

Rapid publications

Human eosinophils are activated by
cysteine proteases and release
inflammatory mediatorsSatoshi Miike, MD, and Hirohito Kita, MD *Rochester, Minn*

Background: Recent studies suggest that serine proteases are involved in various biological responses through activation of protease-activated receptors (PARs). However, the functions of other proteases, such as cysteine proteases, are poorly understood and need elucidation.

Objective: We examined the effects of an authentic cysteine protease, papain, and a protease derived from the mite allergen, Der f 1, on functions of human eosinophils.

Methods: Purified eosinophils were incubated with papain or Der f 1. Eosinophil activation was monitored by superoxide anion generation and by degranulation. Intracellular signaling pathways were investigated through use of pharmacologic approaches.

Results: We found that papain potently induces human eosinophils to degranulate and to produce superoxide anion. A cysteine protease inhibitor, E-64, abolished the stimulatory effects of papain, which suggests that the protease activity of papain is necessary to trigger eosinophil responses. The eosinophil's response to papain was enhanced by IL-5 and mediated by activation of the phosphatidylinositol 3-kinase/Akt pathway. Interestingly, whereas a serine protease, trypsin, activated eosinophils through PAR2, the effects of papain were not inhibited by an antibody to PAR2, which suggests another novel mechanism for the eosinophils' response to cysteine proteases. It is likely that these observations are clinically important, because eosinophils were activated by a natural cysteine protease allergen, Der f 1, and released granule proteins.

Conclusion: Human eosinophils are probably equipped with machineries that recognize and respond to cysteine proteases, such as those found at allergic inflammation sites; the result is active release of proinflammatory mediators. (*J Allergy Clin Immunol* 2003;111:704-13.)

Key words: Eosinophils, allergy, inflammation

Eosinophils likely play important roles in the pathophysiology of bronchial asthma and other allergic diseases.¹ In such diseases, T cells and other inflammatory cells release cytokines and chemokines that induce migra-

Abbreviations used

EDN:	Eosinophil-derived neurotoxin
E-64:	<i>Trans</i> -epoxysuccinyl-L-leucylamido (4-guanidino)-butane
Fmoc-2-Abz-OH:	<i>N</i> - α -Fmoc-2-aminobenzoic acid
Fmoc-Lys(Dnp):	<i>N</i> - α -Fmoc-Lys-dinitrophenol
PAF:	Platelet-activating factor
PAR:	Protease-activated receptor
PI3K:	Phosphatidylinositol 3-kinase
PKC:	Protein kinase C
PMA:	Phorbol-12-myristate-13-acetate
RIA:	Radioimmunoassay
Suc-Leu-Leu-Val-Tyr-AMC:	Succinyl-leucyl-leucyl-valyl-tyrosine-7-amino-4-methylcoumarin
Z-Phe-Arg-AMC:	Benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin

tion of eosinophils from blood into the affected tissues.² At these inflammation sites, eosinophils are exposed to a milieu of inflammatory mediators that can activate eosinophils and induce the local release of lipid metabolites,³ superoxide anion,⁴ and toxic cationic granule proteins.⁵ Mediators able to activate eosinophils include immunoglobulins (eg, secretory IgA),⁵ lipids (eg, platelet activating factor [PAF]),⁶ cytokines (eg, IL-5 and GM-CSF),⁷ and complement fragments (eg, C5a).⁸ Furthermore, proteases derived from allergens (eg, mites, fungi, pollens)⁹⁻¹² or released by inflammatory cells (eg, mast cells)^{13,14} are abundant at the sites of allergic inflammation.¹⁵ However, the precise mechanisms to explain eosinophil activation in human diseases, such as allergic diseases and bronchial asthma, are not well understood.

Proteases, especially serine proteases, activate various hematologic and interstitial cells through a family of G protein-coupled protease-activated receptors (PARs) and induce production of a number of pro-inflammatory mediators.¹⁶ Four members of this receptor family have been cloned. For example, a serine protease, thrombin, activates PAR1 expressed on platelets and induces production of serotonin and chemokines.¹⁷ Another serine protease, trypsin, stimulates PAR2 and induces production of neuropeptides from spinal afferent neurons; it also induces edema formation in rat paw *in vivo*.¹⁸ In addition, serine proteases derived from mite allergens such as

From the Departments of Medicine (Division of Allergic Diseases) and Immunology, Mayo Clinic Rochester.

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Reprint requests: Hirohito Kita, MD, Departments of Medicine and Immunology, Mayo Clinic and Foundation, Rochester, MN 55905.

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Der p 3 and Der p 9 induce cytokine production by airway epithelial cells that is partially mediated by PAR2.¹⁹ We showed previously that trypsin activates human eosinophils through PAR2 and induces eosinophils to release their products.²⁰

Despite this information on serine proteases, our knowledge is extremely limited for other proteases, such as cysteine proteases, with respect to their similar biological capacities. Indeed, the receptors or binding molecules for proteases other than serine proteases have not been identified. However, a cysteine protease, Der p 1, cleaves CD23 on B cells²¹ and CD25 on T cells,²² enhancing the IgE-mediated response²¹ and reducing the T_H1-cytokine response,²² respectively. Thus, certain allergen-derived cysteine proteases might have immunomodulatory properties that could facilitate a T_H2-type immune response. Because both allergens and eosinophils are localized abundantly at allergic inflammation sites, we aimed to investigate (a) whether cysteine proteases induce human eosinophils to become activated and release their products and (b) whether allergen-derived cysteine proteinases contribute to exacerbation of allergic diseases through their pro-inflammatory properties. We addressed these questions by using an authentic cysteine protease, papain, and an allergen-derived cysteine protease, Der f 1. We also studied the molecular mechanisms involved in the eosinophil's response to these cysteine proteases.

METHODS

Materials

Papain (P3125), trypsin (T8658), *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino)-butane (E-64; E3132), and wortmannin (W1628) were purchased from Sigma-Aldrich (St Louis, Mo). Der f 1 (#290451), a purified natural mite allergen, was purchased from Seikagaku Corporation (Tokyo, Japan). IL-5 was a generous gift from the Schering-Plough Research Institute (Kenilworth, NJ). Phorbol 12-myristate 13-acetate (PMA; #524400) and 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-[(1H-indol-3-yl)-maleimide] (GF109203X; #203290) were purchased from Calbiochem (San Diego, Calif). A PhosphoPlus Akt antibody kit (#9270) was purchased from Cell Signaling Technology (Beverly, Mass). Polyclonal antibody to human PAR2 was generated by immunizing rabbits with receptor peptides, and specific antibody was isolated from antiserum by affinity chromatography with the immunization peptide used to generate the PAR2 antibody.²⁰ The 2 reagents *N*- α -Fmoc-2-aminobenzoic acid (Fmoc-2-Abz-OH; #23478) and *N*- α -Fmoc-Lys-dinitrophenol (Fmoc-Lys(Dnp)) resin (#23856) were purchased from Anaspec Inc (San Jose, Calif) to synthesize the FRET peptide (Abz-Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys-Dnp)²³ in the Mayo Protein Core Facility. Benzoyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin (Z-Phe-Arg-AMC) and succinyl-leucyl-leucyl-valyl-tyrosine-7-amino-4-methylcoumarin (Suc-Leu-Leu-Val-Tyr-AMC) were purchased from Bachem (Torrance, Calif); the Z-Phe-Arg-AMC and the Suc-Leu-Leu-Val-Tyr-AMC were used to measure the cysteine protease activity in papain²⁴ and Der f 1,²⁵ respectively.

Eosinophil isolation

Human eosinophils were purified from normal individuals or patients with mild allergy by Percoll density gradient centrifugation and magnetic cell sorting through use of MACS anti-CD16

microbeads.²⁶ Briefly, after peripheral blood was overlaid on an isotonic Percoll solution (1.084 g/mL; Sigma), the blood was centrifuged at 1000g for 30 minutes at 4°C. Mononuclear cells at the interface were removed, and erythrocytes in the sediment were lysed by 2 cycles of hypotonic water lysis. Isolated granulocytes were washed twice in PIPES buffer (25 mmol/L PIPES, 50 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L NaOH, 5.4 mmol/L glucose, pH 7.4) containing 1% bovine calf serum (HyClone Laboratories, Logan, Utah). Cells were then incubated with an equal volume of anti-CD16 mAb MACS microbeads for 60 minutes at 4°C with occasional gentle mixing. After that, cells were loaded onto the separation column positioned in the MACS magnetic field. Cells were eluted 3 times with 5 mL PIPES buffer with 1% bovine calf serum. The purity of eosinophils was >98%. The contaminating cells were neutrophils, and no mononuclear cells or basophils were present. Purified eosinophils were used immediately for experiments.

Eosinophil superoxide production and degranulation assays

To monitor eosinophil function in response to proteases, we used superoxide anion generation by and degranulation of human eosinophils. Superoxide generation was measured by superoxide dismutase-inhibitable reduction of cytochrome *c*, as previously described⁷ (with slight modifications). In brief, freshly isolated eosinophils were washed and resuspended in HBSS with 25 mmol/L HEPES and 0.01% gelatin (Sigma) and 100 μ mol/L cytochrome *c* at 5×10^5 cells/mL. Cell suspension (100 μ L) was dispensed onto the wells of 96-well tissue culture plates, followed either by 100 μ L papain (0.01–10 μ mol/L), purified natural Der f 1 (1 μ mol/L), trypsin (5 nmol/L), or PAF (100 nmol/L) as a positive control or by medium alone. Der f 1 was preincubated with 5 mmol/L cysteine at 37°C for 30 minutes to restore protease activity. Immediately after the addition of stimuli, the reaction wells were measured for absorbance at 550 nm in a microplate autoreader (Thermomax, Molecular Devices, Menlo Park, Calif); this was followed by repeated readings. Between absorbance measurements, the plate was incubated at 37°C. Each reaction was carried out in duplicate and compared with an identical control reaction that contained 20 μ g/mL superoxide dismutase (Sigma). Superoxide anion generation was calculated with an extinction coefficient of 21.1×10^3 cm⁻¹ mol⁻¹ L for reduced cytochrome *c* at 550 nm and was expressed in nanomoles of superoxide produced per 10⁵ cells.

After incubation and repeated measurements of superoxide production at 37°C and 5% CO₂ for 4 hours, cell-free supernatants from 96-well tissue culture plates were collected, and stored at -20°C until assayed for eosinophil degranulation. To quantitate eosinophil degranulation, the concentrations of eosinophil-derived neurotoxin (EDN) in the sample supernatants were measured by specific radioimmunoassay (RIA), as previously described.^{5,7} The RIA is a double-antibody competition assay in which radioiodinated EDN, rabbit anti-EDN antibody, and burro antirabbit IgG are used.⁵ The sensitivity of the EDN RIA was 2.0 ng/mL. All assays were performed in duplicate.

To examine the role of protease activity in eosinophil activation, papain was preincubated with or without 1 μ mol/L cysteine protease inhibitor, E-64, for 30 minutes at 37°C before addition to eosinophils. To examine the involvement of PAR2 in the eosinophil response to proteases, eosinophils were preincubated with or without 1.0 μ g/mL PAR2 antibody or rabbit IgG for 60 minutes at 37°C before stimulation with agonists. To examine the mechanisms of eosinophil activation, eosinophils were pretreated with wortmannin (10 nmol/L) or GF109203X (2.5 μ mol/L) for 15 minutes at 37°C before stimulation. Finally, to examine the effects of IL-5 on

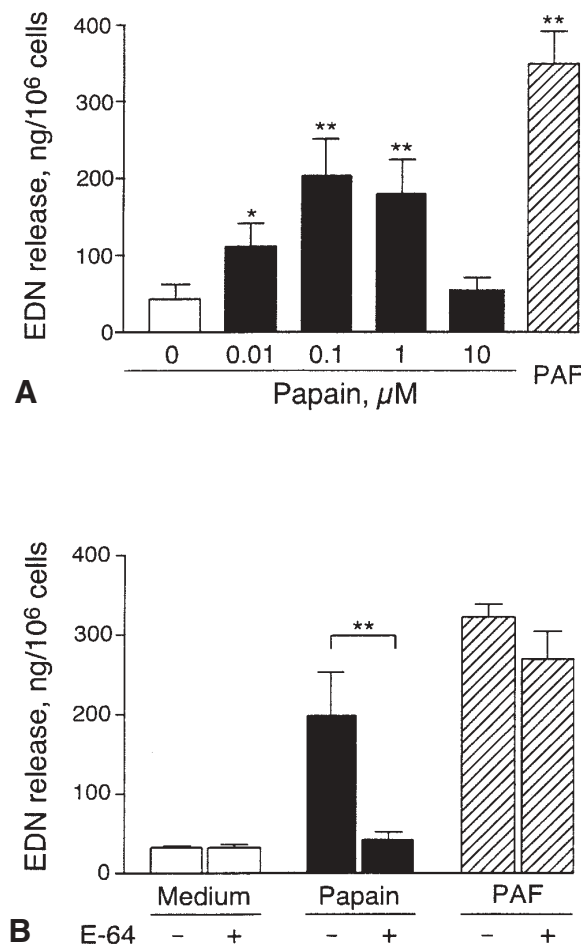


FIG 1. Eosinophil degranulation induced by papain. **A**, Eosinophils were stimulated with papain (0.01 to 10 $\mu\text{mol/L}$), PAF (100 nmol/L), or medium alone for 4 hours at 37°C. After incubation, cell-free supernatants were collected and the concentrations of EDN were measured by RIA, as described in the Methods section. Data are presented as means $\pm$ SDs from 5 separate experiments using different eosinophil preparations. *Significant difference ($P < .05$) in comparison with EDN release without papain. **Significant difference ($P < .01$) in comparison with EDN release without papain. **B**, To test pretreatment of papain with E-64, medium alone, papain (100 nmol/L), and 100 nmol/L PAF were preincubated without (minus) or with (plus) E-64 (1 $\mu\text{mol/L}$) for 30 minutes at 37°C. Eosinophils were then stimulated with treated medium or agonists for 4 hours at 37°C. Data are presented as means $\pm$ SDs from 4 separate experiments using different eosinophil preparations. **Significant difference ($P < .01$) between papain with and papain without E-64 pretreatment.

papain-induced eosinophil degranulation, cells were preincubated with or without IL-5 (10 pg/mL) for 60 minutes at 37°C before stimulation with papain.

Preparation of eosinophil lysates and immunoblotting

Eosinophils were preincubated with 10 ng/mL IL-5 for 60 minutes, stimulated with 1 $\mu\text{mol/L}$ papain for an indicated period, and then lysed with SDS-PAGE sample buffer (20% glycerol, 2% SDS, 125 mmol/L Tris-HCl [pH 6.8], 100 mmol/L dithiothreitol, and

0.15% bromophenol blue). Samples were boiled for 5 minutes and clarified by centrifugation at 3000g at 4°C for 15 minutes with a microcentrifuge. Ten μg of cell lysate per lane was applied to SDS-PAGE and transferred electrically onto polyvinylidene difluoride transfer membranes. The membranes were blocked with PBS containing 0.1% Tween-20 and 5% skim milk, probed with the anti-phospho-Akt or anti-Akt antibodies, and visualized with an enhanced chemiluminescence system (Amersham, Piscataway, NJ). Densitometric analysis was carried out with National Institutes of Health image software. The increased densities were compared with the density of samples preincubated with IL-5 but not stimulated with papain; they are reported as ratios.

Quantitation of the protease activity

The enzymatic activities of proteases were measured through use of fluorescence substrates. The substrates, Z-Phe-Arg-AMC ($\lambda_{\text{ex}} = 360 \text{ nm}$; $\lambda_{\text{em}} = 460 \text{ nm}$)²⁴ and Suc-Leu-Leu-Val-Tyr-AMC ($\lambda_{\text{ex}} = 360 \text{ nm}$; $\lambda_{\text{em}} = 460 \text{ nm}$)²⁵ were used to measure the activities of papain and Der f 1, respectively. After samples were preincubated with or without 5 mmol/L cysteine at 37°C for 30 minutes, 6 to 8 different concentrations of substrate were added to the buffers: 100 mmol/L phosphate buffer (pH 6.0) or HBSS with 25 mmol/L HEPES and 0.01% gelatin (pH 7.4). The kinetics of enzymatic activity was measured spectrofluorometrically on a CytoFluor Multi-Well Plate Reader (PerSeptive Biosystems, Framingham, Mass). Kinetic constants for the substrates were obtained through use of the Michaelis-Menten equation, performed with GraphPad Prism (San Diego, Calif) software.

To investigate whether trypsin or papain cleaves PAR2 protein on eosinophils, we tested the effect of these proteases on the cleavage of a substrate corresponding to the extracellular domain of the PAR2 cleavage site.^{23,27} The ability of a protease to cleave the substrate Abz-Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys-Dnp was assessed by the method used for the quantitation of the cysteine protease activity following the procedure, as described previously.²³

Statistical analysis

Data from at least 3 experiments using different eosinophil preparations from different donors were summarized and presented as means $\pm$ SDs or means $\pm$ SEMs, as noted in the figure legends. Statistical analyses were performed through use of the Student *t* test and the Mann-Whitney test; *P* values less than .05 were considered significant.

RESULTS

A cysteine protease, papain, induces degranulation from eosinophils

To address whether cysteine proteases activate and induce production of inflammatory mediators from eosinophils, we incubated isolated human eosinophils with a cysteine protease, papain, and measured the EDN released. Fig 1, A shows that papain induced eosinophil degranulation in a concentration-dependent manner. At 0.1 $\mu\text{mol/L}$, papain induced $206.8 \pm 45.6 \text{ ng EDN release per } 10^6 \text{ cells}$ (mean $\pm$ SD; $n = 5$), which corresponds to 58% of the EDN release induced by 100 nmol/L PAF, one of the most potent secretagogues for eosinophils.^{6,7} Interestingly, a higher concentration of papain (ie, 10 $\mu\text{mol/L}$) induced less EDN release than 0.1 $\mu\text{mol/L}$ papain, which suggests that eosinophil degranulation induced by papain is probably not due to nonspecific cytotoxic effects.

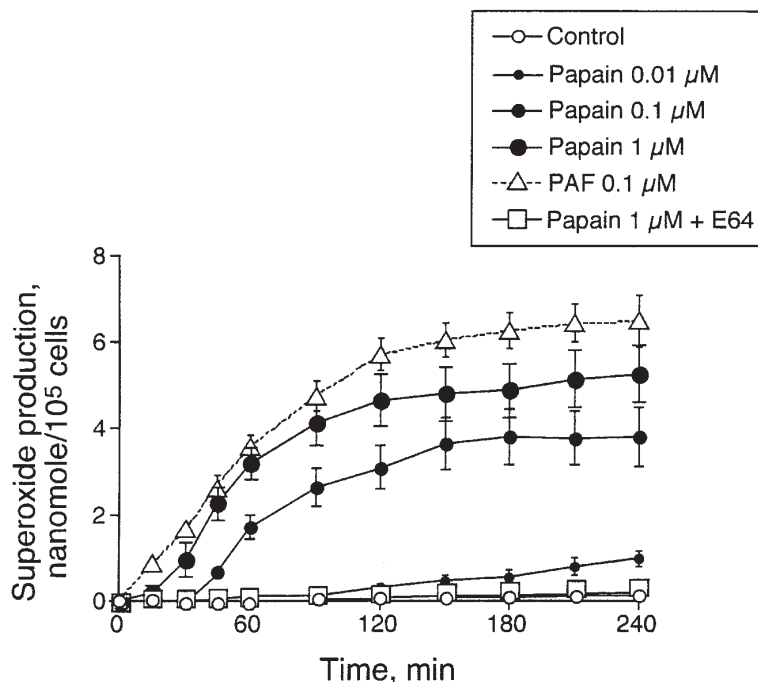


FIG 2. Eosinophil superoxide anion production induced by papain. Eosinophils were stimulated with papain (0.01 to 1 μmol/L), PAF (100 nmol/L), or medium alone (*control*) for up to 4 hours at 37°C. Eosinophils were also stimulated with 1 μmol/L papain that had been pretreated with a cysteine protease inhibitor, E-64 (1 μmol/L), for 30 minutes at 37°C. The kinetics of superoxide production was measured through use of superoxide dismutase-inhibitable reduction of cytochrome *c*, as described in the Methods section. Data are presented as means ± SDs from 5 experiments using different eosinophil donors.

To address whether this papain-induced eosinophil degranulation is due to papain's specific protease activity or to any unknown contaminants, we examined the effects of a cysteine protease inhibitor. In preliminary experiments with a protease activity assay, it was found that incubating 0.1 μmol/L papain with a potent cysteine protease inhibitor, 1 μmol/L E-64, for 30 minutes at 37°C abolishes its protease activity (data not shown). Fig 1, *B* shows that after 0.1 μmol/L papain was pretreated with 1 μmol/L E-64 it induced significantly less EDN release from eosinophils than untreated papain ($P < .001$; $n = 4$). Indeed, pretreatment of papain with E-64 nearly abolished EDN release (mean inhibition, 81%; $n = 4$), which suggests that the cysteine protease activity of papain is necessary to induce eosinophil degranulation. In contrast, EDN release after stimulation with 100 nmol/L PAF was essentially not affected by pretreatment of PAF with E-64 (mean inhibition, 13%; $n = 4$), which suggests that E-64 does not adversely affect the eosinophils' ability to respond to other agonists or to release granule proteins.

Papain induces superoxide anion production from human eosinophils

We used superoxide anion production to investigate whether eosinophil activation induced by papain leads to other effector functions of human eosinophils. Fig 2 shows that eosinophils incubated with 0.01 μmol/L papain produced superoxide; production increased in a concentration-dependent manner up to 1 μmol/L. Super-

oxide production induced by 0.1 μmol/L papain was approximately 60% of that induced by 100 nmol/L PAF, which is consistent with the degranulation results (Fig 1, *A*). Furthermore, pretreatment of 1 μmol/L papain with 1 μmol/L E-64 completely abolished superoxide production ($P < .001$; $n = 5$), which suggests that the cysteine protease activity of papain is involved in eosinophil activation and superoxide production. PAF-induced superoxide production was not affected by E-64 (data not shown).

PAR2 and protein kinase C are not involved in papain-induced eosinophil activation, but phosphatidylinositol 3-kinase is

The foregoing results suggest that papain induces degranulation and superoxide production by eosinophils, which depend on its cysteine protease activity. We next investigated the receptors or binding molecules for papain that mediate eosinophil activation and functional response(s). Serine proteases, such as thrombin and trypsin, cleave a family of G protein-coupled PARs, thus activating the receptor.¹⁶ To our knowledge, PAR2 is the only member of the known PAR family of molecules that involves the effector functions of human eosinophils in response to serine proteases.²⁰ Therefore, we examined the PAR2 response to papain with a blocking antibody to PAR2. Fig 3, *A* shows that preincubation of eosinophils with anti-PAR2 significantly inhibited trypsin-induced superoxide anion production by 41% ($P < .05$; $n = 5$),

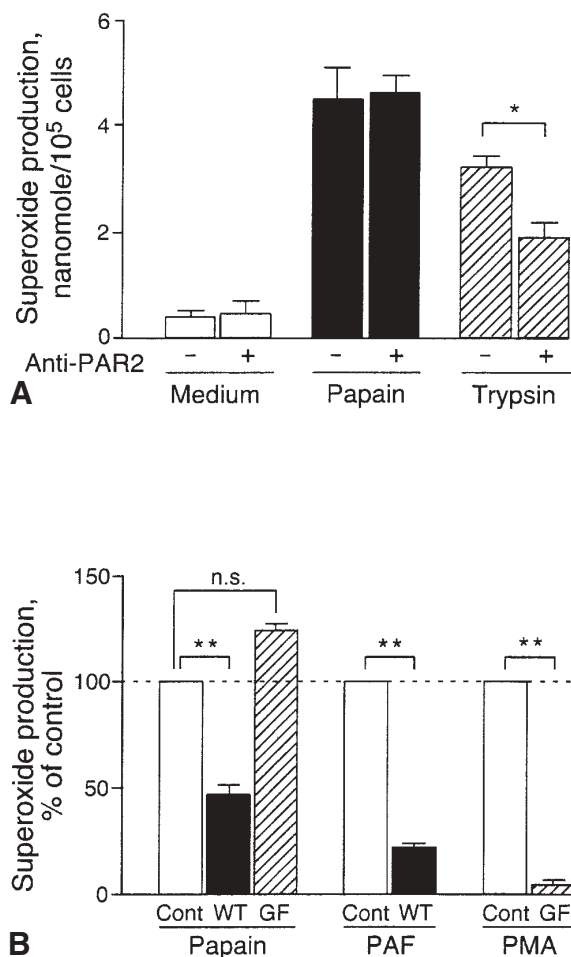


FIG 3. Effects of anti-PAR2 antibody, the PI3K inhibitor wortmannin, and the PKC inhibitor GF109203X on papain-induced eosinophil superoxide anion production. **A**, Eosinophils were preincubated with 1 μ g/mL anti-PAR2 antibody (*plus*) or control rabbit IgG (*minus*) for 15 minutes at room temperature and stimulated with medium alone, 1 μ mol/L papain, or 10 nmol/L trypsin for 4 hours at 37°C. Superoxide anion production was measured as described in the legend to Fig 2. Data are presented as means $\pm$ SEMs from 5 experiments using different eosinophil donors. *Significant difference between control rabbit IgG and anti-PAR2 antibody ($P < .05$). **B**, Eosinophils were preincubated with medium alone (*Cont*), 10 nmol/L wortmannin (*WT*), or 2.5 μ mol/L GF109203X (*GF*) for 15 minutes at 37°C and then stimulated with papain, PAF, or PMA. Data were normalized to values without inhibitors as 100%. Data are presented as means $\pm$ SEMs from 5 experiments using different eosinophil donors. **Significant difference ($P < .01$) between cells with and cells without inhibitors. *n.s.*, Not significant.

which suggests that PAR2 is partially involved in the eosinophil's response to a serine protease, trypsin. In contrast, superoxide anion production induced by papain was not affected by anti-PAR2. In addition, we used a synthetic peptide corresponding to a sequence of the PAR2 cleavage sites to examine whether papain cleaves PAR2 protein. As a positive control, trypsin cleaved the PAR2-sequence peptide (Abz-Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys-Dnp) at pH 7.4 ($K_m = 23.2 \mu\text{mol/L}$; $k_{cat} =$

118/s; $k_{cat}/K_m = 5.1/\mu\text{mol/L} \cdot \text{s}$); however, papain at 10 nmol/L to 1 $\mu\text{mol/L}$ did not cleave the peptide under the same conditions. In contrast, papain at pH 7.4 effectively cleaved a peptide for cysteine protease, Z-Phe-Arg-AMC ($K_m = 80.0 \mu\text{mol/L}$; $k_{cat} = 3.0/\text{s}$; $k_{cat}/K_m = 3.8 \times 10^{-2}/\mu\text{mol/L} \cdot \text{s}$), which suggests that the papain we used is enzymatically active. Thus, papain-induced activation of eosinophils is probably not mediated by a known protease receptor for eosinophils—namely, PAR2.

We next used pharmacologic inhibitors to investigate whether superoxide production by eosinophils in response to papain is mediated by cellular activation and through the induction of one or more intracellular signaling pathways. Although eosinophil effector functions involve several signaling pathways, we focused on the phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) pathways because they are involved in the eosinophils' functional response to PAF.^{28,29} Fig 3, *B* shows that preincubation of eosinophils with a PI3K inhibitor, wortmannin, inhibited PAF-induced superoxide production by 79%, which is consistent with previous observations.³⁰ Under identical conditions, wortmannin significantly inhibited papain-induced superoxide production by 53% ($P < .01$; $n = 5$), which suggests that the PI3K pathway is also involved in papain-induced eosinophil activation. GF109203X is a specific inhibitor of PKC³¹ and blocks the PMA-induced cellular responses³² as well as PAF-induced functions of eosinophils.³³ Fig 3, *B* shows that GF109203X inhibited PMA-induced superoxide production by eosinophils; however, GF109203X did not inhibit papain-induced superoxide production, which suggests that the PKC pathway is not involved in papain-induced eosinophil activation. Thus, papain likely activates the PI3K pathway, but not the PKC pathway, of eosinophils and provokes their effector functions.

To verify the activation of the PI3K pathway, we stimulated eosinophils with papain and examined phosphorylation of a downstream signaling molecule—namely, Akt. When anti-phospho-Akt antibody was used, the immunoblot results were inconsistent, probably because of limitations in assay sensitivity (data not shown). Bates et al³⁴ reported that incubation of eosinophils with IL-5 substantially enhances immunoblot detection of phosphorylated proteins. Therefore, we preincubated eosinophils with IL-5 (10 ng/mL) for 60 minutes and then examined the effect of papain on Akt phosphorylation. Fig 4, *A* shows that after preincubation with IL-5 there was minimal phosphorylation of Akt (time, 0 minutes). Cells were next stimulated with 1 $\mu\text{mol/L}$ papain, and a rapid induction of Akt phosphorylation was observed, reaching a plateau at 5 minutes. The Akt phosphorylation decreased subsequently, reaching a baseline level after 30 to 60 minutes. These experiments were repeated with eosinophils from 5 different donors; the results are summarized in Fig 4, *B*. As shown, papain reproducibly induced phosphorylation of Akt in these IL-5-primed eosinophils. In sum, papain likely activates the PI3K pathway, leading to degranulation and superoxide production by human eosinophils.

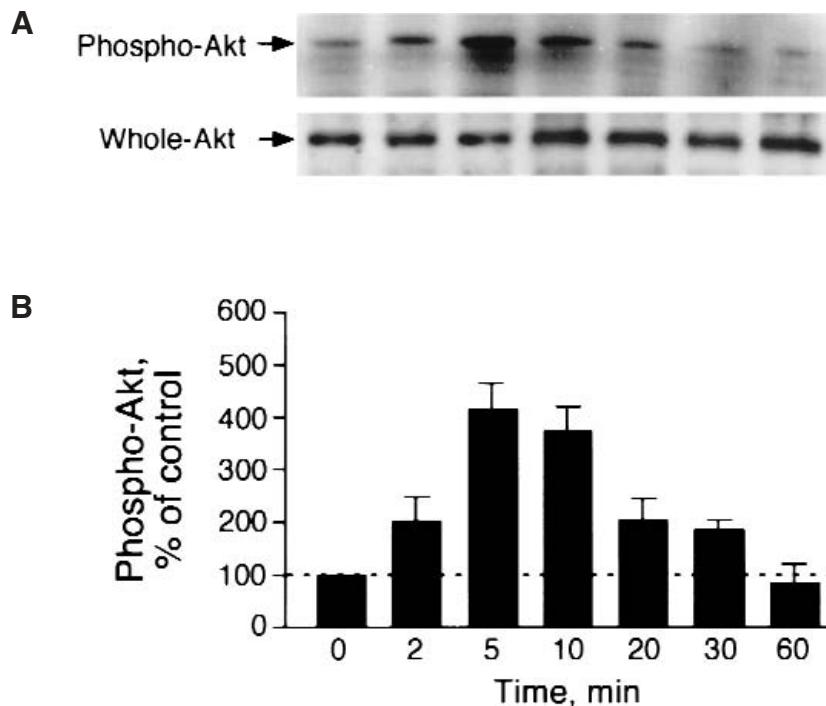


FIG 4. Papain-induced phosphorylation of Akt in eosinophils. **A**, Eosinophils were preincubated with 10 ng/mL IL-5 for 60 minutes and then stimulated with 1 μ mol/L papain for up to 60 minutes. Samples were analyzed by immunoblot through use of antibody to phospho-Akt antibody (*upper panel*); the membrane was reblotted with antibody to whole-Akt antibody (*lower panel*). The figure is representative of 5 experiments showing similar results. **B**, The intensities of the phospho-Akt bands were quantitated by densitometric analyses. Data were normalized to the values immediately before addition of papain (time, 0 minutes) as 100%. Data are presented as means $\pm$ SEMs from 5 separate experiments.

The cysteine protease Der f 1 induces degranulation of eosinophils

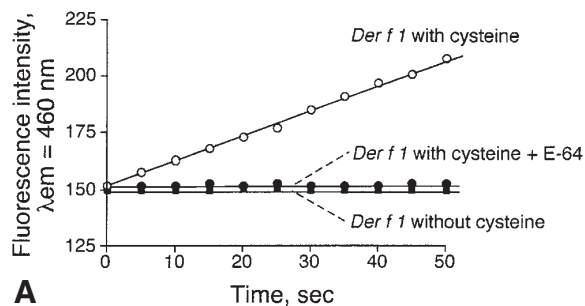
To examine the physiological relevance of these observations, we tested the effects of a purified natural mite allergen, Der f 1, on eosinophil degranulation. Before testing eosinophils, we confirmed the cysteine protease activity of Der f 1 using a peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC. Fig 5, *A* shows that when Der f 1 was preincubated with cysteine it cleaved the substrate and the substrate fluoresced. Kinetic constants for the substrate are as follows: $K_m = 80.0 \mu\text{mol/L}$; $k_{cat} = 4 \times 10^{-3}/\text{s}$; $k_{cat}/K_m = 1 \times 10^{-5}/\mu\text{mol/L} \cdot \text{s}$. Furthermore, E-64 completely blocked the enzymatic cleavage, which confirms that the effect of Der f 1 is dependent on its cysteine protease activity. Neither Der f 1 without preincubation with cysteine (Fig 5, *A*) nor cysteine alone (data not shown) was active, which suggests that preincubation with cysteine is necessary to restore the cysteine protease activity of this purified natural allergen.

Knowing the optimal conditions for restoration of the cysteine protease activity of purified natural Der f 1, we examined the effect of Der f 1 on eosinophil degranulation. Fig 5, *B* shows that Der f 1 induced significant EDN release from human eosinophils ($P < .01$; $n = 4$). Furthermore, the effect of Der f 1 was completely abolished when it was pretreated with E-64, which suggests that the

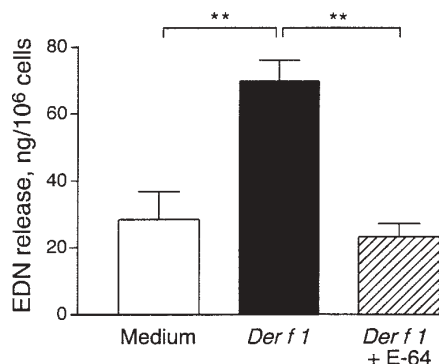
cysteine protease activity of Der f 1 is involved in inducing eosinophil degranulation. Neither Der f 1 without cysteine preincubation nor cysteine alone induced EDN release (data not shown).

IL-5 shows a synergistic effect on papain-induced degranulation

We also took another approach to examine the physiological relevance of our observations. IL-5 plays important roles in recruitment and activation of eosinophils.³⁵ Furthermore, as shown in Fig 4, IL-5 enhances the eosinophil's response to papain. Therefore, we examined the effects of IL-5 on papain-induced degranulation of eosinophils. Fig 6, *A* shows that a suboptimal concentration (10 pg/mL) of IL-5 alone induced minimal EDN release (37.2 ± 4.0 ng EDN release/ 10^6 cells [mean $\pm$ SEM]; $n = 4$); papain (1 $\mu\text{mol/L}$) alone induced approximately 133 ng EDN release per 10^6 cells. Importantly, preincubation with IL-5 significantly enhanced by 78% the eosinophil degranulation induced by 1 $\mu\text{mol/L}$ papain, resulting in 236.4 ± 39.6 ng EDN release per 10^6 cells ($P < .05$; mean $\pm$ SEM; $n = 4$). Similarly, as shown in Fig 6, *B*, preincubation of eosinophils with IL-5 significantly enhanced by 141% the eosinophil degranulation induced by 100 $\mu\text{g/mL}$ Der f 1 ($P < .05$; $n = 3$). Thus, the eosinophil's response to cysteine proteases is synergistically enhanced by a small amount of IL-5.



A



B

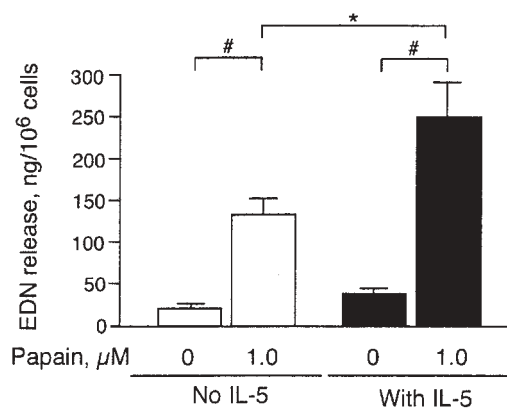
FIG 5. Protease activity of the purified natural allergen Der f 1 and its effect on eosinophil degranulation. **A**, Purified natural mite allergen, Der f 1, was preincubated with medium alone (solid squares), 5 mmol/L cysteine (open circles), or cysteine plus 1 μ mol/L E-64 (solid circles) for 30 minutes at 37°C and then added to the fluorescence substrate, 250 μ mol/L Suc-Leu-Leu-Val-Tyr-AMC. Fluorescence intensity monitored the enzymatic activity of Der f 1 at 5-second intervals, as described in the Methods section. **B**, Der f 1 was preincubated with 5 mmol/L cysteine at 37°C for 30 minutes to restore the enzymatic activity. Eosinophils were incubated with medium alone or 1 μ mol/L Der f 1 with or without 1 μ mol/L E-64 for 4 hours. Amounts of EDN in the supernatants were determined as in Fig 1. The results are presented as means $\pm$ SDs from 4 experiments using different eosinophil donors. **Significant difference ($P < .01$) between the samples.

DISCUSSION

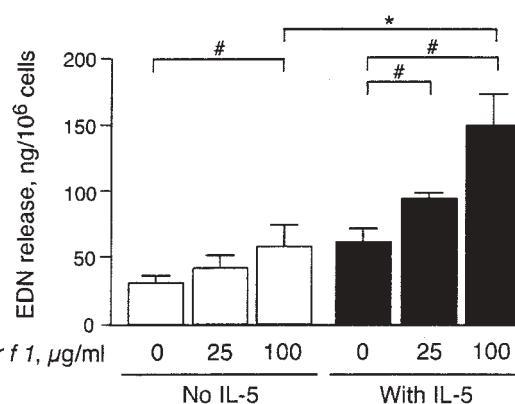
Human eosinophils are activated by cysteine proteases and release inflammatory mediators. This conclusion is based on the following observations:

1. Papain and Der f 1 induced significant superoxide production and degranulation from human eosinophils.
2. Papain induced eosinophil effector functions that involved the PI3K-Akt pathway, and the eosinophil's response to papain was enhanced synergistically by IL-5.
3. Because a cysteine protease inhibitor, E-64, completely abolished the eosinophil degranulation induced by papain and Der f 1, their effects are totally dependent on cysteine protease activity.

Previous reports show that another cysteine protease, Der p 1, cleaves CD23 on B cells²¹ and CD25 on T



A



B

FIG 6. Effect of IL-5 on papain- and Der f 1-induced eosinophil degranulation. Eosinophils were preincubated with or without (No) a suboptimal concentration of IL-5 (10 pg/mL) for 60 minutes at 37°C and then stimulated with papain (1 μ mol/L; **A**) or Der f 1 (25 and 100 μ g/mL; **B**) for 4 hours at 37°C. Amounts of EDN in the supernatants were determined as in Fig 1. Data are presented as means $\pm$ SEMs from 4 (panel A) or 3 (panel B) experiments using different eosinophil donors. *Significant difference ($P < .05$) in comparison with no IL-5. #Significant difference ($P < .05$) in comparison with no papain or no Der f 1.

cells,²² enhancing the IgE-mediated response²¹ and reducing the T_H1 -cytokine response,²² respectively; this suggests the immunomodulatory effects of certain cysteine proteases. As summarized in Fig 7, our observations now provide evidence that cysteine proteases can activate the effector functions of an inflammatory cell (ie, the eosinophil) through their protease activity. Thus, certain cysteine proteases likely have pro-inflammatory as well as immunomodulatory effects.

Our results might have important clinical relevance in allergic diseases and bronchial asthma. We used papain to investigate cysteine proteases because a number of proteases from natural allergens (eg, mite) and parasite organisms (eg, *Schistosoma mansoni*) belong to this group (family C1 in clan CA³⁶⁻³⁸; see the MEROPS database at <http://www.merops.co.uk>) and have similar struc-

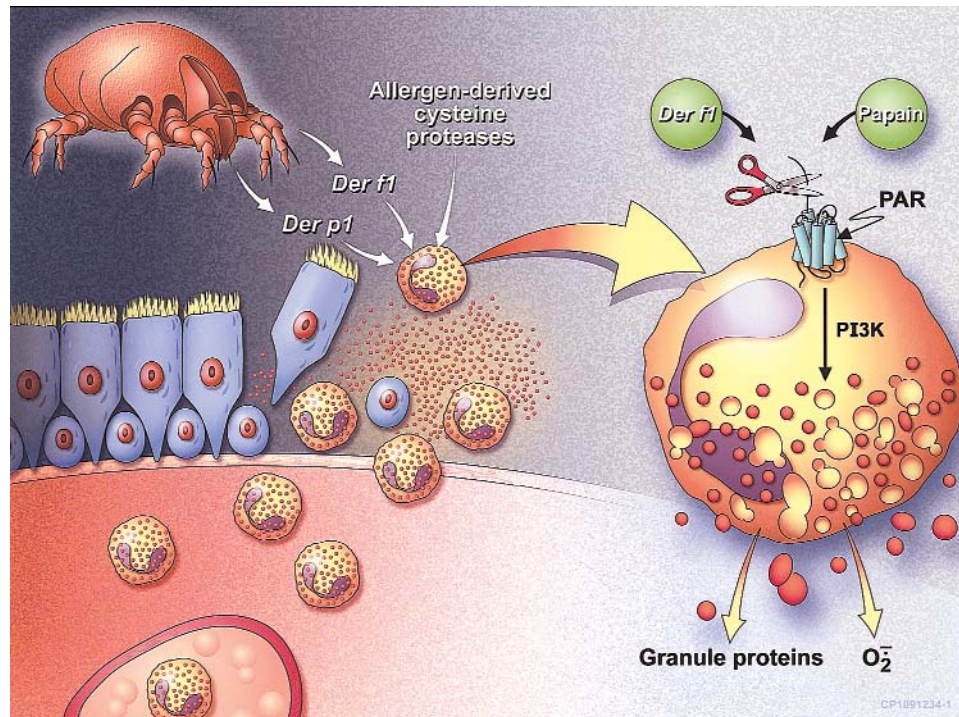


FIG 7. Activation of eosinophils by allergen-derived cysteine proteases and potential implications for allergic diseases and asthma. Exposure of human eosinophils to cysteine proteases, such as papain and Der f 1, activates the PI3K-Akt pathway of the cell and induces degranulation and superoxide production. The mechanism likely involves proteolytic cleavage and subsequent activation of a PAR or one or more related molecules on the eosinophil's surface. Thus, in allergic diseases and asthma, cysteine proteases derived from various allergens, such as Der p 1 and Der f 1, might activate eosinophils and provoke inflammatory mediator production, the result being exacerbation of inflammation and tissue damage.

tures and biochemical characteristics.³⁹ Furthermore, these papainlike C1 cysteine proteases are distributed among all kingdoms of organisms. Many cysteine proteases have their optimum pH values comparatively acidic and below the physiological pH values; however, as shown in this report and in others,²² certain cysteine proteases from allergens and papain exert their enzymatic activities over a broad pH range. Therefore, these cysteine proteases might affect eosinophils and other immune cells under physiological conditions. Furthermore, mite proteases disrupt the tight junctions that seal the gaps between epithelial cells, increasing the permeability of proteins from the airway into the tissues.^{40,41} Therefore, once these allergen-derived proteases disrupt the epithelial cell barrier, eosinophils in inflamed tissues might be constantly exposed to and activated by these proteases. In addition, IL-5 synergistically enhances the eosinophil's response to these proteases (Fig 6). Therefore, cysteine proteases in various allergens might modulate and exacerbate allergic inflammation in allergic diseases and asthma (Fig 7). To clarify the mechanism, the *in vivo* effects of cysteine protease in the airways—especially the effect(s) of chronic exposure to low-dose protease on airway inflammation—need to be elucidated.

The mechanisms of cellular activation induced by serine proteases have been studied in several investigations.

For example, serine proteases induced the activation of c-Jun N-terminal kinase⁴² and p42/p44-MAP kinases^{43,44} and increased intracellular Ca^{2+} ^{45,46} through PAR2. Furthermore, serine proteases stimulated PAR1 on rat myocytes and phosphorylated Akt.⁴⁷ However, similar studies using cysteine proteases are rare: in this report, we have demonstrated for the first time that papain, a cysteine protease, activates the PI3K-Akt pathway. Unfortunately, we were unable to identify receptors for these cysteine proteases. The phosphorylation of Akt both by PAR1 activation⁴⁷ and by papain (Fig 4 in this report) suggests that PAR might also be involved in the eosinophils' response to cysteine proteases as well as to serine proteases. However, PAR2 is the only known functional PAR expressed by human eosinophils, yet anti-PAR2 antibody, which recognizes the receptor site responsible for trypsin-induced activation, did not inhibit papain-induced superoxide production by eosinophils (Fig 3). Recently, it has been shown that the Der p 1-mediated calcium response in A549 cells is mediated by PAR2 but not by PAR1.⁴⁸ Furthermore, HeLa cells transfected with PAR2 showed a calcium response and receptor internalization when stimulated with Der p 1.⁴⁸ These findings strongly suggest that the cellular response to another cysteine protease, Der p 1, is mediated by PAR2 in these cell types.

Accordingly, several possibilities remain as potential effector mechanisms for cysteine proteases; these include (1) cleavage and activation of known surface molecules other than PAR, (2) cleavage and activation of one or more unknown specific receptors or unidentified PAR-like molecules, and (3) cleavage and activation of PAR2 but at different sites from those used for serine proteases. Der p 1 cleaves known receptors, such as CD23 and CD25,^{21,22} and modulates the functions of immune cells. Our observation that papain rapidly phosphorylates Akt in eosinophils also suggests that the cysteine protease directly activates one or more receptors, which positively regulate cell activation and similar to PAR. Therefore, for a better understanding of the role of exogenous cysteine proteases in cell function it will be extremely important to fully elucidate the molecular mechanisms, including receptors and signaling mechanisms, that are involved in the cellular response. Furthermore, the distinctive characteristics that differentiate the cellular responses to cysteine proteases and serine proteases need to be elucidated; this will clarify the roles of these proteases and their potential functional dichotomies in immunity and inflammation.

The potential implications of our observations for eosinophil biology are substantial. They suggest that eosinophils are equipped with machineries for recognizing papain-like proteases, which are widely expressed by natural allergens and parasite organisms. In the past, perhaps, this cysteine protease-dependent activation mechanism was used by the eosinophilic leukocyte to protect human beings from parasitic infections. In modern society, however, the same mechanism might have detrimental outcomes through unnecessary eosinophil activation by various allergens. The synergistic effect of cysteine proteases and IL-5 might further aggravate the problem. The validity of this paradoxical concept needs to be examined in clinical studies of human parasitic infections and allergic diseases. The answers to these questions might lead to a better understanding of the immunobiology of human eosinophils and might elucidate the roles of cysteine proteases in the complex inflammatory networks of human immunity and diseases.

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