) into the mouse ear elicited an edematous skin reaction in a biphasic manner, with a transient reaction peaking at 1 hr, followed by a delayed response persisting for at least 24 hr. The kinetics of this reaction was analogous to the biphasic skin reaction induced by ascaris extract in actively sensitized mice. A similarity between the two dermatitis models was also shown by histological analysis, i.e. accumulation of inflammatory cells was observed exclusively in the later phases of the skin reaction. A chymase inhibitor, SUN-C8077 [3-(3-aminophenylsulfonyl)-7-chloroquinazorine 2,4(1*H*, 3*H*)-dione], significantly inhibited both the early- and late-phase responses of the skin reaction induced by ascaris extract. These findings suggest that chymase may play an important role in the allergen-induced biphasic skin reaction. A histamine receptor antagonist, homochlorcyclizine, inhibited the early-phase but not the late-phase of the chymase-induced skin reaction. In addition, human chymase showed chemotactic activity to human polymorphonuclear leukocytes *in vitro*. Mast cell chymase may participate in the two phases of allergic skin inflammation by two distinct mechanisms, i.e. histamine- and leukocyte-dependent mechanisms, respectively.

Keywords: Chymase; Atopic dermatitis; Biphasic reaction; Mast cell; Allergy; Leukocyte infiltration

1. Introduction

Atopic dermatitis is a common form of allergic skin disease associated with high IgE production and positive immediate-type hypersensitivity to various allergens including mite particles and molds. Following challenge with a relevant antigen, sensitized animals as well as atopic patients exhibit an acute biphasic skin reaction [1,2]. The first reaction, termed the early-phase reaction, peaks 1 hr after antigen challenge, and seems to be mediated by mast cell degranulation, since receptor antagonists for histamine, the major mediator of mast cells, reduce the reaction [3,4]. The second reaction, the late-phase reaction, appears 6–30 hr after the antigen challenge and is characterized by a marked infiltration of inflammatory cells such as eosinophils and neutrophils [5,6]. However, the mechanism that mediates the biphasic reaction is not completely understood.

Chymase (EC 3.4.21.39) is a chymotrypsin-like serine protease stored within mast cell granules that hydrolyzes a variety of substrates, e.g. angiotensin I, metalloproteases, lipoproteins, and procollagen [7,8]. Although the hydrolytic activity and localization of chymase have suggested a potential relationship to a variety of diseases [8], little is known about its precise function in physiological and pathological states. It has been reported recently that injection of chymase not only increases vascular permeability [9] but also induces leukocyte accumulation *in vivo* [10]. Chymase is also known to participate in the processing of cytokines, IL-1β [11], and SCF [12]. These findings strongly suggest that chymase may play some role in allergic inflammation.

In the present study, we investigated the possible role of mast cell chymase in the allergic biphasic skin reaction, utilizing purified chymase and a chymase inhibitor. The

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Abbreviations: IgE, immunoglobulin E; IL-1β, interleukin-1β; SCF, stem cell factor; PMN, polymorphonuclear; and fMLP, *N*-formyl-methionyl-leucyl-phenylalanine.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01265-0

data demonstrate that chymase may be involved in the two phases of the reaction by different mechanisms.

2. Materials and methods

2.1. Mice

BALB/c mice were purchased from Charles River Japan, Inc. Mast cell-deficient mice (WBB6F$_1$-*W/W^v*) [13] and their littermates (WBB6F$_1$$^{+/+}$) were obtained from Japan SLC, Inc. All animal experiments were performed according to the Guideline for Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987).

2.2. Recombinant human chymase

Human chymase was expressed in Chinese hamster ovary (CHO) cells using the secretion and activation pathway of trypsin II [14]. Briefly, cDNA for mature human chymase (79–756) [15] was amplified by polymerase chain reaction, and the product was cloned into pDE in conjunction with 23 amino acid residues of the human trypsin II precursor that include the signal sequence and cleavage site for enterokinase. The resultant plasmid was transfected into dihydrofolate reductase-deficient CHO cells (CHO*dhfr*$^-$), and the transfectant was selected as described [16]. The fusion protein in the culture supernatant of the transfectant was concentrated by means of a HiTrap Heparin column (Amersham Pharmacia Biotech) and cleaved with recombinant enterokinase (Invitrogen). The mature form of human chymase was then purified using a Heparin 5PW column (Tosoh Corp.), and the purity was assessed by polyacrylamide gel electrophoresis with a 10–20% (w/v) gradient gel (10/20 Multi gel, Daiich Pure Chemicals). The activity of recombinant human chymase was measured using 1 mM Suc-Ala-Ala-Pro-Phe-MCA (Peptide Institute) in 0.1 M Tris/HCl, pH 8.0.

2.3. Allergen-induced biphasic cutaneous reaction

The allergen-induced biphasic cutaneous reaction was elicited as described [17]. Briefly, BALB/c mice were sensitized by i.p. injection of 0.5 mL of ascaris extract (Cosmo Bio Co., Ltd.) (1.6 mg/mL) emulsified in 32 mg/mL of aluminum hydroxide adjuvant (Cosmo Bio Co., Ltd). Two weeks after the sensitization, 10 μL of ascaris extract (1.0 mg/mL) was injected intradermally into the right ear of the mice, and the edematous reaction in the ear was evaluated by weighing an ear punch biopsy (a diameter of 6 mm, Fukui Kiko Shokai). The edema was expressed as the difference in the weight of the ear punch biopsy taken from the right and the left ears of the same mouse. In the control group, the same volume of saline was injected into the sensitized mice. For histological analysis, ear samples were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4 μm, and then stained with hematoxylin and eosin.

2.4. Cutaneous reaction induced by chymase or histamine

Human recombinant chymase and histamine (Sigma–Aldrich Corp.) were dissolved in saline, and 20 μL of each solution was injected intradermally into the right ear of the mice. Doses were 2.0 and 5.0 μg/site for chymase and histamine, respectively, unless otherwise described. The same volume of saline was injected into the control mice. Evaluation of ear edema was carried out as described for the antigen-induced biphasic cutaneous reaction.

2.5. Chymase inhibitor and histamine receptor antagonist

A chymase inhibitor, SUN-C8077 [3-(3-aminophenylsulfonyl)-7-chloroquinazorine 2,4(1*H*, 3*H*)-dione], was synthesized as described previously [18]. Its IC_{50} value for human recombinant chymase was 0.36 μM. The IC_{50} value for murine skin chymase that was prepared as described in Ref. [19] was 0.18 μM. SUN-C8077 does not inhibit trypsin (bovine pancreas, Nacalai Tesque) and human neutrophil elastase (Calbiochem) even at 10 μM. Homochlorcyclizine, an antagonist for histamine receptor H1, was purchased from the Sigma–Aldrich Corp. SUN-C8077 and homochlorcyclizine were suspended in 0.5% (w/v) hydroxy propyl cellulose (Nippon Soda Co., Ltd.) and administered 30 min prior to the elicitation of dermatitis.

2.6. Isolation of PMN cells

Heparinized whole blood from normal healthy volunteers was mixed with 6% dextran (1:5, v/v), and erythrocytes were sedimented by settling for 1 hr at 37°. The clear top fraction containing PMN leukocytes was layered on a Ficoll-Paque (Pharmacia Biotech) gradient in 15-mL polypropylene tubes. Following centrifugation at 500 *g* for 30 min at 4°, the PMN cell pellets were washed twice with PBS and resuspended in RPMI 1640 supplemented with 1% (w/v) bovine serum albumin and 25 mM HEPES. Viability of the PMN leukocytes was assessed by trypan blue.

2.7. Chemotaxis assay

Chemotaxis was measured using a 48-well microchemotaxis chamber, in which the upper and the lower compartments were separated by a polycarbonate filter with a pore diameter of 5 μm (Neuroprobe). Fifty microliters of cell suspension (1×10^6 cells/mL) was placed in the upper chamber, and aliquots of either human chymase or fMLP (Sigma–Aldrich Corp.) were added in the lower chamber. The chamber was incubated for 1 hr at 37° in an atmosphere of 5% CO_2. Next, the filter was removed, and cells on the filter were fixed and stained with Hemacolor (Merck Diagnostics). The migrated cells adhered to the distal part

of the filter were quantitated by counting in three to five high-power fields for each well.

2.8. Statistical analysis

The statistical analysis was performed with Dunnett's multiple comparison test or Student's *t*-test using Super-ANOVA (Abacus Concepts) or Statview (SAS Institute Inc.), respectively. A P value of less than 0.05 was considered significant.

3. Results

3.1. Induction of the biphasic skin reaction by human chymase

As shown in Fig. 1A, an intradermal injection of human chymase elicited a biphasic edematous skin reaction in mice. The first reaction was transient and peaked 1 hr after the chymase injection, whereas the second reaction reached a maximal level at 6 hr and lasted for at least 24 hr. The kinetics of this reaction was analogous to the biphasic skin reaction induced by ascaris extract in actively sensitized mice (Fig. 1B). When histamine, the major chemical mediator of mast cells, was injected similarly, a skin reaction was also induced immediately after the injection, but it disappeared completely within 20 hr (Fig. 1C), in contrast to the chymase- and allergen-induced reactions. These results suggest that chymase may be involved in both the early- and late-phase of the reaction, while histamine has a role only in the early-phase of the reaction.

3.2. Histological analysis of the chymase-induced skin reaction

The chymase-induced skin reaction was examined further by histological analysis. Dermal thickening was apparent 1 hr following the intradermal injection of chymase (Fig. 2D), when compared to the hematoxylin and eosin-stained sections of normal uninjected and saline-injected mice (Fig. 2A and B). Cell infiltration was detectable but not remarkable at 1 hr (Fig. 2D), but it became extensive 24 hr after the chymase injection (Fig. 2E). There was minimal, if any, cellular infiltration 24 hr after saline injection (Fig. 2C). Counting of the cells revealed that

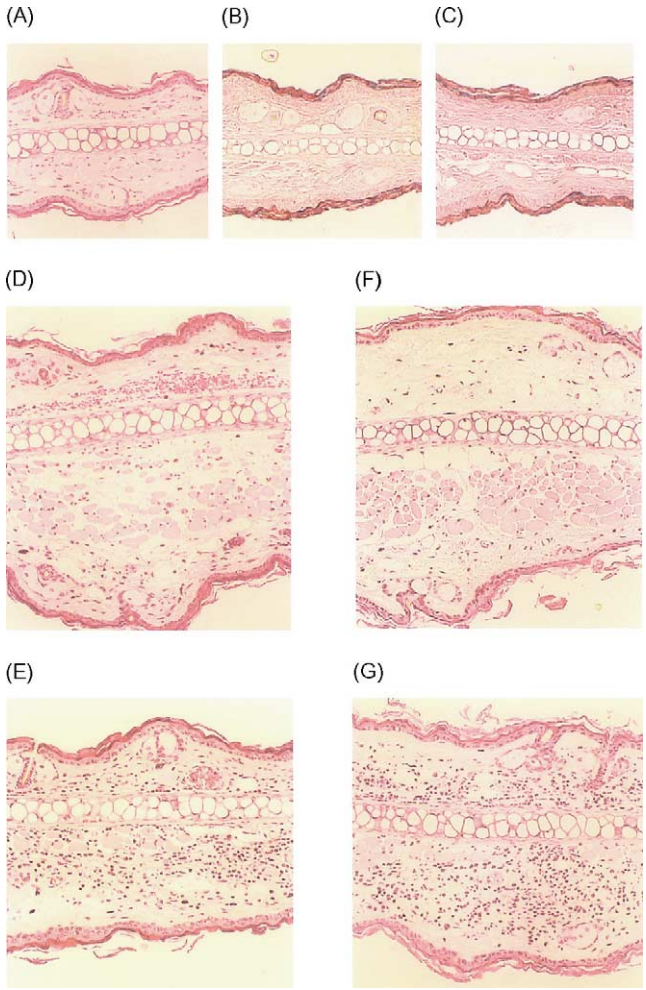

Fig. 2. Histological analysis of skin lesions in chymase- and antigen-induced biphasic reactions. Chymase-induced (D and E) and antigen-induced (F and G) cutaneous reaction was elicited as described in Section 2, and ears were excised at 1 hr (B, D, and F) or at 24 hr (C, E, and G), sectioned, and stained with hematoxylin and eosin. As a control, saline was injected intradermally into the ears of the mice instead of chymase (B and C). Panel A shows the ear section of a normal uninjected mouse. Original magnification, 50×.

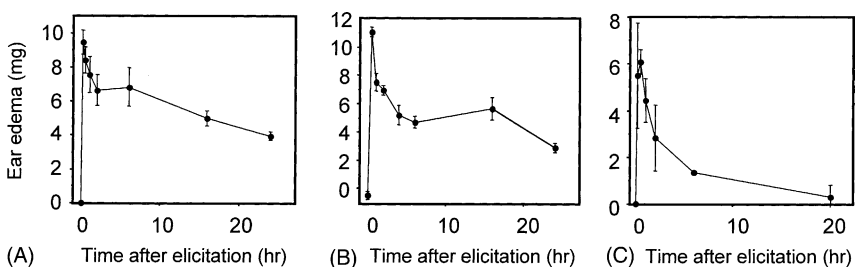

Fig. 1. Skin reaction induced by chymase, allergen, and histamine. Chymase-induced (A) and histamine-induced (C) skin reactions were elicited by intradermal injection of 2.0 μg of human chymase and 5.0 μg of histamine, respectively, in the ears of normal BALB/c mice. An antigen-induced biphasic reaction (B) was elicited by intradermal injection of ascaris extract into the ears of BALB/c mice that had been sensitized with the same antigen 2 weeks before the challenge. Ear edema was evaluated as described in Section 2. Data are means ± SEM; N = 3 (C) or 4 (A and B).

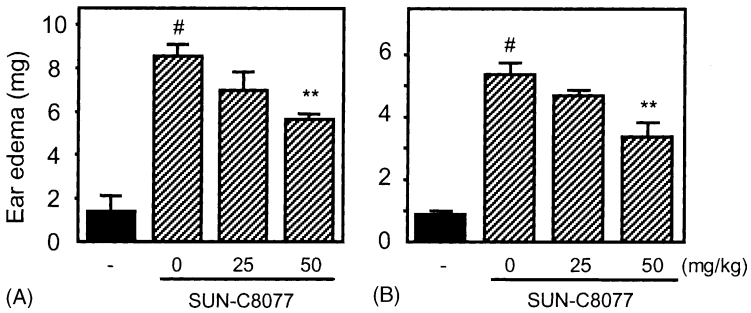

Fig. 3. Effect of a chymase inhibitor on the antigen-induced biphasic skin reaction. An antigen-induced cutaneous reaction was elicited by intradermal injection of ascaris extract into the ears of BALB/c mice that had been sensitized with the same antigen 2 weeks before the challenge. The chymase inhibitor SUN-C8077 was administered i.p. 30 min before the antigen challenge. Ear edema was evaluated at 1 hr (A) or at 16 hr (B) as described in Section 2. In the control group, the same volume of saline was injected instead of ascaris extract to the sensitized mice. Hatched bars, challenged with ascaris extract; solid bars, injected with saline. Data are means ± SEM; N = 7. Key: (#) $P < 0.01$ as compared with the control group (Student's *t*-test); and (**) $P < 0.01$ as compared with the treated group without the inhibitor (0 mg/kg) (Dunnett's test).

chymase injection increases neutrophils, eosinophils, and mononuclear cells by ~135-, ~8.9-, and 3.7-fold, respectively, at 24 hr, which is consistent with the data reported by He and Walls [10]. Similar histopathological changes were observed in the biphasic reaction induced by ascaris extract (Fig. 2F and G). Namely, marked cell accumulation was seen only in the lesion at 24 hr, although the increase in the thickness of the dermis was more prominent at 1 hr.

3.3. Effect of chymase inhibitor on the biphasic reaction

To elucidate further the importance of chymase in the biphasic skin response, the effect of a nonpeptide inhibitor for chymase, SUN-C8077, was examined in an allergen-induced skin reaction model. As shown in Fig. 3, injection of SUN-C8077 inhibited both the early- and late-phase reactions in this model. The inhibitory effect of SUN-C8077 was dose-dependent, and statistical significance was shown at 50 mg/kg for both reactions. This result supports the idea that chymase plays a significant role in the two phases of the biphasic skin reaction.

3.4. Induction of ear edema by recombinant human chymase in mast cell-deficient mice

Rat chymase has been shown to stimulate mast cell degranulation *in vitro* [20]. Thus, the chymase-induced skin reaction might be mediated by mast cell degranulation. To examine this point, the ability of human chymase to induce a skin reaction was examined in mast cell-deficient mice (WBB6F$_1$-*W/W^v*). As shown in Fig. 4, there was no difference in the chymase-induced skin reaction between WBB6F$_1$-*W/W^v* mice and their littermates, WBB6F$_1$$^{+/+}$.

3.5. Effect of a histamine receptor antagonist on chymase-induced ear edema

The effect of homochlorcyclizine, a histamine receptor antagonist, was examined on the chymase-induced skin reaction. As shown in Fig. 5A, the early-phase reaction of the chymase-induced dermatitis was inhibited significantly by the injection of 30 mg/kg of homochlorcyclizine. Homochlorcyclizine did not affect ear thickness in the saline-injected mice (data not shown), showing that it

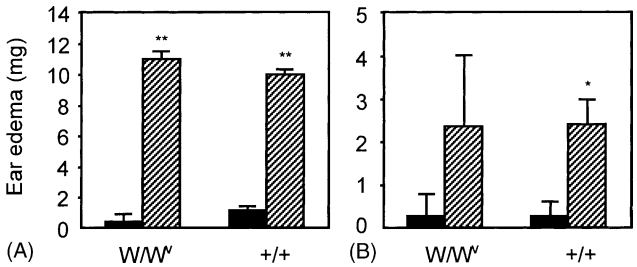

Fig. 4. Chymase-induced skin reactions in mast cell-deficient mice (WBB6F$_1$-*W/W^v*) and their littermates (WBB6F$_1$$^{+/+}$). Cutaneous reactions were induced by the intradermal injection of 2.0 μg of human chymase into the ears of WBB6F$_1$-*W/W^v* and WBB6F$_1$$^{+/+}$ mice. Ear edema was evaluated at 1 (A) or 16 (B) hr as described in Section 2. In the control group, the same volume of saline was injected instead of chymase. Hatched bars, chymase-injected; solid bars, saline-injected. Data are means ± SEM; N = 3 (B) or 4 (A). Key: (*) $P < 0.05$; and (**) $P < 0.01$ as compared with the control group (Student's *t*-test).

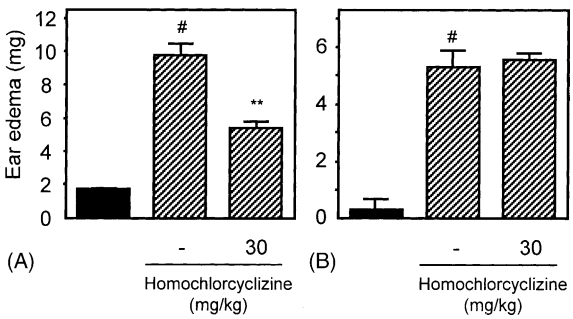

Fig. 5. Effect of homochlorcyclizine on chymase-induced skin reaction. Cutaneous reactions were elicited by human chymase, and ear edema was evaluated at 1 hr (A) (N = 7) or at 16 hr (B) (N = 6) as described in Section 2. In the control groups, the same volume of saline was injected instead of chymase (N = 3). Homochlorcyclizine or vehicle was administered p.o. 30 min before the chymase injection. Hatched bars, chymase-injected; solid bars, saline-injected. Data are means ± SEM. Key: (#) $P < 0.01$ as compared with the control group (Student's *t*-test); and (**) $P < 0.01$ as compared with the vehicle group (Dunnett's test).

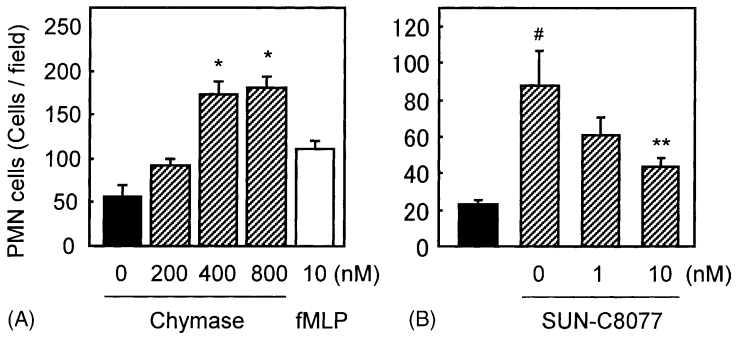

Fig. 6. Chemotactic effect of human chymase on human PMN leukocytes. Isolation of human PMN cells and the chemotactic assay were carried out as described in Section 2. Data are expressed as migrated cells per high power field (means ± SEM). (A) Effect of human chymase on the migration of PMN cells. Solid bar, control; hatched bars, human chymase; open bar, fMLP. Key: (*) $P < 0.05$ as compared with the control (without chymase) (N = 3) (Dunnett's test). (B) Effect of the chymase inhibitor SUN-C8077 on the chemotactic activity of human chymase for PMN cells. Solid bar, control; hatched bars, human chymase (400 nM) with or without SUN-C8077. Key: (#) $P < 0.01$ as compared with the control group (Student's *t*-test); and (**) $P < 0.01$ as compared with the control (chymase alone) (N = 5) (Dunnett's test).

inhibits the response to chymase. In contrast, the ear swelling in the late-phase was not affected by the injection (Fig. 5B). A similar result was obtained when mice were administered another histamine antagonist, diphenhydramine (data not shown). These results suggest that the two phases of the chymase-induced reaction may be induced by different mechanisms: one mediated at least in part by histamine, the other unrelated to the action of histamine.

3.6. Chemotactic activity of human chymase for human leukocytes

To clarify the mechanism by which chymase develops the late-phase reaction, the ability of human chymase to promote migration of human PMN leukocytes was investigated *in vitro*. As shown in Fig. 6A, human chymase stimulated the migration of PMN cells in a concentration-dependent manner, consistent with the data reported by Tani *et al*. [21]. The chemotactic activity of 200 nM human chymase for PMN leukocytes was likely equivalent to that of 10 nM fMLP in this study. Heat-inactivated chymase did not stimulate PMN cell migration (data not shown), indicating that the chemotactic activity of chymase is dependent upon enzymatic activity and unrelated to endotoxin contamination. The chymase inhibitor SUN-C8077 inhibited chymase-stimulated PMN leukocyte migration (Fig. 6B). These findings suggest that chymase participates in the late-phase skin reaction by acting as a chemoattractant for the recruitment of inflammatory cells.

4. Discussion

An intradermal injection of human chymase caused a biphasic skin reaction (Fig. 1A), reminiscent of the reaction generally observed in allergic subjects [2] and the antigen-induced skin reaction in sensitized mice (Fig. 1B). Recombinant murine chymase MMCP-4 also produced a similar skin reaction (data not shown), showing that the effect of human chymase is not due to its antigenicity. A similarity between chymase- and antigen-induced reactions was also shown by histological analysis (Fig. 2), i.e. inflammatory cells infiltrated in the late-phase reactions but not in the earlier reactions in both models. These data suggest that chymase may participate in allergic biphasic skin reactions, since chymase would be released by activated mast cells. This idea is strongly supported by the result that the chymase inhibitor SUN-C8077 inhibited the antigen-induced biphasic skin reaction (Fig. 3).

The second peaks of the chymase- and antigen-induced edematous reactions were miniscule, raising the notion that the reactions are not biphasic; the first reactions might be just prolonged and last for many hours. However, cellular infiltration started to occur at 1 hr and continued to increase for at least 24 hr (Fig. 2) in chymase- and antigen-induced skin reactions, whereas the edematous reactions showed a rapid onset (Fig. 1A and B). These results suggest that the cellular infiltration may be associated with the delayed edematous reactions but not the earlier reactions. Thus, both the chymase- and antigen-induced skin edematous reactions are probably due to more than one type of reaction, and are, therefore, appropriately termed biphasic.

Histamine receptor antagonists inhibit the early-phase but not the late-phase of the IgE-mediated biphasic cutaneous reaction [4]. Similarly, homochlorcyclizine, a histamine antagonist, inhibited only the early-phase of the chymase-induced reaction (Fig. 5). These findings indicate that histamine is involved, at least in part, in the early-phase reactions of the two dermatitis models. However, chymase elicited an early-phase as well as a late-phase reaction in mast-cell-deficient mice (Fig. 4), in contrast to the IgE-mediated skin reaction that was not observed in the mutant mice [22]. These findings indicate that the early-phase of allergic skin reactions is mediated by mast cell-derived histamine, but that induced by chymase is mast cell histamine-independent. Histamine-producing cells other than mast cells [23] may take part in the early-phase of

the chymase-induced inflammation. Further studies are needed to clarify how chymase induces histamine release in these cells.

The inhibition of the chymase-induced early-phase reaction by the histamine antagonist was partial (Fig. 5A), and, therefore, it is possible that another mechanism is involved. It has been reported that infusion of rat chymase (RMCP-II) into the vasculature of the *ex vivo* perfused jejunum induces translocation of macromolecules to the gut lumen [24]. Injection of human chymase into the skin of guinea pigs provokes an increase in microvascular leakage within 20 min, a reaction that vanishes within 6 hr [9]. In addition, rat chymase (RMCP-II) promotes rapid and reversible permeability to macromolecules in a pulmonary epithelial cell line [25,26]. Thus, the ability of chymase to increase vascular permeability may also play a part in the chymase-induced early-phase reaction.

The mechanism involved in the late-phase reaction of allergic dermatitis is poorly understood, although accumulation of inflammatory cells appears to contribute to its development [3,4]. The data of Tani *et al*. [21] and that of the present study (Fig. 6) clearly indicate that human chymase is a chemoattractant for human PMN leukocytes, suggesting that the chymase-induced and possibly allergen-induced late-phase reactions may be mediated, at least in part, by the chemotactic activity of chymase. It was shown recently that cathepsin G, an enzyme closely related to chymase, may act directly on platelets by interacting with protease-activated receptor-4 (PAR-4) [27]. The chemotactic activity of chymase may also be mediated through such a receptor(s) on leukocytes.

Numerous studies have shown the importance of mast cells in the development of a late-phase reaction. For example, isolated mast cell granules have been shown to induce a late-phase reaction in addition to an immediate response [28]. It is also known that intradermal injection of a stimulant for mast cell degranulation, such as substance P or compound 48/80, causes a biphasic reaction in the skin [28,29]. In addition, mast cell-deficient mice do not exert IgE-mediated early-phase as well as late-phase reactions [22]. These findings are in agreement with our data showing that chymase stored in and secreted from mast cells may be important in the late-phase reaction in allergic skin inflammation.

The late-phase reaction induced by chymase in *W/W*^{v} mice and their littermates was less than that of BALB/c mice, while the early-phase reactions were similar in the three strains. These results suggest that skin reactions induced by chymase may be distinct between BALB/c and C57BL/6 mice, since the extent and mechanism of allergic response seem to be different among mouse strains [30,31]. A comparison of the cell types accumulated in the dermis by chymase injection of BALB/c and C57BL/6 mice may clarify not only the mechanism of the chymase-induced skin reaction but also the difference in the response to chymase among the strains.

In conclusion, the intradermal injection of exogenous chymase evoked a biphasic cutaneous reaction that resembled that of an allergen-induced skin reaction. Our data demonstrate that mast cell chymase may play an important role in the pathogenesis of allergic dermatitis and may be a novel target for the therapy of this skin disorder.

Acknowledgments

We are grateful to Drs. Tatsuro Nishihara and Toshihiro Nakanishi for their helpful advice and valuable discussion during this study.

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