Tryptase Activates the Mitogen-Activated Protein Kinase/Activator Protein-1 Pathway in Human Peripheral Blood Eosinophils, Causing Cytokine Production and Release

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Tryptase Activates the Mitogen-Activated Protein Kinase/Activator Protein-1 Pathway in Human Peripheral Blood Eosinophils, Causing Cytokine Production and Release

Vladislav Temkin,* Boris Kantor,2† Vivian Weg,2‡ Mor-Li Hartman,* and Francesca Levi-Schaffer3*‡

We have previously shown that mast cells enhance eosinophil survival and activation. In this study we further characterized mast cell activity toward eosinophils. Sonicate of both rat peritoneal mast cells and the human mast cell line 1 (HMC-1) induced a concentration-dependent IL-6 and IL-8 release from human peripheral blood eosinophils (ELISA). HMC-1-induced IL-6 release was significantly reduced by the tryptase inhibitors GW-45 and GW-58 (90 and 87%, respectively, at an optimal concentration) but not by anti-stem cell factor, anti-TNF-α, or anti-IFN-γ neutralizing Abs or by the antihistamine drugs pyrilamine and cetirizine. In a manner similar to HMC-1, human recombinant tryptase induced the expression of mRNA for IL-8 (RT-PCR) and caused IL-8 release from the eosinophils. Addition of cycloheximide, actinomycin D, dexamethasone, PD 98059, curcumin, or SB 202190 completely inhibited the tryptase-induced IL-6 and IL-8 release. In contrast, cyclosporin A had no effect on tryptase-induced IL-8 release. Tryptase caused phosphorylation of extracellular signal-regulated kinases 1 and 2, c-Jun N-terminal kinases 1 and 2, and p38 (Western blot). Tryptase also induced the translocation of c-Jun from the cytosol to the nucleus (confocal microscopy) and enhanced AP-1 binding activity to the DNA (EMSA). Eosinophils were found to express proteinase-activated receptor 2 (FACS). When eosinophils were incubated with tryptase in the presence of anti-proteinase-activated receptor 2 antagonist Abs a significant decrease in the IL-6 and IL-8 release occurred. In summary, we have demonstrated that the preformed mast cell mediator tryptase induces cytokine production and release in human peripheral blood eosinophils by the mitogen-activated protein kinase/AP-1 pathway. The Journal of Immunology, 2002, 169: 2662–2669.

D uring allergic inflammatory reactions mast cells and eosinophils can interact when the eosinophils have infiltrated into the tissues in the late phase response or when the inflammation becomes chronic (1, 2). Eosinophil cross-talk is important in regulating the severity, duration, and outcome of the allergic response. Within this framework we demonstrated that human peripheral blood eosinophils induce IgE-desensitized rat peritoneal mast cells to release histamine (3). We previously reported that rat peritoneal mast cells enhance eosinophil survival in vitro by the induction of GM-CSF autocrine production (4, 5). Mast cell-derived TNF-α was found to be the preformed mediator predominantly responsible for this effect (4). Another mast cell preformed mediator tryptase, present in virtually all mast cell types (6), was found to cause the release of eosinophil cationic protein from eosinophils (7) and to act as their chemoattractant (8) as well as to induce IL-8 production by human epithelial cells and neutrophils (9, 10). Tryptase is a serine protease with trypsin-like activity that cleaves several proteins and peptides such as fibrinogen, kininogen, and vasoactive intestinal peptide (11, 12). The cleavage of proteinase-activated receptor (PAR)1-2 by tryptase induces receptor-mediated signaling in human vascular endothelial cells (13), leading to inositol 1,4,5-triphosphate production. Furthermore, it has recently been found that human peripheral blood eosinophils from normal and mild asthmatics express PAR-2 (14).

Tissue eosinophilia is a constant feature of allergic inflammation and correlates with high tissue levels of eosinophil granule proteins and eosinophil-derived cytokines (15, 16). Two cytokines with proinflammatory properties, IL-6 and IL-8, are produced by eosinophils (16, 17). IL-8 is a potent granulocyte chemoattractant and can serve as a marker of tissue eosinophilia (18, 19). IL-6, in turn, regulates acute phase protein production (20), B cell proliferation, and final differentiation (21).

The production of these cytokines is regulated by some transcription factors, such as NFAT, NF-κB, and AP-1 (22–24). The latter is actually a family of transcription factors and is composed of members of the Jun, Fos, and activating transcription factor (ATF) subfamily, which are sequestered in the cytoplasm (25). Upon activation by Jun N-terminal mitogen-activated protein kinase (MAPK), AP-1 is phosphorylated and translocates to the nucleus where it regulates the activity of many genes involved in the inflammatory response (26). The initial enhancement of cytosolic calcium by tryptase, as a result of inositol 1,4,5-triphosphate production (13), might be required for the involvement of MAPKs that, in turn, activate AP-1. Therefore, the MAPK/AP-1 pathway could play an important role in mediating mast cell-derived tryptase effects in allergic inflammation.

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2 B.K. and V.W. contributed equally to this work.
3 Address correspondence and reprint requests to Dr. Francesca Levi-Schaffer, Department of Pharmacology, School of Pharmacy, Faculty of Medicine, Hebrew University of Jerusalem, P.O. Box 12065, Jerusalem 91120, Israel. E-mail address: fls@cc.huji.ac.il.

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0022-1767/02/$02.00

4 Abbreviations used in this paper: PAR, proteinase-activated receptor; HMC-1, human mast cell line 1; SCF, stem cell factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/ERK kinase; EM, enriched medium; ATF, activating transcription factor; PI, propidium iodide.
In the present work we evaluated the ability of mast cells to induce IL-6 and IL-8 production and release by eosinophils. We also investigated the role of tryptase and its signal pathway in this event.

Materials and Methods

Eosinophil purification

Eosinophils were purified according to a previously published procedure (27) from the peripheral blood of mildly atopic volunteers (20–44 years old, with blood eosinophilia ranging from 4 to 10%) according to the guidelines established by the Hadassah-Hebrew University Human Experimentation Helsinki Committee. None of the volunteers had been taking any medication during the previous 3 mo. Venous blood, collected in heparinized syringes, was subjected to dextran (Pharmacia Biotech, Uppsala, Sweden) and mononuclear leukocytes were centrifuged on Ficoll-Paque (density = 1.077, Sigma-Aldrich, St. Louis, MO) for 25 min at 700 × g. Neutrophils and T cells in the granulocyte-enriched pellet were tagged with micromagnetic beads to anti-CD16 and anti-CD3 Abs, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany). Eosinophils were collected at a purity of >99%, as assessed by Kimura’s staining, and at a viability of >99%, as assessed by trypan blue (Sigma-Aldrich) exclusion test.

Mast cells

Rat mast cells were isolated by a sterile procedure from the peritoneal cavity of “Sabra” rats, an outbred strain of the Hebrew University. Rat peritoneal lavage was performed with Tyrode buffer containing 0.1% gelatin (TG buffer) and mast cells were purified on 22.5% metrizamide (Sigma-Aldrich) in TG buffer. Mast cells were collected at a purity of 97–100%, as assessed by toluidine blue staining (Sigma-Aldrich), and at a viability of >99% (28).

Human mast cell line 1 (HMC-1; a kind gift from Dr. J. Butterfield, Mayo Clinic, Rochester, MN) was cultured in Iscove’s medium supplemented with 10% v/v iron-enriched calf serum, 1.2 mM α-monothioglycolate, 200 U/ml penicillin, 200 μg/ml streptomycin, and 2 mM gentamicin (Biological Industries, Beit Haemek, Israel) at 37°C. Cells were passaged every 5 days (29).

To obtain mast cell sonicate, isolated rat peritoneal mast cells or HMC-1 were resuspended in medium containing RPMI 1640, 200 U/ml penicillin, 200 μg/ml streptomycin, 2 mM gentamicin, 0.1 mM nonessential amino acids, and 5% v/v heat-inactivated FCS (enriched medium (EM); Biolog-Xa vs San Diego, CA), 105 cultures were incubated with one of the following additions in 100 μl EM: cytokines, factor Xa, thrombin, curcumin (Sigma-Aldrich), or 1 μM pyrilamine, 0.1 mM actinomycin D, 1 μM cyclosporin A (Sigma-Aldrich), be-700,000yond 1.4 pg/ml for IL-8 and IL-6, respectively. The limit of assay sensitivity is 10 and 1.4 pg/ml for IL-8 and IL-6, respectively.

RNA isolation and RT-PCR amplification

Total RNA was purified using the commercial reagent Tri-Reagent (Sigma-Aldrich) based on the acid guanidinium-phthiocerane RNA extrac- tion technique (31). The first-strand cDNA synthesis was cata- lyzed by SuperScript II RNase H Reverse Transcriptase and oli-go(dt)12–18 primer (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. The generated complementary DNA was amplified using 1.25 U of Taq DNA polymerase and dNTP mixture and IL-8 (5′-ATGACTCTCAAGGTCGCGTCTGC and 3′-TCTCAGCGCTCTTC AAAAATCCTT) primers (Clontech Laboratories, Palo Alto, CA) in the presence of 10% glycerol (Sigma-Aldrich) as a specificity enhancer. Prim- ers for GDPDH, used as a control to test the efficiency of cDNA synthesis, were 5′-ACCAAGCTTCCATGACCTACGTC and 3′-CATGTTGGGCG ATTAGGTCCAC (Clontech Laboratories). The specificity of the prim- ers was confirmed by the manufacturer. DNA templates for IL-8 provided by the manufacturer were used as a positive control. The products, amplified by thermocycler, were electrophoresed on 1.8% agarose gel stained with ethidium bromide (Sigma-Aldrich) and photographed under UV light.

Western blot analysis and EMSA

Protein isolation, electrophoresis, and blotting using specific anti-active Abs (for c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK)1/2, Promega) were essentially as described (32). Lysis buffer (50 mM Tris, 25 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM Na3VO4 containing a protease inhibitor mixture (Sigma-Aldrich) was added to the samples. Cell debris was removed from lysates by centrifugation (15,300 × g for 10 min) after vortex mixing and sonication (10 s bursts at intervals of 10 s using a W-380 sonicator (Heat Systems Ultrasounds) at 50% duty cycles, output 5). All procedures were performed on ice or at 4°C. Protein concentrations were assessed by modified Bradford assay before loading the samples. Samples were analyzed on 10% SDS-PAGE.

The gel was electrotransferred (90 min) to nitrocellulose filter paper blocked in PBS containing 5% BSA and 0.1% Brij (Sigma-Aldrich). The filter was then incubated with rabbit anti-human MAPKs (2.5 μg/ml; AB-255 NA polyclonal Abs; R&D Systems) overnight at 4°C, washed in PBS/ Brij, and incubated with secondary peroxidase-conjugated immunoprobe donkey anti-rabbit Abs (1/5000; Pierce, Rockford, IL) and finally with the reagents of the chemiluminescence system ECL detection kit (Amer sham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.).

EMSA was performed as follows on nuclear extract of eosinophils pre- pared as described (33). The AP-1 oligonucleotide (5′-GTCTAGAAT GACTCGGCCC-3′, underlined oligonucleotides are representative of AP-1-recognized recognition motif) was labeled in a 20-μl reaction mixture containing 30 ng of the double-stranded oligonucleotide as listed below, 1 μl of the DNA polymerase, and 5 μl of 10 μC/ml [α-32P]dCTP (Amersham). Labeled oligonucleotides (30–100 pg/20/μl104 cpm) were incu- bated at 30°C for 30 min, with the nuclear extract (10 μg protein) in a buffer containing 12 mM HEPES (pH 7.2), 60 mM KCl, 0.6 mM Na3EDTA, 0.6 mM DTT, 5 mM MgCl2, and 1 μg poly(dIdC). The reaction mixtures were electrophoresed on 4% polyacrylamide gels and photo- graphed under UV light.

Confocal laser microscopy

Cytospins of eosinophils (1 × 105 cells) incubated with either 50 nM tryptase or 2.5 ng/ml PMA for 10 min were prepared (3 min at 1000 × g). Cells were fixed in 3.8% paraformaldehyde (Sigma-Aldrich) for 10 min at

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Mast cell number (x10³)

Cytokine (pg/ml)

Histamine (pg/ml)

Confocal laser microscopy

Statistical analysis

Results

Characterization of the mast cell mediator(s) that induces IL-8 release: the role of tryptase

Table I. Tryptase inhibitors reduce HMC-1-induced IL-8 release

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IL-8 (pg/ml)</th>
<th>HMC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM</td>
<td>22.6 ± 3.4</td>
<td>78.7 ± 5.8</td>
</tr>
<tr>
<td>GW-455378A (1 μM)</td>
<td>19.8 ± 3.1</td>
<td>69.8 ± 6.1</td>
</tr>
<tr>
<td>GW-585361A (1 μM)</td>
<td>22.0 ± 2.1</td>
<td>72.6 ± 5.1</td>
</tr>
<tr>
<td>GW-455378A (10 μM)</td>
<td>23.2 ± 1.4</td>
<td>46.7 ± 3.7*</td>
</tr>
<tr>
<td>GW-585361A (10 μM)</td>
<td>22.6 ± 1.4</td>
<td>54.7 ± 4.1*</td>
</tr>
<tr>
<td>GW-455378A (20 μM)</td>
<td>20.1 ± 0.7</td>
<td>27.6 ± 4.2*</td>
</tr>
<tr>
<td>GW-585361A (20 μM)</td>
<td>18.9 ± 3.1</td>
<td>32.4 ± 3.6*</td>
</tr>
<tr>
<td>GW-455378A (50 μM)</td>
<td>19.7 ± 4.2</td>
<td>29.7 ± 3.1</td>
</tr>
<tr>
<td>GW-585361A (50 μM)</td>
<td>23.1 ± 2.6</td>
<td>33.5 ± 2.6</td>
</tr>
</tbody>
</table>

* The tryptase inhibitors were preincubated with HMC-1 sonicate (mast cell:eosinophil ratio of 1:1) or with EM for 2 h in ice and then added to eosinophil culture for an additional 18 h. At this time point supernatants were evaluated for IL-8 presence. Values are means ± SEM of six experiments.

* p < 0.001.
To evaluate the potential role of SCF, TNF-α, and IFN-γ on IL-8 release, HMC-1 sonicate was preincubated with different concentrations of neutralizing Abs for these cytokines and then added to the eosinophil cultures. None of these treatments influenced HMC-1-induced IL-8 release (69.7 ± 7.1 pg/ml IL-8 from eosinophils incubated with HMC-1 sonicate alone vs 64.1 ± 4.3 pg/ml from eosinophils incubated with anti-SCF, 73.7 ± 3.4 eosinophils with anti-TNFα, and 74.8 ± 5.6 pg/ml from eosinophils incubated with anti-IFN-γ).

In contrast, when HMC-1 sonicate was preincubated with the specific tryptase inhibitors GW-455378A or GW-585361A and then added to the eosinophil cultures, inhibition of IL-8 release was observed (see Table I). At an optimal concentration (20 μg/ml), these compounds decreased HMC-1-induced IL-8 release by 89.8 ± 7.6 and 82.5 ± 4.4%, respectively (n = 4; p < 0.001). These data clearly indicate the involvement of tryptase in IL-8 release from human eosinophils.

**Tryptase induces IL-6 and IL-8 production and release by eosinophils**

To investigate the direct role of tryptase in IL-8 production by human eosinophils, the cells were incubated with human recombinant tryptase. As shown in Fig. 2, tryptase induced IL-8 release in a concentration-dependent manner with a maximal release of 81.25 ± 5.2 pg/ml IL-8 at a concentration of 50 ng/ml (n = 6; p < 0.001). Higher tryptase concentrations did not further increase IL-8 release. The tryptase-induced IL-8 release was inhibited to a similar extent by the two tryptase inhibitors GW-455378A and GW-585361A, i.e., by 92.9 and 89.5%, respectively (20 μM).

**Table II. Effect of inhibitors of mRNA and protein synthesis on tryptase-induced IL-6 and IL-8 release from eosinophils**

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>IL-8</th>
<th>HMC-1</th>
<th>IL-6</th>
<th>IL-8</th>
<th>Tryptase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EM</td>
<td>HMC-1</td>
<td>Tryptase</td>
<td>EM</td>
<td>HMC-1</td>
<td>Tryptase</td>
</tr>
<tr>
<td>EM</td>
<td>6.7 ± 3.2</td>
<td>12.6 ± 4.2</td>
<td>46.7 ± 5.7</td>
<td>64.3 ± 6.9</td>
<td>41.3 ± 4.2</td>
<td>53.2 ± 4.3</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>3.1 ± 1.9</td>
<td>5.2 ± 2.6</td>
<td>5.9 ± 3.9*</td>
<td>4.3 ± 4.1*</td>
<td>4.6 ± 3.0*</td>
<td>5.1 ± 2.0*</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>2.6 ± 1.2</td>
<td>3.2 ± 1.7</td>
<td>4.8 ± 2.2*</td>
<td>2.1 ± 1.2*</td>
<td>3.1 ± 2.4*</td>
<td>5.3 ± 3.2*</td>
</tr>
</tbody>
</table>

*Eosinophils (1 × 10^7/200 μl) were incubated with HMC-1 (mast cell: eosinophil ratio of 1:1) or with human recombinant skin I-β-trypsinogen (50 nM) or with EM in the presence or absence of actinomycin D (10 μM) or cycloheximide (0.1 μg/ml). After 18 h of culture, eosinophil supernatants were assessed for IL-6 or IL-8 presence. Values are means ± SEM of six experiments.

*p < 0.001.
18 h with tryptase (50 ng/ml) and with 35 μM PD 98059 (mitogen-activated protein/ERK kinase (MEK) inhibitor), 25 μM curcumin (JNK inhibitor), or 10 μM SB 202190 (p38 inhibitor). As shown in Fig. 4, these three compounds partly inhibited the tryptase-induced IL-8 release. When added together they completely abrogated IL-6 and IL-8 release from both tryptase- and medium-incubated eosinophils. In contrast, cyclosporin A had no effect on tryptase-induced IL-8 release but slightly enhanced IL-8 release in medium alone (p < 0.01; n = 4).

Direct tryptase-induced activation of MAPKs was assessed by Western blot using specific anti-active JNK1/2, p38, and ERK1/2 Abs. Tryptase caused phosphorylation of ERK1/2, JNK1/2, and p38 MAPKs in the eosinophils after 3 min of incubation, indicating the specific activation of these MAPKs (Fig. 5).

Interestingly, tryptase also induced IL-8 release in human fetal lung fibroblasts (745.3 ± 43.1 pg/ml in 50 ng/ml tryptase vs 481.7 ± 36.2 pg/ml in medium alone; n = 3; p < 0.001). Similarly to the eosinophils, the tryptase-induced release of IL-8 from the fibroblasts was abrogated by the MAPK inhibitors PD 98059 (35 μM), curcumin (25 μM), and SB 202190 (10 μM) added together (346.8 ± 21.8 pg/ml in tryptase-incubated cultures and 311.4 ± 41.7 in medium alone; n = 3; p < 0.001).

**Tryptase activates AP-1: confocal microscopy and EMSA analysis**

To investigate whether AP-1 transcription factor mediates the tryptase-induced IL-8 release, eosinophils were preincubated with dexamethasone (10^-6 M) for 45 min and then cultured with tryptase for 18 h. Dexamethasone completely abrogated HMC-1- (from 46.7 ± 5.7 to 4.1 ± 1.2 pg/ml; n = 4; p < 0.001) and tryptase-induced (from 41.3 ± 4.2 to 4.7 ± 3.6 pg/ml; n = 4; p < 0.001) IL-6 release. Similarly, IL-8 release induced by HMC-1 and tryptase was inhibited by dexamethasone, i.e., for HMC-1 from 64.3 ± 6.9 to 1.7 ± 1 pg/ml (n = 4; p < 0.09) and for tryptase from 53.2 ± 4.3 to 4.7 ± 3.6 pg/ml (n = 4; p < 0.001).

Confocal microscopy analysis of eosinophils incubated for 10 min with either tryptase or PMA (as a control) showed the translocation of the c-Jun from the cytoplasm to the nucleus (Fig. 6). In fact, at this time point yellow regions corresponding to overlapping green (FITC anti-p65 Abs) and red images (PI) indicated c-Jun localization in the nucleus (Fig. 6, green (FITC anti-p65 Abs) and red images (PI) indicated c-Jun localization in the nucleus (Fig. 6). In contrast, freshly isolated eosinophils and eosinophils incubated with EM for 10 min displayed a green cytoplasmic staining, indicating the presence of c-Jun in the cytoplasm (Fig. 6, A and B).

Epicardial smooth muscle cells (EM) were isolated from the myocardium of a 4-month-old fetus. These cells were then incubated with 50 ng/ml tryptase (lane 2) or with EM alone (lane 1) or with EM alone (lane 3) or with EM alone (lane 2) were prepared. Protein levels were assessed by the Bradford assay. PC12 cells cultured with sorbitol (lane 5) or medium alone (lane 4) for 10 min were used as control. Blots with transferred samples were exposed to rabbit anti-human active ERK or anti-active JNK or anti-active p38 Abs. Each image is representative of three different experiments.

**PAR-2 mediates tryptase-induced cytokine release from the eosinophils**

PAR-2 was found to mediate tryptase effects in many cell types (36). Recently, PAR-2 was found to be expressed by peripheral blood eosinophils obtained from normal healthy and mild asthmatic patients (14). In our study, flow cytometric analyses of human peripheral blood eosinophils incubated with anti-PAR-2 Abs showed a strong staining for this receptor (Fig. 8). No difference in fluorescence was observed in intact eosinophils and in permeabilized eosinophils (data not shown). When added to the eosinophils 30 min before tryptase, anti-PAR-2 Abs, but not control Abs, reduced the tryptase-induced IL-6 and IL-8 release in a concentration-dependent manner. Maximal inhibitory effect was observed at an Ab concentration of 1 μg/ml. At this concentration, tryptase-induced IL-6 and IL-8 release was reduced by 54.7 and 59.4%, respectively (Table III; n = 3; p < 0.001).

**Discussion**

Mast cell-eosinophil interactions can take place during allergic inflammatory reactions, especially once the eosinophils have infiltrated into the inflamed tissues. We have previously reported that rat peritoneal mast cells enhance eosinophil survival through their activation to produce and release the autocrine survival cytokine GM-CSF (4, 5). The mediator responsible for this event is mast cell preformed TNF-α. In addition, mast cells cause the release of eosinophil peroxidase and eosinophil adherence to plastic wells. By a proteomics analysis we have recently detected that [35S]methionine-labeled eosinophils are induced by HMC-1 soniccate to produce a large number of proteins. This shows that mast cells are a potent stimulus for eosinophils and that human peripheral blood eosinophils are highly biosynthetically active cells (35).

In this study we have further investigated the influence of mast cells on eosinophil activation by assessing whether mast cells could induce IL-6 and IL-8 production and release from eosinophils. Both these proinflammatory
cytokines are produced by eosinophils in vitro (16, 17) and participate in the allergic inflammatory responses (15, 20, 21).

Rat peritoneal mast cell and the human mast cell line HMC-1 were used as a source of mast cells. Rat peritoneal mast cells are a readily available source of a large number of mast cells with connective tissue phenotype similar in many aspects to human skin mast cells. Even though HMC-1 lacks FcεRI it is still a very useful tool for in vitro studies on a large number of human mast cells of the mucosal phenotype. In fact, it contains mostly β-tryptase and only traces of, if any, chymase (29). In addition, HMC-1, like rat peritoneal mast cells, contains other mediators, such as preformed histamine and heparin, TNF-α, and SCF (29, 32, 34).

In our study, both types of mast cell sonicate caused IL-6 and IL-8 production and release by human peripheral blood eosinophils in a concentration-dependent fashion. This would indicate that both connective tissue and mucosal mast cells can interact with eosinophils and that this interaction is conducted by a common mediator seemingly specific for mast cells because PBMC sonicate was ineffective.

The next aim of our study was to determine this mast cell mediator(s). Among mast cell preformed mediators, histamine, TNF-α, SCF, IFN-γ, and tryptase might be good candidates to cause IL-6 and IL-8 release. Histamine has been shown to stimulate eosinophil superoxide production (37) and, together with PGD₂, to increase their cytosolic calcium (38). In addition, histamine induces IL-6 and IL-8 production in different cell types (39–41). As we and others have previously reported, TNF-α causes IL-8 and GM-CSF production by human eosinophils via NF-κB activation (5, 42). SCF through c-Kit receptor induces very late Ag-4-mediated eosinophil adhesion to endothelial cells (43). IFN-γ enhances eosinophil survival and eosinophil-mediated cytotoxicity (44), and we recently found that IFN-γ preformed in HMC-1 partially enhances eosinophil survival (V. Temkin and F. Levi-Schaffer, unpublished data). Tryptase, in turn, induces IL-8 production and release by many cell types, such as endothelial cells and neutrophils (9, 10). Moreover, tryptase causes eosinophil degranulation (7) and contraction of bronchial smooth muscle (45).
Tryptase inhibitors suppress not only the early phase but also the late phase of allergic inflammation, indicating tryptase influences on infiltrated inflammatory cells and on cytokine/chemokine production (46, 47).

In our study, cimetidine (H1 antagonist), pyrilamine (H2 antagonist), anti-SCF, anti-TNF-α, and anti-IFN-γ neutralizing Abs had no influence on HMC-1-induced IL-8 release. In contrast, the tryptase-specific inhibitors GW-455378A and GW-585361A inhibited the HMC-1-induced IL-8 release. There are at least two tryptase isoforms, α and β, that share a sequence identity of ~98%. The β-isofrom appears to be activated intracellularly and stored in the secretory granules of most human mast cells, including the HMC-1 cell line (29, 48). We found that both the human recombinant skin I-β tryptase and the HMC-1 sonicate induce both IL-8 release and its mRNA production.

Human peripheral blood eosinophils can both store preformed IL-6 and IL-8 (49, 50) and several other cytokines in their secondary granules and synthesize them in response to specific stimuli (16, 50). Therefore, we assessed whether tryptase could induce not only the release of preformed IL-6 and IL-8 but also their mRNA and the protein syntheses. Eosinophils were incubated with tryptase or HMC-1 in the presence of cycloheximide, a protein synthesis inhibitor, and the protein syntheses. Eosinophils were cultured for an additional 18 h and at this time point supernatants were assessed for cytokine release. Values are means ± SEM of three experiments.

* EM tryptase EM tryptase

<table>
<thead>
<tr>
<th>IL-6 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
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<tbody>
<tr>
<td>5.8 ± 2.4</td>
<td>44.2 ± 5.6</td>
</tr>
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<td>4.9 ± 3.1</td>
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<td>5.1 ± 3.1</td>
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<td>7.9 ± 1.9</td>
<td>5.6 ± 3.1</td>
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<tr>
<td>28.4 ± 4.3</td>
<td>5.7 ± 3.6</td>
</tr>
<tr>
<td>58.4 ± 6.1</td>
<td>54.5 ± 5.7</td>
</tr>
</tbody>
</table>

Eosinophils were incubated with rabbit anti-human PAR-2 polyclonal antibodies (20 µg/ml) for 30 min at 37°C prior to the addition of tryptase (50 nM) or EM. Eosinophils were cultured for an additional 18 h and at this time point supernatants were assessed for cytokine release. Values are means ± SEM of three experiments.

*p < 0.001.

In summary, we have provided evidence that tryptase activates human eosinophils through PAR-2 binding/activation. Fibroblasts have been shown to express PAR-2s that mediate tryptase-induced fibroblast proliferation (54), and we presently found that tryptase causes their IL-8 production. Recently, PAR-2 was found to be expressed by peripheral blood eosinophils obtained from normal healthy and mildly asthmatic patients (14). In our study, PAR-2 was also shown to be present on the plasma membrane of eosinophils isolated from the peripheral blood of atop dermatitis and rhinitis patients. Furthermore, Abs that are antagonists to PAR-2 partly reduced tryptase-induced IL-6 and IL-8 release in a concentration-dependent manner indicating that, at least in part, PAR-2 mediates these tryptase effects on the eosinophils. PAR-2 belongs to the thrombin receptor family. It is a G protein-coupled receptor activated with proteolytic cleavage of the extracellular domain (55). Thrombin cleaves PAR-1, PAR-3, and PAR-4, while PAR-2 is preferentially cleaved by trypsin and tryptase (55, 56) and cleaves, to a lesser extent, PAR-1 (54). Interestingly, mRNA for PAR-3 was recently found to be expressed by eosinophils (14). The finding that PAR-2 mediates tryptase-induced IL-8 production by eosinophils and by fibroblasts is in line with the observations from other cell types such as epithelial cells (9), neutrophils (10), and endothelial cells (57).

It is interesting to point out that in PAR-2 knockout mice the early phase of allergy is not affected, while the onset of inflammation is delayed (58). This could be explained by the fact that various mast cell mediators released at the early phase, such as tryptase, are responsible for inducing cytokine release from eosinophils and other inflammatory cells in the late phase.

In summary, we have provided evidence that tryptase activates the MAPK/AP-1 pathway. This is probably mediated by the cleaving of PAR-2s, which results in the production of IL-6 and IL-8 by human peripheral blood eosinophils.
We believe that these findings further elucidate mast cell-eosi-
nophil interactions and underline the important role of tryptase in this cross-talk as well as in the overall allergic reaction.

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