

Enhancement of Antigen-specific Functional Responses by Neutrophils from Allergic Patients

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Summary

It has been demonstrated that neutrophils from healthy donors or from patients with inflammatory disorders can bind immunoglobulin (Ig) E proteins through binding to Mac-2/εbp. Functional responses to allergens were assessed by measuring the respiratory burst and intracellular Ca²⁺ levels, and binding of allergens to neutrophils was assessed by flow cytometry analysis and fluorescence microscopy. In this article, we demonstrate that neutrophils sensitized to specific allergens (from allergic patients), but not from healthy donors, are sensitive to allergens of the same type as those that produce clinical allergic symptoms. The activation of neutrophils was analyzed by the induction of a respiratory burst that was detected with luminol-dependent chemiluminescence. Intracellular Ca²⁺ levels increased parallel to those of the inducing allergens. In addition, the specific binding of allergens on the cell surface was revealed by flow cytometry and allergen-FITC-labeled staining analyses. The present data suggest a restricted recognition of allergen by sensitive neutrophils, probably associated with the specific binding of the allergen to its corresponding IgE molecule, which is bound to the Mac-2/εbp structure. These findings demonstrate a functional role of allergen-associated neutrophils during the allergic state.

The development of an immediate hypersensitivity reaction involves various biochemical events leading to the release of histamine, arachidonic acid metabolites, anion superoxide, and other effector molecules (1, 2). All inflammatory cells (macrophages, lymphocytes, mast cells, eosinophils, neutrophils, and platelets) have been shown to be involved in asthma, but the relative role of each cell is not completely understood (1). Because of the chronic nature of allergic diseases, leukocytes that persist in the lung tissue, especially eosinophils and lymphocytes, have become more strongly associated with the disease. For this reason, neutrophils have often been neglected in studies on allergic diseases. However, there is increasing evidence of neutrophil participation in asthma and the allergic process. For instance, plasma chemotactic activity of neutrophils increases, and peripheral-blood neutrophils show markers of activation, during active asthma (3), after exercise-induced asthma (4), and during early and late asthmatic reactions induced by allergens (5) and toluene diisocyanate (6). In subjects with pollen-induced asthma, seasonal antigenic exposure is associated with an increase in the number of neutrophils shown in bronchial biopsies (7). In other atopic asthmatics, local endobronchial allergen challenge leads to increased neutrophil infiltration of the airway mucosa (8). Further-

more, activated neutrophils can induce airway hyper-responsiveness (9). Most of the cells, such as eosinophils, epidermal Langerhans cells, macrophages/monocytes, and bronchial epithelial cells, selectively express two types of IgE receptors, namely, receptors of high affinity (FcεRI)¹ and of low affinity (FcεRII) (10–15). Although neutrophils express neither FcεRI nor FcεRII receptors for IgE (10, 16), evidence has been presented that neutrophils express a new family of IgE-binding receptors, which belong to the S-type lectins. Among them, Mac-2/εbp is the best characterized, and its presence in neutrophils has been demonstrated (17, 18). The presence of this new family of IgE receptors has been previously demonstrated in other cells (19–21). Stimulation of human neutrophils induces several phenomena known collectively as respiratory burst, characterized by an increase in the production of superoxide anion (22, 23). Although the generated inflammatory mediators are critical to the host defense role of the neutrophils, they can cause severe tissue damage when excessively or inappropriately produced (24).

¹Abbreviations used in this paper: CL, chemiluminescence; FcεRI and FcεRII, high and low affinity IgE Fc receptors, respectively.

Despite this evidence, it is not known whether neutrophils can directly interact with the allergens, and if so, what the functional response is. The present work was undertaken to analyze these questions. The rationale is that neutrophils from allergic patients possess specific IgE pre-attached to the surface of these cells. Challenging with an allergen could trigger the activation of neutrophils through the IgE- ϵ b ν complex in the membrane. Here we present evidence that specific allergens were able to activate respiratory burst from asthmatic patients sensitized to those allergens, and we have found a relationship between the two parameters (that is, the respiratory burst is closely related to sensitized neutrophils). Moreover, we found that increased respiratory burst was associated with enhanced antigen-dependent Ca^{2+} elevation by neutrophils from allergic patients. In addition, sensitized neutrophils from allergic patients specifically bound FITC-labeled allergens, as measured by fluorescence microscopy and flow cytometry.

Materials and Methods

Materials. The allergens studied included D₁ (*Dermatophagoides pteronyssinus*), G₃ (*Dactylis glomerata*), T₉ (*Olea europea*), and W₆ (*Artemisia vulgaris*). They were purchased from Ifidasa-Aristegui (Bilbao, Spain). Mezerein was from Sigma-Aldrich Co. Ltd. (Poole, Dorset, England). Other biochemicals were from Sigma or Merck (Barcelona, Spain).

Patients and Controls. The group studied included adult atopic patients with bronchial asthma and healthy adult nonatopic volunteer controls. The asthmatic patients had positive skin prick test (Ifidasa-Aristegui) and specific IgE (CAP, Pharmacia Ibérica, Barcelona, Spain) to at least one common allergen (house dust mites and pollens). The subjects received neither treatment nor specific hyposensitization. None of them had experienced episodes of asthma for at least 3 mo, and none had had respiratory tract infections for 4 wk before blood sampling. The healthy controls had no history of allergy or bronchial symptoms and had negative skin prick test (Ifidasa-Aristegui) and specific IgE to a battery of inhalant allergens (house dust mites, pollens, molds, and animal danders).

Preparation of Polymorphonuclear Leukocytes. Human neutrophils were purified (25) from freshly drawn heparinized (10 U/ml) venous blood in two steps: first, the blood (20 ml) was mixed with 1.5 ml of 10% Dextran T 500 (final concentration of 0.7%), dissolved in PBS. The supernatant fraction was centrifuged through a Ficoll-Hypaque. The neutrophil-rich pellet was then removed by plastic pipette and red cells were eliminated by one hypotonic lysis in water. Neutrophils were resuspended to $\sim 10^7$ cells/ml in PBS and 10 mM of glucose and were kept at room temperature.

Measurement of Intracellular Ca^{2+} . Freshly prepared neutrophils from allergic or healthy donors were incubated in a medium (Ca^{2+} medium) composed of 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM Hepes at pH 7.4, at a concentration of 10^7 cells/ml at 37°C for 45 min in the presence of 2 μ M Fura-2 acetoxymethyl ester (26). The excess of the dye was then washed away, and the neutrophils were kept in the same medium. Batches of 10^7 cells were resuspended in 0.6 ml of Ca^{2+} medium at 37°C, and the allergens to be studied were added to the cellular suspension. Fura-2 fluorescence (λ_{exc} at 340 nm and λ_{em} at 505 nm) was recorded on a Perkin-Elmer (Norwalk, CT) LS-5 spectrofluorimeter. Ca^{2+} was calibrated with a value of 224 for the K_d of Fura-2.

Measurement of Chemiluminescence. Luminol-amplified chemiluminescence (CL) was measured in a Berthold luminometer at 37°C basically as described in reference 27. Briefly, neutrophils (10^6 cells/ml) were incubated in 1 ml of PBS buffer supplemented with 10 mM glucose, 500 μ M CaCl₂, 5 μ M luminol, 100 μ M sodium azide for 5 min. Then allergens as stimulants at the indicated doses were added, to give a final volume of 1 ml. The CL response was measured every 1 min in each tube. Data were expressed as the number of cpm recorded each minute over a 15-min period.

Coupling of FITC to Allergen Protein. Coupling was carried out with a conjugation kit in accordance with the instructions of the manufacturer (Quicktag FITC Conjugation Kit; Boehringer Mannheim Corp., Indianapolis, IN).

Coupling of Allergen Labeled with FITC to Neutrophils. This coupling was carried out as previously described (28), with minor modifications. In brief, after fixing in 3% paraformaldehyde, 7×10^6 cells in PBS with 10 mM glucose were incubated with allergen labeled with FITC (100 μ g/ml) for 16–18 h at 4°C. The cells were spun (500 rpm, 4°C, 5 min), and were decanted, 10 ml of PBS, 1% HSA was added, and the cells were resuspended, and spun again. This washing was repeated three more times. Finally the cells were resuspended in 500 μ l of PBS, 10 mM glucose.

Fluorescence Staining for Microscopy Analysis. The method previously described (29), with minor modifications, was followed. Neutrophils were fixed for 20 min in 3% paraformaldehyde in PBS buffer, pH 7.4. After washing, cells (1.5×10^5 cells per 40 μ l) were suspended in PBS with 10 mM glucose and were attached to polylysine coated slides for 30 min at 25°C. Cells were incubated with specific allergen, labeled with FITC, for 16–18 h at 4°C in the dark and in a humid chamber. The slides were rinsed with PBS with 1% HSA once and rinsed again with PBS alone. Slides were processed for epifluorescence microscopy using 10% PBS, 90% glycerol as mounting medium.

Flow Cytometry Analysis. Flow cytometry was performed on an Epics Elite flow cytometer (Coulter Corp., Hialeah, FL) and calibrated by standard techniques. For each stained sample, 2×10^4 cells were analyzed for FITC fluorescence intensity. The fluorescent distribution for both control (cell autofluorescence) and test cells with allergen labeled with FITC was analyzed with a Coulter Elite software workstation.

Results

Respiratory Burst. Fig. 1, A and B, illustrates the kinetic effect of G₃ allergen on respiratory burst for neutrophils obtained from two patients who expressed high levels of specific IgE for the G₃ pollen. The onset of luminescence began 2 min after the G₃ additions in both cases. Samples from all patients studied ($n = 12$) gave a similar response. The kinetic study shows that maximum levels for each dose of allergen are obtained 7–8 min after the addition of G₃ to cells. Plotting the luminescence data from Fig. 1 against the dose of G₃ shows a sigmoid relationship between the two parameters (Fig. 2). This could suggest some type of cooperation in the occupation by the allergen of the IgE-binding sites located on the surface of cells. Neutrophils from healthy donors were always simultaneously assayed and taken as controls. After G₃ challenge in these cells, no difference in values was detected (data not shown).

Fig. 3 shows the specificity of the response to allergens.

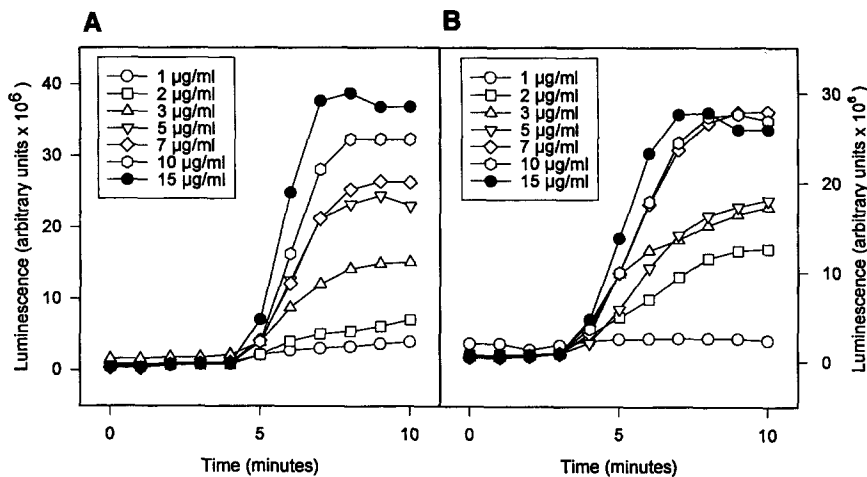


Figure 1. Activation of neutrophils by a specific allergen (G_3). Neutrophils isolated from two allergic patients (each represented separately in *A* and *B*) were suspended (10^6 cells/ml) in 1 ml of PBS buffer supplemented with 10 mM glucose, 500 μ M $CaCl_2$, 100 μ M sodium azide and the addition of different concentrations of G_3 , at the following final concentrations: 1 (\circ), 2 (\square), 3 (\triangle), 5 (∇), 7 (\diamond), 10 (\circ), and 15 μ g/ml (\bullet). At the same time, 5 μ M luminol was added. The suspension was incubated at 37°C, and the chemiluminescence response was recorded for 10 min. Similar results were obtained in at least eight other experiments using neutrophils from different patients with clinical allergies to G_3 allergen.

Neutrophils from a patient who was specifically allergic to G_3 show a clear response when cells were incubated with this pollen. However, when cells were incubated with D_1 or T_9 (to which the patient was not sensitized), no respiratory burst was detected. In other experiments, neutrophils from patients allergic to T_9 or D_1 were incubated with the specific pollen. Fig. 4 illustrates a dose-dependent response in the activation of respiratory burst when cells were challenged with different doses of T_9 (*A*) or D_1 (*B*). These data show evidence that the activation of respiratory burst in sensitized neutrophils is not restricted by the type of allergen. The specificity of the response to allergens was further assessed with neutrophils from subjects without any allergic pathology. Fig. 5 illustrates the absence of response to G_3 , T_9 , D_1 , and W_6 in neutrophils from a healthy donor who is not sensitive to those allergens. Another seven separate experiments performed on neutrophils from healthy donors gave similar results (data not shown). The respiratory burst was normal when cells were treated with mezerein. This indicates that the lack of response to these pollens was due

to some alteration in the binding of pollen to the membrane (e.g., no recognition of the stimulus or absence of receptor [IgE] for the antigen) but not in the mechanism of the NADPH-oxidase complex.

Next, experiments were performed to analyze whether unspecific anti-IgE could induce the stimulation of respiratory burst. To this end, neutrophils from allergic or healthy donors were incubated with 0.8 mg/ml or 1.2 mg/ml of anti-IgE. Fig. 6 illustrates that both preparations of cells (i.e., allergic [*A*] and nonallergic [*B*]) produced a clear and rapid increase in respiratory burst. This finding seems in agreement with the fact that neutrophils express IgE molecules in a significant percentage (18).

Ca²⁺ Mobilization and Binding of Allergens to Surface Cells. Early work indicates that mobilization of intracellular Ca^{2+} plays a role in the onset of respiratory burst (30), although this has been discussed by others (31). In the present study, we address the issue of whether Ca^{2+} participates in allergen-induced respiratory burst. Fig. 7 illustrates that in neu-

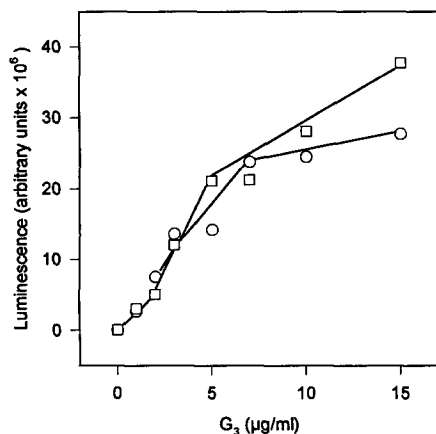


Figure 2. Dose-dependence of a grass (G_3) on respiratory burst by sensitized neutrophils. Shown values were taken from Fig. 1 at 7 min of incubation.

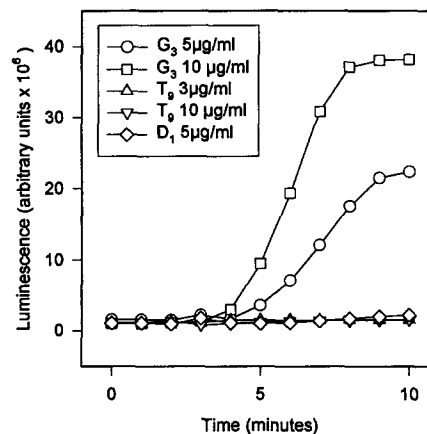


Figure 3. Specific activation of sensitized neutrophils by different allergens. Neutrophils from a patient allergic to G_3 were challenged with G_3 , T_9 and D_1 allergens. Other experimental conditions were as in Fig. 1. Lack of response to allergens different than those producing clinical symptoms in patients was observed in 9 cases out of 10 studied.

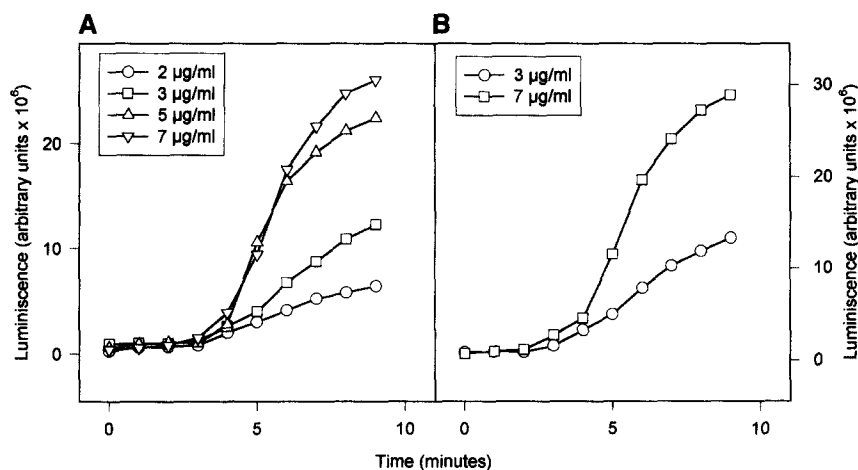


Figure 4. Specific activation of sensitized neutrophils by T₉ (A) or D₁ (B) allergens. (A) Neutrophils from a patient allergic to T₉ were challenged with different concentrations of T₉, as indicated in the figure. (B) Neutrophils from a patient allergic to D₁ were incubated with two concentrations of D₁, as indicated in the figure. Other experimental conditions were as in Fig. 1. Typical result of two other experiments is shown.

trophils from allergic patients, Ca²⁺ levels increased when the cells were challenged with the specific allergens. Thus, neutrophils from patient 1, sensitized to D₁ and G₃ (Fig. 7, 1-a and 1-b) showed a clear mobilization of Ca²⁺ levels when they were incubated with D₁, at 30 µg/ml, or G₃ at indicated concentrations. In this last case, a dose-dependent response was observed. In neutrophils from patient 2 (Fig. 7, 2-a and 2-b), sensitized to D₁ and T₉, and challenged with these pollens, a similar increase of Ca²⁺ was detected, although at a lower level. In patient 3 (Fig. 7, 3-a and 3-b), who gave a negative result for D₁ and positive to T₉ in the

skin test, these allergens altered the Ca²⁺ levels according their allergic capacity. With neutrophils from healthy donors, no Ca²⁺ mobilization after the addition of these allergens was observed (data not shown). In addition, there was a high degree of specificity in the response, since it was not altered either by other allergens (to which the patients were not sensitive) or by neutrophils from healthy donors. Fig. 8 illustrates that the addition of anti-IgE Ab to neutrophils from an allergic patient and from healthy donors produced an increase in Ca²⁺ levels. This was corroborated in four separate experiments. Challenging neutrophils with anti-

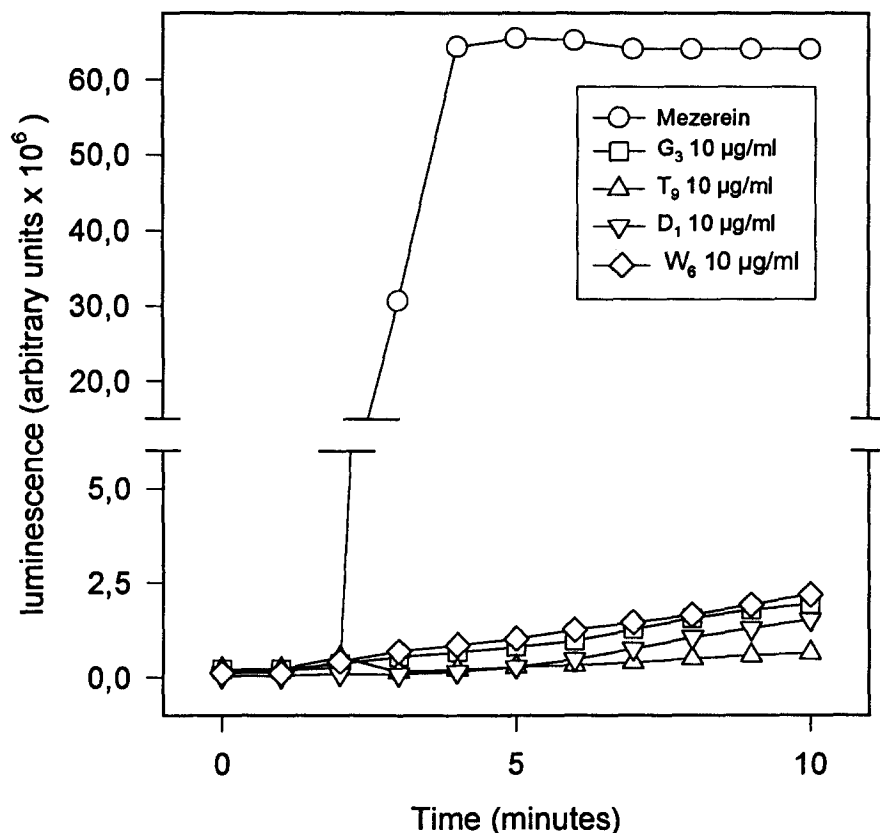


Figure 5. Lack of response to allergens by neutrophils from healthy donors. Neutrophils (10⁶ cells/ml) from healthy donors were incubated with allergens, as indicated in the figure or with 100 µM-mezeroin (○). Similar results were systematically obtained in 10 other experiments performed with cells from different donors.

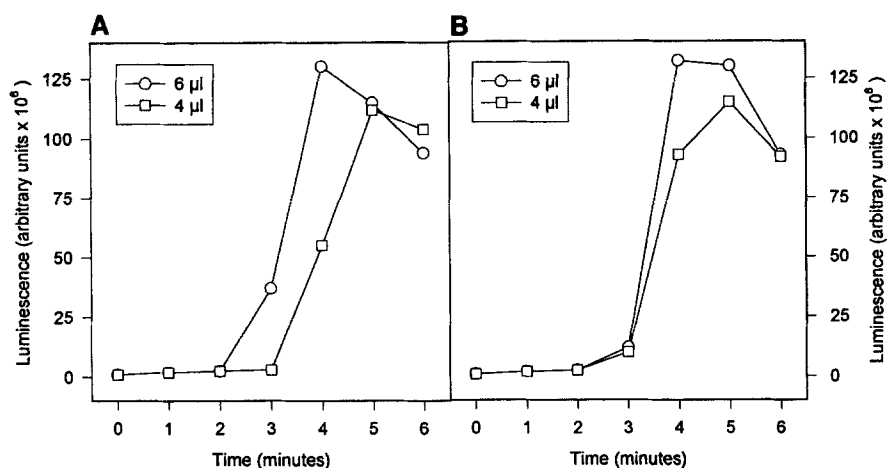


Figure 6. Effect of anti-IgE on neutrophil activation. Neutrophils (10^6 cells/ml) from an allergic (A) or a healthy (B) donor were incubated in triplicate with the indicated volumes of anti-IgE (from a preparation at $1 \mu\text{g/ml}$). Chemiluminescence was recorded. Three separate experiments gave the same results.

IgE Ab, we systematically observed greater Ca^{2+} levels in cells from allergic patients than from healthy donors.

To determine whether allergens can specifically bind to neutrophils, we searched for an FITC-labeled allergen capable of staining the cells isolated from allergic subjects. As observed in Fig. 9, flow cytometry studies demonstrated an increased binding of FITC-labeled G_3 and T_9 (c and d) in neutrophils from patients with allergy to these pollens, compared with FITC-labeled D_1 (to which the patients were not sensitive [b]) or from cell autofluorescence (a). These data verify the linking of allergens to structures located on the surface of sensitized neutrophils and, in addition, the specificity of such linking. These structures capable of binding allergens are presumably the Mac-2/εbp receptor-IgE complex, as was demonstrated previously (21). The attachment of neutrophils from allergic patients to FITC-labeled G_3 was further assessed by microscopy analysis of stained cells. Fig. 10 illustrates the interaction of

labeled allergens and neutrophils. As expected from flow cytometry data, only cells from patients sensitized to G_3 were positively stained (Fig. 10 A). By contrast, D_1 added to neutrophils from a subject who was not sensitive to this allergen did not give any fluorescence signal, or it was very weak (Fig. 10 C). Also, neutrophils from patients sensitized to T_9 (Fig. 10 E) or D_1 (Fig. 10 G) were positively stained when they were challenged with the specific FITC-labeled allergen.

Discussion

Activated neutrophils produce oxyradicals that are essential for microbicidal and cytotoxic activities and play an important role in the host defense system (32). However, the overproduction of oxyradicals by neutrophils, as in chronic inflammatory diseases, may lead to severe local tissue damage.

The experiments described here provide the first evi-

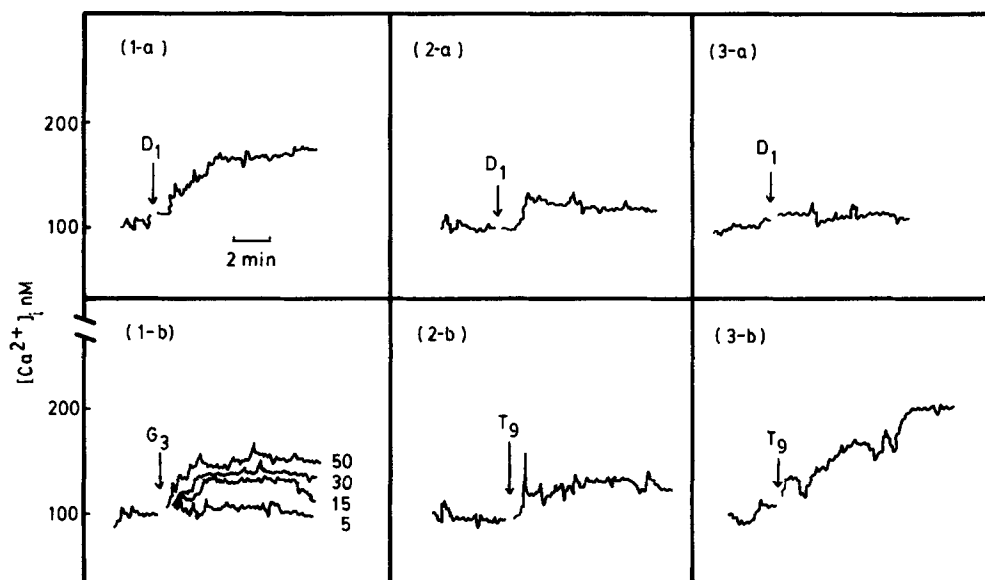


Figure 7. Ca^{2+} increase in neutrophils from different allergic patients challenged with their specific allergens. Neutrophils (10^7 cells/ml) from three allergic patients who presented clinical symptoms as indicated: patient 1 to D_1 and G_3 (profiles 1-a and 1-b); patient 2 to D_1 and T_9 (profiles 2-a and 2-b); and patient 3 to T_9 , only (profiles 3-a and 3-b). The cells were loaded with Fura-2AM, and intracellular Ca^{2+} was analyzed. The added dose of allergens was $30 \mu\text{g/ml}$ in all cases, except in 1-b, where it is indicated in the margin (in micrograms per milliliter).

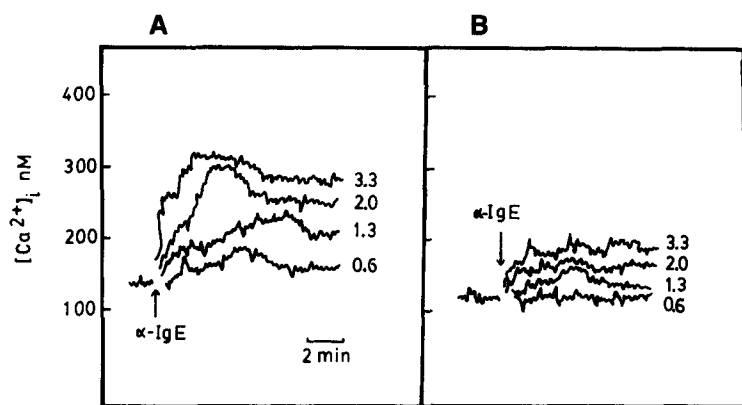


Figure 8. Ca^{2+} mobilization induced by anti-IgE. Loaded Fura-2AM neutrophils (10^7 cells/ml) from allergic (A) or healthy (B) donors were incubated with the indicated doses of anti-IgE, and changes in Ca^{2+} levels were analyzed.

dence that neutrophils from allergic patients are sensitive in vitro to specific allergens that lead to their activation. Neutrophil activation was initiated when specific allergens bound to preattached surface IgE molecules. This activated state was evidenced by a clear increase in the respiratory burst of the neutrophils from allergic patients when they were incubated in the presence of allergens. The apparent half-maximal dose ($\sim 30 \mu\text{g/ml}$) calculated for G_3 allergen from data presented in Fig. 2 is in keeping with data observed for other biochemical systems. Thus, in T cell proliferation studies, the effective doses of allergens were in the range of $0.1\text{--}30.0 \mu\text{g/ml}$ (33), and in the analysis of IL-4 production by CD4^+ T cell clones, the concentration of $50 \mu\text{g/ml}$ of allergen was studied (34). Also, in mononuclear cells, the synthesis of IL-4 and IFN- γ was induced by $10 \mu\text{g/ml}$ of allergen (35). Our results indicate that in vitro activation of neutrophils was very specific for certain aller-

gens. Thus, allergens other than those that produce clinical symptoms did not stimulate the respiratory burst, and also, allergens were ineffective on neutrophils from healthy donors. Present data also show that the activation of cells by allergens was rapid (~ 1 min) and that the magnitude of respiratory burst was dependent on the dosage of allergen.

The nature of the mechanisms whereby allergens promote the respiratory burst is not fully understood. Our results suggest that allergens are bound to preformed structures on the neutrophil membrane. The pivotal observation was made recently by Truong et al. (18). Besides the well-known receptors (e.g., $\text{Fc}\epsilon\text{RI}$ and $\text{Fc}\epsilon\text{RII}$) for IgE (10–15), a third type of IgE-binding structure has been described by these authors, the so-called IgE-binding protein, ϵbp (18). cDNA coding for rat and human ϵbp revealed that this protein is distinct from $\text{Fc}\epsilon\text{RI}$ and $\text{Fc}\epsilon\text{RII}$ (36). Subsequently, it was demonstrated that ϵbp is identical to CBP35

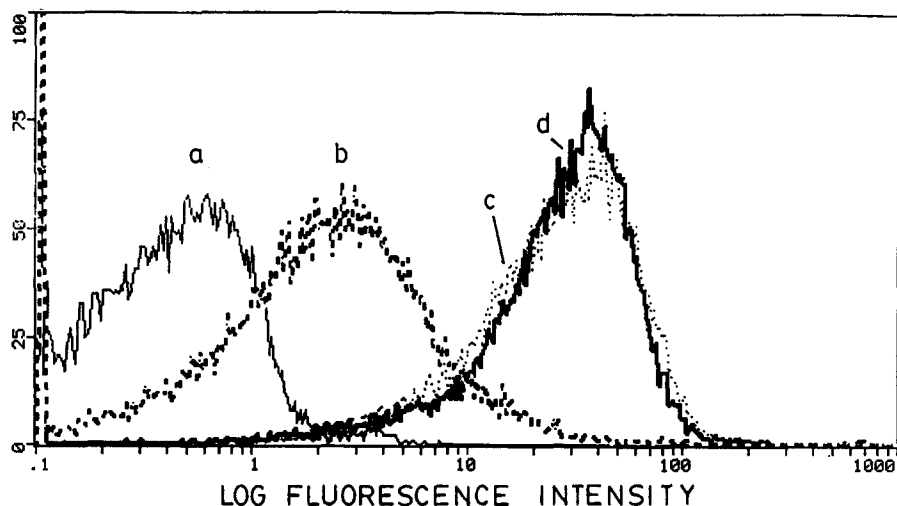
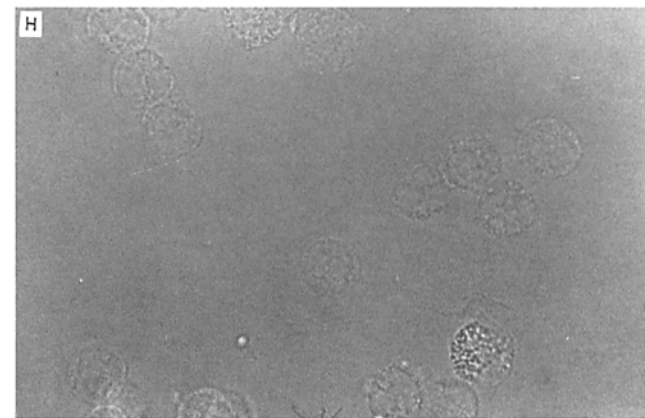
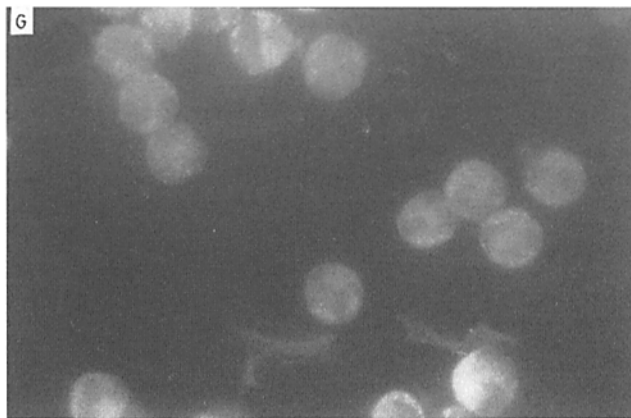
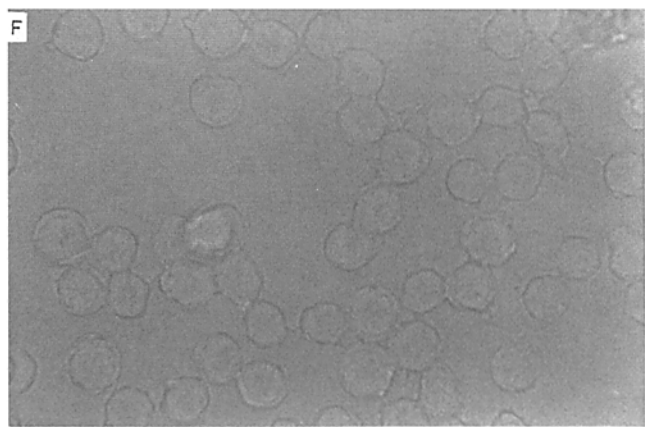
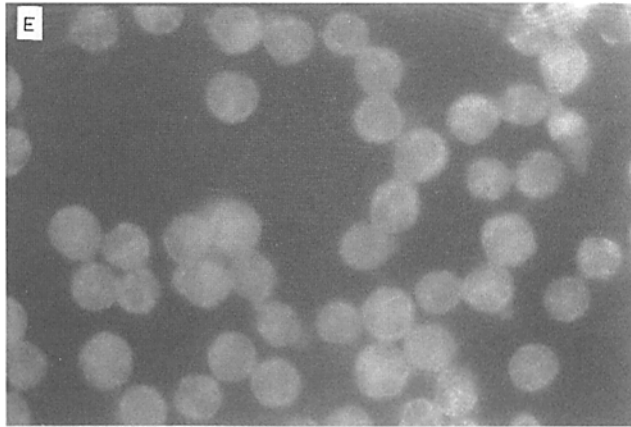
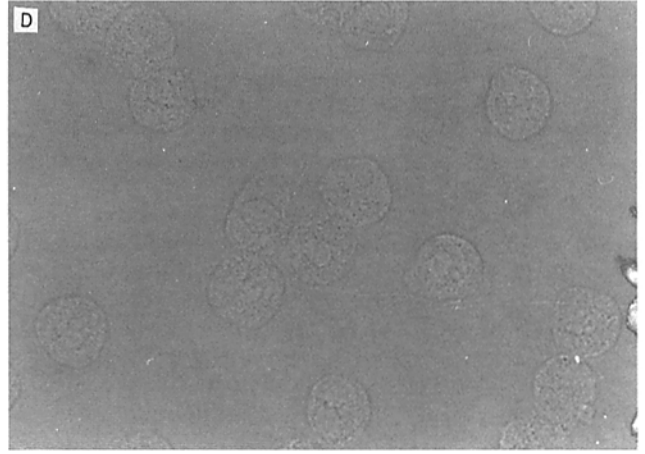
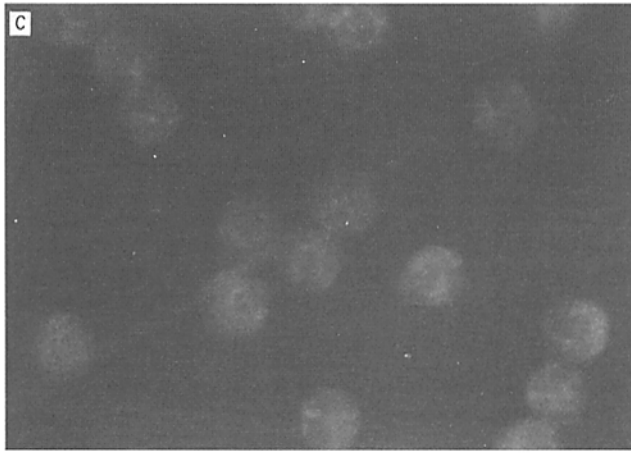
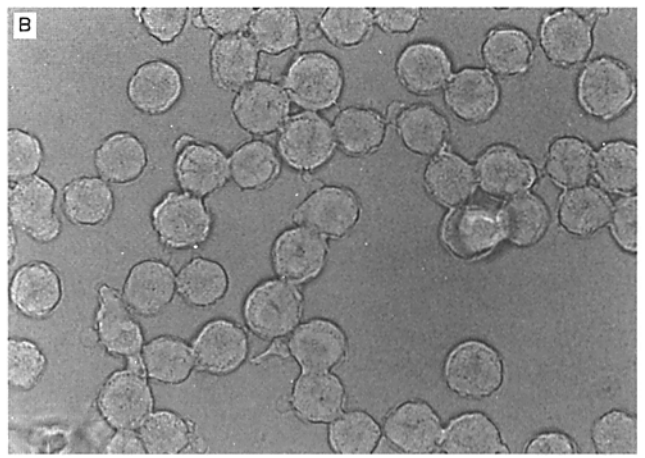
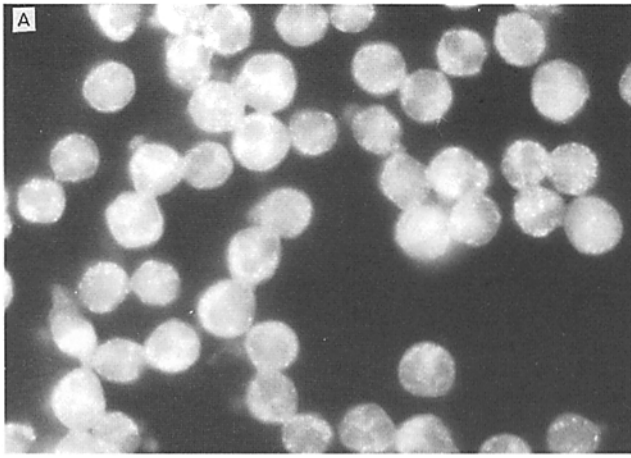


Figure 9. Binding of FITC-labeled allergens to neutrophils analyzed by flow cytometry. After fixing in 3% (wt/vol) paraformaldehyde, neutrophils (7×10^6 cells/ml) from a patient allergic to G_3 and T_9 were incubated at 4°C for 18 h, either without additions (a) or in the presence of FITC-labeled D_1 (b), G_3 (c) and T_9 (d), and were further processed as indicated in Materials and Methods. Data correspond to an experiment representative of three performed.

Figure 10. Fluorescence microscopy location of FITC-labeled allergens bound to neutrophils. Cells from G_3 (A–D), T_9 (E and F), or D_1 (G and H) allergic donors were fixed and plated to polylysine-coated slides. Slides were incubated with FITC-labeled G_3 (A and B) and FITC-labeled D_1 (C and D) (to which the patient was not sensitive), FITC-labeled T_9 (E and F), or FITC-labeled D_1 (G and H) for 18 h at 4°C and processed as indicated in Materials and Methods for fluorescence microscopy (A, C, E, and G) and light microscopy (B, D, F, and H).



(37), Mac-2 (19), and HL-29 (38). All these structures share common characteristics, including a molecular mass of 29–35 kD (39). It is noteworthy that neutrophils represent the only blood cell population that does not express conventional Fc receptors for IgE. Truong et al. (18) also observed that the addition of polyclonal IgE antibodies increased the respiratory burst in neutrophils from healthy donors. Present data (Fig. 8) are in agreement with this observation and, in addition, extend the study to populations of neutrophils from allergic patients.

On the basis of previous data (17), it may be hypothesized that allergens could bind to specific IgE antibodies that are previously linked to its receptors. This mechanism, based on the specificity of the binding between allergens and IgE molecules, would explain why neither neutrophils from healthy donors nor the presence of other allergens different to those that have previously sensitized the neutrophils stimulates respiratory burst. In these cases, allergens are not specifically bound to the preexistent IgE molecules on the cell surface, and the neutrophils remain inactive. Conceivably the addition of polyclonal anti-IgE antibodies triggers a response that is the same as the one produced by its natural ligands (e.g., allergens).

With regard to the intracellular molecular events trig-

gered by the interaction of allergens, our data show evidence that Ca^{2+} is mobilized. Previously it had been shown that intracellular Ca^{2+} mobilization is a requirement for respiratory burst (30). The mechanism by which an allergen induces Mac-2-mediated signal transduction in neutrophils is unknown at the present. By analogy with the high-affinity receptor for IgE (FcεRI), it may be postulated that the covalent modification by phosphorylation through protein-tyrosine kinase as well as through serine/threonine kinases is an early molecular event occurring after cross-linking of the Mac-2 receptor. It is noteworthy that the observed response in Ca^{2+} production was very rapid after challenge of neutrophils from allergic patients with a specific allergen.

Recently, Yamaoka et al. (40) described recombinant Mac-2/εbp exogenously added to human neutrophils as enhancing superoxide production. This observation suggests an extracellular function of this protein once it has been exocytosed (41). Comparing these with the present data (e.g., stimulation by allergens, reported in the present study, or by Mac-2/εbp, reported in [40]) may suggest that the molecular pathways involved in the stimulation of NADPH-oxidase presumably act throughout IgE binding on the cell surface.

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