

Specific Allergens Enhance Elastase Release in Stimulated Neutrophils from Asthmatic Patients

Javier Monteseirín^a Inés Bonilla^a M. Jesús Camacho^a Pedro Chacón^a
Antonio Vega^a Antonio Chaparro^a José Conde^a Francisco Sobrino^{a, b}

^aDepartamento de Medicina, Servicio Regional de Immunología y Alergia, Hospital Universitario Virgen Macarena,

^bDepartamento de Bioquímica Médica, Facultad de Medicina, Universidad de Sevilla, Sevilla, España

Key Words

Allergy · IgE-dependent mechanism · Lung function · FEV₁

Abstract

Background: The presence of the three forms of IgE receptor – the heterotrimeric high-affinity receptor for IgE (FcεRI), the low-affinity receptor for IgE (FcεRII/CD23) and the Mac-2/IgE-binding protein (εBP) – has been demonstrated on human neutrophils. We have previously shown that specific allergens are able to activate functional responses by neutrophils from allergic patients sensitized to those allergens. Neutrophils are present at the sites of allergic inflammation. The primary (azurophilic) granules of neutrophils contain a variety of enzymes, such as elastase, that might potentiate inflammation. It is not known whether specific allergens are able to elicit elastase release by neutrophils from allergic patients. In addition, we attempted to evaluate the relationship between neutrophil degranulation and lung function of the patients, measured as FEV₁. **Methods:** Neutrophils were challenged in vitro with the specific allergens that produced clinical symptoms in asthmatic patients. The cells were also challenged with allergen to which the

patients were not sensitive. Neutrophils from normal subjects were challenged with allergens as control. **Results:** The in vitro challenge of neutrophils with allergens to which the patients were sensitive elicited a release of elastase by these cells. The in vitro activation of neutrophils was highly allergen specific; allergens other than those accounting for clinical symptoms did not evoke elastase release, and allergens were ineffective on neutrophils from healthy donors. A significant inverse correlation was observed between elastase release and patients' lung function, measured as FEV₁. **Conclusion:** An IgE-dependent mechanism might promote elastase release by neutrophils at allergic sites. There is a significant inverse relationship between levels of elastase released by neutrophils from allergic patients and lung function, as assessed by FEV₁.

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Introduction

There is increasing evidence of neutrophil participation in asthma and the allergic process [1]. For instance, peripheral blood neutrophils are activated during active asthma [2], after exercise-induced bronchospasm [3] and

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Correspondence to: Dr. J. Monteseirín
Asunción 27, 3^o Izda
ES–41011 Sevilla (Spain)
Fax +34 954 907 041
E-Mail fmonteseirinm@meditex.es

during both early and late asthmatic reactions induced by allergens [4]. Several studies have reported evidence linking the presence of neutrophils with airway damage and dysfunction [5–7]. In particular, the number of neutrophils detected in bronchoalveolar lavage and airway mucosa has been correlated with the degree of lung dysfunction [8, 9]. Elastase is a proteolytic enzyme contained in the azurophilic granules of neutrophils, and has been reported to cause epithelial damage [10], vascular hyperpermeability [11], mucus hypersecretion [12], mucus gland metaplasia [13], reduction in mucociliary clearance [14], bronchoconstriction and airway hyperresponsiveness [15]. Previous studies have demonstrated the presence of the three forms of IgE receptor – the heterotrimeric high-affinity receptor for IgE (FcεRI) [16–19], the low-affinity receptor for IgE (FcεRII/CD23) [20–22] and galactin-3 [23–26] – on human neutrophils. We have previously shown that specific allergens were able to activate functional responses by neutrophils from allergic patients sensitized to allergens of the same type that produced clinical allergic symptoms [27]. IgE molecules have been demonstrated to be present on the surface of neutrophils [23]. Our group also revealed the specific binding of FITC-labeled allergens on the surface of neutrophils, using flow cytometry and microscopy analysis [27]. Despite all this evidence, it is not known whether neutrophil-elicited release of elastase might be modulated directly by an IgE-dependent mechanism. The present work is an attempt to throw light on the issue. Here, we present evidence that specific allergens were able to elicit elastase release by neutrophils from allergic patients.

Patients and Methods

Materials

The allergens were commercially available antigen extracts, including D₁ (*Dermatophagoides pteronyssinus*), G₃ (*Dactylis glomerata*), T₉ (*Olea europaea*) and W₆ (*Artemisia vulgaris*). They were purchased from Bial-Aristegui (Bilbao, Spain). Other biochemicals were from Sigma (Madrid, Spain) or Merck (Barcelona, Spain). All cultured reagents had endotoxin levels of ≤ 0.01 ng/cm³, as tested by the *Limulus* lysate assay (Coatest, Chromogenix, Mölndal, Sweden).

Patients and Controls

The studied group included adult atopic patients with intermittent [28] bronchial asthma and healthy adult nonatopic volunteer controls. The asthmatic patients had positive skin prick test (Bial-Aristegui) and positive specific IgE (CAP System, Pharmacia & Upjohn Diagnostics AB, Barcelona, Spain) to at least one common allergen (house-dust mites and pollens). They received neither treatment nor specific hyposensitization, and were not allowed to take any bronchodilators within the 8 h before challenge of neutrophils in

vitro. Oral bronchodilators were withheld for 24 h, and none of the subjects had taken corticosteroids, cromolyn sodium or nedocromil sodium in the previous week. The healthy controls had no history of allergy or bronchial symptoms, and had negative skin prick test (Bial-Aristegui) and negative specific IgE (CAP System) to a battery of inhalant allergens (house-dust mites, pollens, molds and animal danders). The Hospital Ethics Committee approved the study, and each subject gave informed consent.

Preparation of Polymorphonuclear Leukocytes

Human neutrophils were purified from freshly drawn heparinized (10 U/cm³) venous blood [27]. Neutrophils were resuspended to about 10^7 cells/cm³ in PBS and kept at room temperature. These cells were $>96\%$ neutrophils as evaluated by morphological analysis. Eosinophil contamination was $\leq 2\%$. Trypan blue exclusion showed greater than 96% viability in neutrophils.

Lung Function

FEV₁ was measured using a dry spirometer (Vitalograph, Buckingham, UK). The best value of three maneuvers was expressed as a percentage of the predicted value.

Neutrophil Stimulation

Cells, resuspended at 1×10^6 in PBS buffer supplemented with 500 μ M CaCl₂, in a volume of 300 mm³, were stimulated with a dose of 10 μ g/cm³ allergens [27] at 37°C for 30 min. After preincubation with 5 μ g/cm³ cytochalasin B for 10 min at 37°C under agitation, elastase released with fMLP at a dose of 10^{-7} mol/dm³ at 37°C for 30 min was the positive control [29].

Elastase Assay

After stimulation with allergens or fMLP, the cell suspension was immediately placed on ice to prevent further release, and centrifuged at $2,000$ rpm for 5 min. Elastase activity in the collected supernatant was measured by Elastase-EIA method (Immundiagnostik, Bensheim, Germany).

Dissociation of Neutrophil-Bound IgE

IgE was dissociated from the surface of neutrophils as described previously [30], with minor modifications. Briefly, after isolation, cells (1×10^8) were resuspended in 1 cm³ of acetate buffer (50 mM sodium acetate, pH 4, 85 mM NaCl, 5 mM KCl, 0.03% human serum albumin) and incubated on ice for 3 min. An equal volume of gelatin veronal buffer (GVB; 1.8 mM sodium barbital, 3.1 mM barbituric acid, 0.1% gelatin, 0.05 mM MgCl₂, 141 mM NaCl, 0.15 mM CaCl₂, pH 7.3–7.4) was then added to the treated cells, and the mixture was centrifuged at 500 g for 10 min. The IgE-containing supernatant was neutralized with 1 N NaOH and used for specific IgE determination or immunoblotting.

Immunoblotting

Allergenic extracts, resuspended in 1 cm³ of PBS, were mixed with an equal volume of $2 \times$ sample buffer [$1 \times$: 62.5 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 5% β -mercaptoethanol, 8.5% glycerol, 0.025% bromophenol blue] and boiled for 5 min at 95°C . Proteins (80 μ g/lane) were separated on 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes using a semidry device (Bio-Rad, Richmond, USA). After transference, the membranes were washed with distilled water, soaked in methanol and blocked by drying [31] at 37°C for 30 min.

Then, they were probed with IgE-containing supernatant, diluted 1:7 in GVB, for 12 h at room temperature. Next, the membranes were washed twice with PBS, and immunoreactive bands were visualized using the enhanced chemiluminescence assay as previously described [32]. Briefly, the membranes were incubated for 1 min in 10 cm³ of fresh luminescent reagent solution composed of 10 mM Tris-HCl, pH 8.5, 2.25 mM luminol, 0.015% (v/v) H₂O₂ and 0.45 mM 4-iodophenol. Luminol and 4-iodophenol were freshly prepared in 10 cm³ of 10 mM Tris-HCl, pH 8.5. Luminol was previously dissolved in 50 mm³ of 1 M NaOH. After 1 min of incubation, the membranes were placed on paper filter, covered with Saran Wrap and exposed on X-ray films (X-Omat, Eastman Kodak Co.) in the dark for 1–5 min.

Statistical Analysis

Data are expressed as means $\pm$ SEM. A Student's unpaired t test or one-way ANOVA was used to make comparisons between groups. Regression analysis was performed using Pearson rank correlation coefficients. A p value <0.05 was considered significant.

Results

The presence of specific IgE from the neutrophil cell surface was examined in human neutrophils. Cells from the sensitive asthmatic group, from the nonsensitive asthmatic group or from healthy donors were treated as indicated in Patients and Methods, and supernatants obtained were used for specific IgE determination by immunoblotting and/or CAP to D₁, T₉, G₃ and W₆ allergens. Tables 1–3 illustrate the results of the specific IgE eluted from the neutrophil surface compared with those of the skin prick test and serum-specific IgE from the same donors. As can be seen, only patients with positive skin prick test and positive serum-specific IgE to those allergens show positive specific IgE from the neutrophil cell

Table 1. Results of specific IgE eluted from the neutrophil surface compared with those of the skin prick test and serum-specific IgE from the same donors (sensitive asthmatic group)

Patient No.	Skin test/blood specific IgE				Neutrophil surface IgE eluted			
	D ₁	G ₃	T ₉	W ₆	D ₁	G ₃	T ₉	W ₆
1	+	–	–	–	+I/C	–I/C	–I/C	–C
2	+	–	–	–	+I/C	–C	–I/C	–C
3	–	+	–	–	–I/C	+I/C	–C	–I/C
4	–	–	+	–	–C	–C	+I/C	–C
5	+	–	–	–	+I/C	–C	–I/C	–C
6	–	–	–	+	–C	–C	–C	+I/C
7	–	+	–	–	–C	+I/C	–I/C	–C
8	+	–	–	–	+I/C	–C	–C	–C
9	–	–	+	–	–C	–C	+C	–C
10	–	+	–	–	–C	+I/C	–C	–C
11	–	–	–	+	–I/C	–C	–C	+I/C
12	–	–	+	–	–C	–I/C	+I/C	–C
13	–	+	–	–	–C	+C	–C	–C
14	–	–	+	–	–C	–C	+I/C	–C
15	–	–	–	+	–C	–C	–C	+I/C
16	–	–	–	+	–C	–C	–C	+C
17	+	–	–	–	+C	–C	–C	–C
18	+	–	–	–	+I/C	–I/C	–C	–C
19	–	+	–	–	–C	+I/C	–I/C	–C
20	–	–	+	–	–C	–C	+C	–C
21	–	+	–	–	–I/C	+I/C	–C	–C
22	–	–	–	+	–C	–C	–I/C	+C
23	–	–	+	–	–C	–C	+C	–C
24	+	–	–	–	+C	–C	–C	–C
25	–	–	+	–	–C	–I/C	+I/C	–C
26	–	+	–	–	–C	+I/C	–C	–C
27	–	–	–	+	–C	–C	–I/C	+I/C
28	+	–	–	–	+I/C	–I/C	–C	–C
29	–	–	+	–	–C	–I/C	+I/C	–I/C
30	–	+	–	–	–C	+I/C	–C	–C

+ = Positive; – = negative; I = immunoblotting; C = CAP.

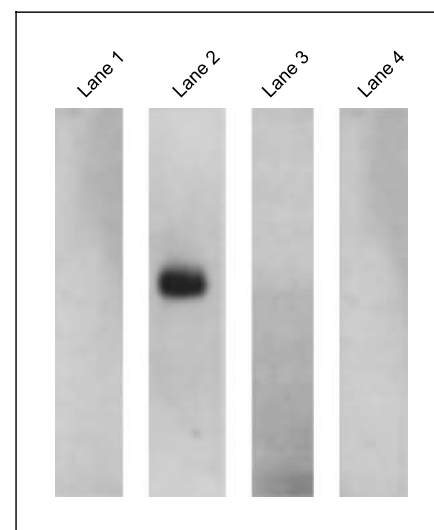


Fig. 1. Reactivity of specific IgE eluted from the neutrophil cell surface. Allergenic extracts (D₁: lanes 1 and 2; G₃: lane 3; T₉: lane 4) were resolved by SDS-PAGE and transferred to PVDF membranes. Then, lanes 1–4 were probed with different supernatants diluted in GVB buffer. Lane 1: supernatant from a patient with D₁-positive specific IgE without IgE cell surface elution; lane 2: supernatant from a patient with D₁-positive specific IgE with IgE cell surface elution; lane 3: supernatant from a patient with G₃-negative specific IgE with IgE cell surface elution; lane 4: supernatant from a patient with T₉-negative specific IgE with IgE cell surface elution. After washing, membranes were incubated with anti-human IgE, and the immunoreactive bands were revealed with an enhanced chemiluminescence assay.

Table 2. Results of specific IgE eluted from the neutrophil surface compared with those of the skin prick test and serum-specific IgE from the same donors (nonsensitive asthmatic group)

Patient No.	Skin test/blood specific IgE				Neutrophil surface IgE eluted			
	D ₁	G ₃	T ₉	W ₆	D ₁	G ₃	T ₉	W ₆
1	–	–	–	–	–I/C	–C	–C	–C
2	–	–	–	–	–I/C	–C	–C	–C
3	–	–	–	–	–C	–I/C	–C	–C
4	–	–	–	–	–C	–C	–I/C	–C
5	–	–	–	–	–C	–C	–C	–I/C
6	–	–	–	–	–C	–C	–C	–C
7	–	–	–	–	–C	–I/C	–I/C	–C
8	–	–	–	–	–C	–C	–C	–I/C
9	–	–	–	–	–C	–C	–C	–C
10	–	–	–	–	–I/C	–I/C	–C	–C
11	–	–	–	–	–C	–C	–I/C	–C
12	–	–	–	–	–C	–C	–C	–I/C
13	–	–	–	–	–C	–C	–I/C	–C
14	–	–	–	–	–C	–C	–C	–C
15	–	–	–	–	–I/C	–I/C	–C	–C
16	–	–	–	–	–C	–C	–C	–I/C
17	–	–	–	–	–C	–C	–C	–C
18	–	–	–	–	–I/C	–C	–C	–C
19	–	–	–	–	–C	–C	–I/C	–C
20	–	–	–	–	–C	–I/C	–C	–I/C
21	–	–	–	–	–I/C	–C	–C	–C
22	–	–	–	–	–C	–C	–I/C	–C
23	–	–	–	–	–C	–C	–C	–C
24	–	–	–	–	–C	–C	–C	–I/C
25	–	–	–	–	–C	–I/C	–C	–C
26	–	–	–	–	–C	–C	–C	–C
27	–	–	–	–	–I/C	–C	–I/C	–C
28	–	–	–	–	–C	–I/C	–C	–C
29	–	–	–	–	–C	–I/C	–I/C	–C
30	–	–	–	–	–C	–I/C	–C	–C

+ = Positive; – = negative; I = immunoblotting; C = CAP.

Table 3. Results of specific IgE eluted from the neutrophil surface compared with those of the skin prick test and serum-specific IgE from the same donors (healthy donors)

Subject No.	Skin test/blood specific IgE				Neutrophil surface IgE eluted			
	D ₁	G ₃	T ₉	W ₆	D ₁	G ₃	T ₉	W ₆
1	-	-	-	-	-C	-I/C	-C	-I/C
2	-	-	-	-	-I/C	-C	-C	-C
3	-	-	-	-	-C	-C	-C	-C
4	-	-	-	-	-C	-C	-I/C	-C
5	-	-	-	-	-C	-C	-C	-C
6	-	-	-	-	-C	-C	-C	-I/C
7	-	-	-	-	-C	-C	-C	-C
8	-	-	-	-	-I/C	-C	-C	-C
9	-	-	-	-	-C	-C	-I/C	-C
10	-	-	-	-	-C	-C	-C	-I/C
11	-	-	-	-	-I/C	-C	-C	-C
12	-	-	-	-	-C	-C	-C	-C
13	-	-	-	-	-C	-I/C	-C	-C
14	-	-	-	-	-C	-I/C	-C	-C
15	-	-	-	-	-C	-C	-I/C	-C
16	-	-	-	-	-C	-C	-C	-C
17	-	-	-	-	-I/C	-C	-C	-C
18	-	-	-	-	-C	-C	-C	-I/C
19	-	-	-	-	-C	-I/C	-C	-C
20	-	-	-	-	-C	-C	-I/C	-C
21	-	-	-	-	-C	-C	-C	-C
22	-	-	-	-	-I/C	-C	-C	-C
23	-	-	-	-	-C	-C	-C	-C
24	-	-	-	-	-C	-C	-I/C	-C
25	-	-	-	-	-C	-I/C	-C	-C
26	-	-	-	-	-C	-C	-C	-I/C
27	-	-	-	-	-C	-C	-C	-C
28	-	-	-	-	-C	-C	-C	-C
29	-	-	-	-	-C	-C	-C	-C
30	-	-	-	-	-C	-C	-C	-C

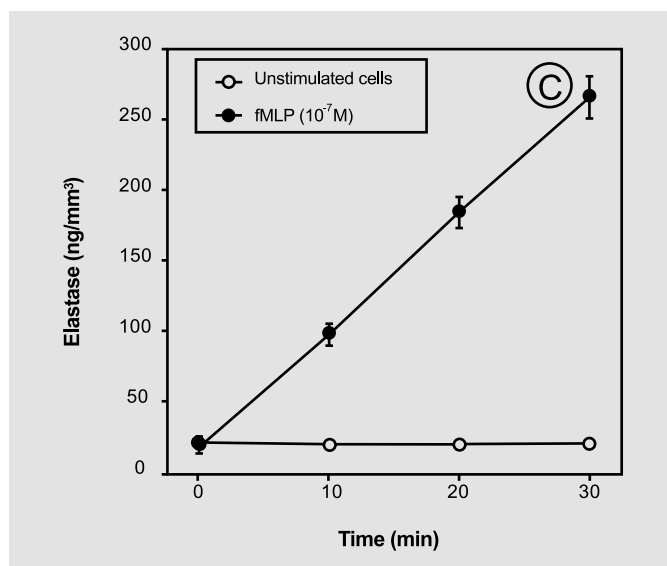
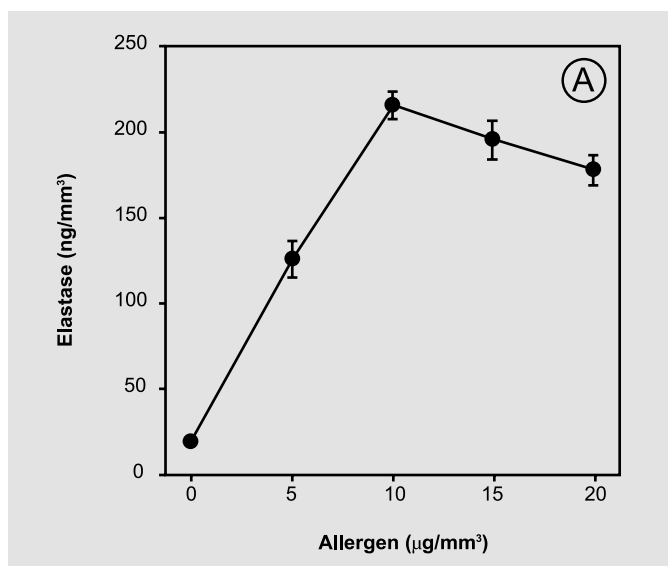
+ = Positive; - = negative; I = immunoblotting; C = CAP.

surface (fig. 1, table 1). None of the subjects with negative skin prick test and negative serum-specific IgE to these allergens (patients and controls) had positive specific IgE from the neutrophil cell surface (tables 1–3). The reactivity of the specific IgE to allergens from the neutrophil surface is shown in figure 1. In this case, the specific IgE eluted from the surface of neutrophils from a D₁-sensitized patient shows reactivity only to this allergen. No immunoreactivity was observed to T₉ or G₃ (to which the patient was not sensitive). After in vitro allergen challenge of neutrophils, only patients with positive allergen-specific IgE from the neutrophil cell surface demonstrated elastase release (data not shown).

Figure 2 illustrates that the treatment of neutrophils with G₃ allergen did elicit elastase release by neutrophils obtained from patients who were specifically allergic to

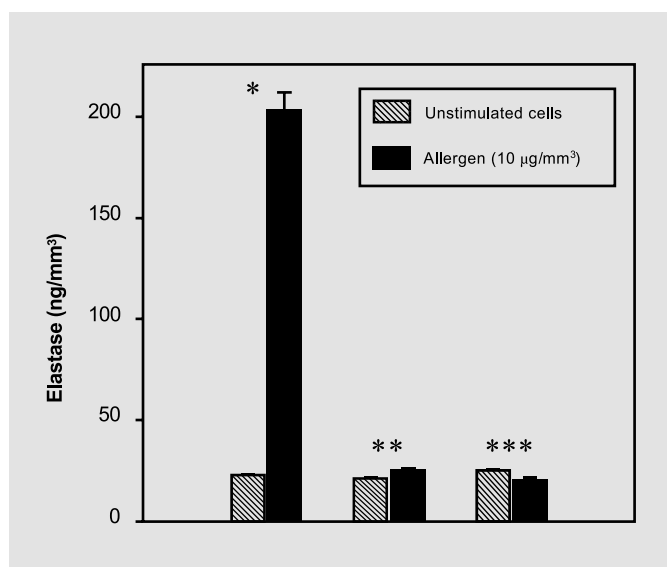
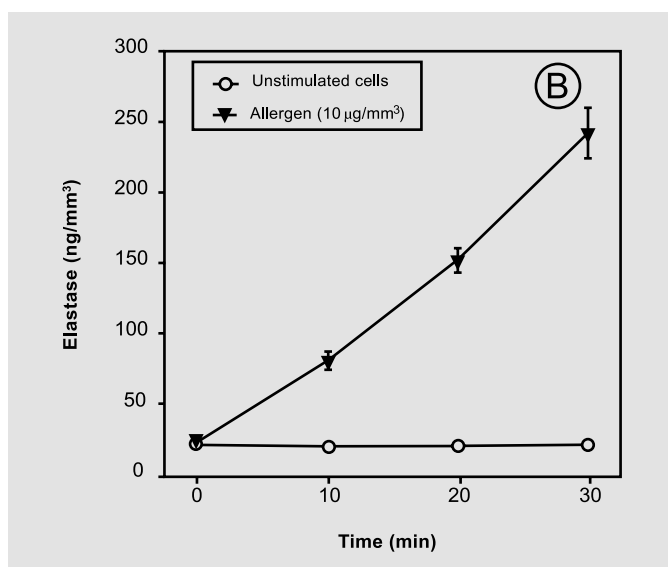
G₃ pollen, in both a dose- (fig. 2A) and time-dependent (fig. 2B) fashion. Similar results were obtained with other allergens, including *D. pteronyssinus*, *O. europaea* and *A. vulgaris* (data not shown). The elastase released by allergens was $34 \pm 9\%$ of total neutrophil elastase. Figure 2C shows the elastase release taking place when these neutrophils were exposed to fMLP (preincubated with cytochalasin B), used as positive control, as well as the levels of elastase released by unstimulated cells. Figure 3 illustrates the specificity of the response to allergens. The allergen-stimulated neutrophils from the group of asthmatic patients stimulated with the allergen to which they were sensitive showed significantly higher production of elastase than did cells from healthy donors, or cells from asthmatic patients incubated with an allergen to which the patients were not sensitized ($p < 0.001$). No statistical

2



2

2



3

Fig. 2. Challenge with G_3 allergen of neutrophils from asthmatic patients ($n = 10$) who were specifically allergic to G_3 pollen elicited release of elastase in a dose- (**A**) and time-dependent (**B**) manner. **C** Levels of elastase from neutrophils preincubated with cytochalasin B and challenged with fMLP were taken as positive controls. Levels of elastase from unstimulated cells are also shown.

Fig. 3. Neutrophils from the asthmatic group ($n = 30$) stimulated with the allergen to which they were sensitive (*) showed a significantly higher production of elastase than did cells from healthy donors ($n = 30$) (**) or cells from asthmatic patients ($n = 30$) incubated with an allergen to which they were not sensitized (***) ($p < 0.001$). No statistical differences were observed between the levels of elastase released by unstimulated cells and the elastase released by cells from healthy donors and by cells from allergic subjects not sensitized to the particular allergen.

differences were observed between the levels of elastase released by unstimulated cells and those released by cells from healthy donors and allergic subjects not sensitized to the particular allergen.

The amount of elastase released by neutrophils after incubation with allergens was evaluated in relation to the patients' lung function, measured as FEV_1 . A significant inverse correlation was observed between elastase secretion and lung function ($r = -0.705$; $p = 0.003$) (fig. 4).

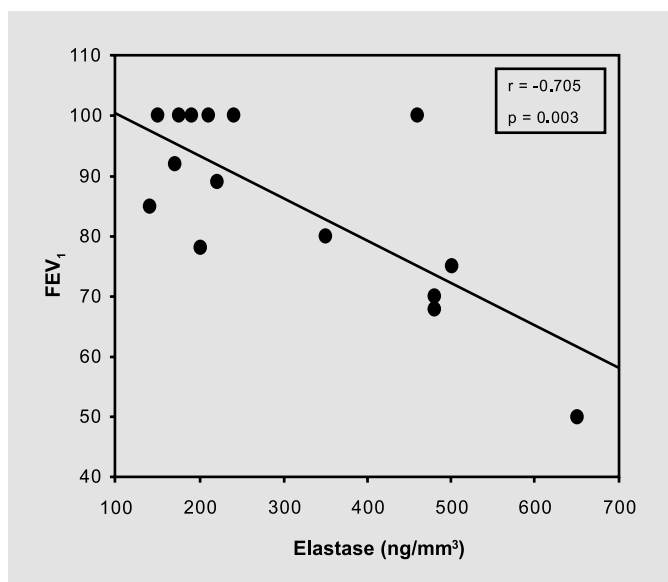


Fig. 4. Relationship between the patients' lung function and the degranulation of neutrophils. The amount of elastase released by neutrophils was evaluated in relation to the patients' lung function, measured as FEV₁. A significant inverse correlation was observed between elastase secretion and lung function ($r = -0.705$; $p = 0.003$).

Discussion

Earlier studies have demonstrated the presence of the three forms of IgE receptor on human neutrophils [16–26]. We have previously shown that specific allergens were able to activate functional responses by neutrophils from allergic patients sensitized to allergens of the same type as those which produce clinical allergic symptoms [27]. The presence of IgE molecules on the surface of neutrophils has been demonstrated [23]. Our group, using flow cytometry and microscopy analysis, also revealed the specific binding of FITC-labeled allergens on the cell surface of neutrophils [27]. Neutrophils are present at the sites of allergic inflammation [1]. The primary (azurophilic) granules of neutrophils contain a variety of enzymes, such as elastase, that might potentiate inflammation [29]. In skin chamber studies, there was a significantly increased release of elastase at allergen-challenged sites versus buffer control sites [33]. Allergen challenge of allergic patients produced a significant increase in the levels of elastase in nasal lavage fluid. In contrast, allergen challenge of nonallergic control individuals produced no such response [34]. Recent evidence has shown the presence of elastic fiber disruption in the bronchi of asthmatic individuals, supporting the concept of an imbalance between

proteases and antiproteases in this disease [35]. Neutrophil elastase has been found in the secretions of patients during exacerbations of asthma [36–39]. Neutrophil elastase can reproduce many of the pathologic features of asthma [10–15]. It can also promote neutrophil recruitment in the lung by inducing production of IL-8 [40], and cause degranulation of eosinophil cationic protein from eosinophils [41]. Despite such evidence, it is not known whether the release of elastase by neutrophils from allergic patients might be initiated directly by an IgE-dependent mechanism. The present experiments provide the first evidence that neutrophils from allergic patients release elastase by an IgE-dependent mechanism. Only patients with positive skin prick tests and positive serum-specific IgE to the studied allergens showed positive specific IgE from the neutrophil cell surface. None of the subjects with negative skin prick test and negative serum-specific IgE to those allergens (patients and controls) had positive specific IgE from the neutrophil cell surface. After in vitro allergen challenge of neutrophils, only neutrophils from patients with positive specific IgE from the neutrophil cell surface demonstrated a release of elastase. The in vitro challenge of neutrophils with allergens to which the patients were sensitive produced a dose- and time-dependent release of elastase. Our results indicate that in vitro activation of neutrophils was highly allergen specific. Allergens other than those which produce clinical symptoms did not elicit elastase release, and allergens were ineffective on neutrophils from healthy donors. Elastase was released, however, when neutrophils from both allergic patients and control individuals were treated with fMLP plus cytochalasin B. There was a significant inverse relationship between levels of elastase released by neutrophils from allergic patients and lung function as assessed by FEV₁. These findings are in agreement with another study showing that in induced sputum, the levels of active and total elastase are inversely correlated with the degree of airway obstruction as assessed from FEV₁ values [36]. It follows that an allergen-dependent mechanism might account for a direct release of elastase by neutrophils into allergic sites.

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