

Human neutrophils synthesize IL-8 in an IgE-mediated activation

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Abstract: It has been demonstrated that neutrophils are responsible for the release of large amounts of the inflammatory chemokine interleukin-8 (IL-8), associated with inflammation. To further define the mechanisms implicated, we have analyzed the response of human neutrophils from allergic patients to specific antigens or challenge with anti-immunoglobulin (Ig)E antibodies. Neutrophils showed a dose- and time-dependent production of IL-8. The release of the cytokine was parallel to expression of IL-8 mRNA analyzed by the polymerase chain reaction. This expression was transient—it occurred after 3 h of anti-IgE treatment and was maintained for 18 h. Trifluoperazine, EGTA, reduced nicotinamide adenine dinucleotide phosphate-oxidase inhibitors, and reactive oxygen species (ROS) scavengers inhibited IL-8 production, indicating a critical dependence of calcium and oxidative stress. Moreover, an inhibitory effect of cyclosporin A, an immunosuppressor that inhibits calcineurin activity, on IL-8 release and IL-8 mRNA expression was observed. This is the first evidence of the involvement of ROS and calcium/calcineurin in IgE-dependent IL-8 production. These findings open new perspectives into the functional role of neutrophils in IgE-associated diseases. *J. Leukoc. Biol.* 76: 692–700; 2004.

Key Words: anti-IgE · ROS · Ca²⁺ · calcineurin

INTRODUCTION

Interleukin (IL)-8, a member of the CXC chemokine family [1], is a cytokine that has a wide spectrum of biological activities on different cell types, such as neutrophils, T cells, and basophils [2–4]. The biological properties of IL-8, including chemotaxis of neutrophils and T lymphocytes [5–7], implicate IL-8 as a key factor in acute inflammatory processes, such as activation of neutrophils [1, 8], up-regulation of adhesion molecules [2], and leukocytosis [9], as well as in the pathogenesis of inflammatory diseases [7]. Furthermore, the administration of a neutralizing antibody (Ab) against IL-8 inhibited neutrophil infiltration and tissue damage in several types of acute

inflammation [10], suggesting a causal role of IL-8 in inflammatory reactions. Although initially believed to be a primary product of monocytes, IL-8 is secreted as a 72- or 77-amino acid protein by a variety of cells, including neutrophils, stimulated with lipopolysaccharide (LPS), inflammatory cytokines [IL-1, IL-6, and tumor necrosis factor α (TNF- α)], microbial toxins, and other conditions that disturb homeostasis [1, 4, 11–14]. As accumulation of neutrophils in tissues is a characteristic feature of inflammation, this capacity to produce IL-8 suggests that these cells can, by themselves, enhance recruitment of new neutrophils at local inflammatory sites. Thus, close regulation of neutrophil migration is necessary to control inflammatory reactions. Excessive production of IL-8 may be involved in the pathogenesis of various types of inflammatory reaction. Moreover, various cytokines and agents have been reported to inhibit IL-8 production. Cytokines such as IL-4 [15], IL-10 [16], transforming growth factor- β , and interferon- β [17] counteract the induction of IL-8 production by IL-1, TNF- α , or LPS. Immunomodulatory drugs such as glucocorticoids [18] and vitamin D3 [19] also act as suppressors of IL-8 production. An immunosuppressive drug, cyclosporin A (CsA), has been reported to suppress the induction of IL-8 mRNA in human T lymphocytes [20].

Although eosinophils and lymphocytes have become more strongly associated with allergic diseases, peripheral blood neutrophils also show markers of activation during active asthma [21] and after exercise-induced asthma [22]. Previous studies have demonstrated the presence on human neutrophils of the three forms of the immunoglobulin (Ig)E receptor, Fc ϵ RI [23–25], Fc ϵ RII/CD23 [26–28], and galectin-3 [29–31]. We have previously shown that specific antigens were able to activate functional responses of neutrophils from allergic patients sensitized to antigens of the same type as those that produce clinical allergic symptoms [32–35]. Our group described the specific binding antigens on the surface of neutrophils, using flow cytometry and microscopy analysis [33]. Other groups [24, 27, 29, 31] have demonstrated the presence of IgE

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molecules bound on the surface of neutrophils. Furthermore, we have shown the specific IgE bound on the surface of neutrophils from allergic patients [32]. In the present work, we set out to analyze whether the attachment of cellular IgE molecules with anti-IgE/specific antigens was able to regulate IL-8 production and its gene expression in human neutrophils from patients sensitized to the antigens. Additionally, we have investigated whether reactive oxygen species (ROS) and calcium/calcieneurin could participate in that regulation. In this context, the evidence here shown points to neutrophils as cells implicated in the IgE-mediated inflammatory and pathological states, such as in allergic diseases.

MATERIALS 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). Dextran T-500 was obtained from Amersham Pharmacia Biotech (Barcelona, Spain). Goat anti-human IgE was from Caltag Laboratories (Burlingame, CA). Goat IgG (Sigma-Aldrich Co., Alcobendas, Madrid, Spain) was taken as negative control. Low endotoxin fetal bovine serum, trifluoperazine (TFP), N-formyl-methionyl-leucyl-phenylalanine (fMLP), LPS (from *Escherichia coli*), diphenylene iodonium (DPI), pyrrolidine dithiocarbamate (PDTTC), 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), and 4-hydroxy-3-methoxyacetophenone (HMAP) were from Sigma-Aldrich Co. Ficoll-Hypaque, RPMI-1640 culture medium, antibiotics, and L-glutamine were from Bio-Whittaker (Verriers, Belgium). CsA was kindly provided by Dr. Jean-François Borel (Sandoz Ltd., Basel, Switzerland). Polymerase chain reaction (PCR) primers were synthesized by Amersham Pharmacia Biotech. Horseradish peroxidase (HRP)-conjugated anti-human IgE Ab, which does not cross-react with other human Igs [36, 37], was from Dako (Copenhagen, Denmark). Other biochemicals were from Merck (Barcelona, Spain). All reagents used in the culture of cells had endotoxin levels of <0.01 ng/ml, as tested by the *Limulus* lysate assay (Coatest, Chromogenity, Mölndal, Sweden).

Preparation of polymorphonuclear leukocytes and cell culture

The group studied included adult atopic patients with bronchial asthma and healthy adult nonatopic volunteer controls. The asthmatic patients had positive skin-prick tests (Bial-Aristegui) and specific IgE (HYTEC 288, IZASA, Barcelona, Spain) to at least one common antigen (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 tests and specific IgE to a battery of inhalant antigens. The Hospital Universitario Virgen Macarena (Sevilla, Spain) Ethics Committee approved the study, and each subject gave informed consent.

Human neutrophils were purified from fresh-drawn, heparinized (10 U/ml) venous blood as follows: The blood (20 ml) was mixed with 1.5 ml 10% Dextran T 500 (final concentration of 0.7%), dissolved in phosphate-buffered saline (PBS). The supernatant fraction was centrifuged through a Ficoll-Hypaque. The neutrophil-rich pellet was then removed with a plastic pipette, and red cells were eliminated by one hypotonic lysis in water [32–35, 38]. Neutrophils were washed twice with PBS, resuspended in RPMI-1640 medium supplemented with 10% fetal serum albumin, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B, and cultured at 37°C under a humidified air atmosphere containing 5% CO₂ and 95% O₂. For further purification, contaminating eosinophils were depleted with mouse anti-human CD9 Ab (Immunotech-IZASA) and goat anti-mouse IgG micro-magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany). The purity of neutrophils was, on average, >99%. Eosinophil contamination was ≤1%.

For stimulation treatments, the cells were incubated with allergens, IgG, or anti-IgE for the indicated times at 37°C. The inhibitors EGTA, TFP, and PDTTC were added 60 min and CsA, DPI, TEMPO, and HMAP, 30 min prior to stimulation. None of these reagents affected the viability of the cells at the concentrations used. Trypan blue exclusion showed greater than 96% viability in neutrophils. In all cases, the cells were maintained in culture for the same time in the presence or absence of stimuli.

Dissociation of neutrophil-bound Igs and immunoblotting methods

Ig molecules were dissociated from the surface of neutrophils as described previously [32]. Briefly, after isolation, cells (1×10⁸ cells) were resuspended in 1 ml acetate buffer (50 mM sodium acetate, pH 4, 85 mM NaCl, 5 mM KCl, supplemented with 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 7.4) was then added to the treated cells, and the mixture was centrifuged at 500 g for 10 min. Supernatants were collected and neutralized with 1 N NaOH and used for specific IgE and IgG analyses. Antigenic extracts were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry device (Bio-Rad, Richmond, VA). After transference and blocking, membranes were probed for 12 h at room temperature with previously obtained IgE-containing supernatants, which were diluted 1:7 in GVB. After washing, filters were incubated with HRP-conjugated anti-human IgE, diluted 1:750 in GVB buffer for 1 h at room temperature, and then washed. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) assays as described previously [38].

Analysis of IL-8 release

Cells were dispensed in 24-well tissue-culture plates (Nunc, Roskilde, Denmark) at a density of 2 × 10⁶ cells/ml and cultured in the absence or presence of additions at the times and doses indicated in each case. Supernatants were harvested and stored at –80°C until measuring and thawed only once for cytokine detection. IL-8 in the supernatants was measured using the enzyme-linked immunosorbent assay (ELISA) kit obtained from Bender MedSystems (Vienna, Austria).

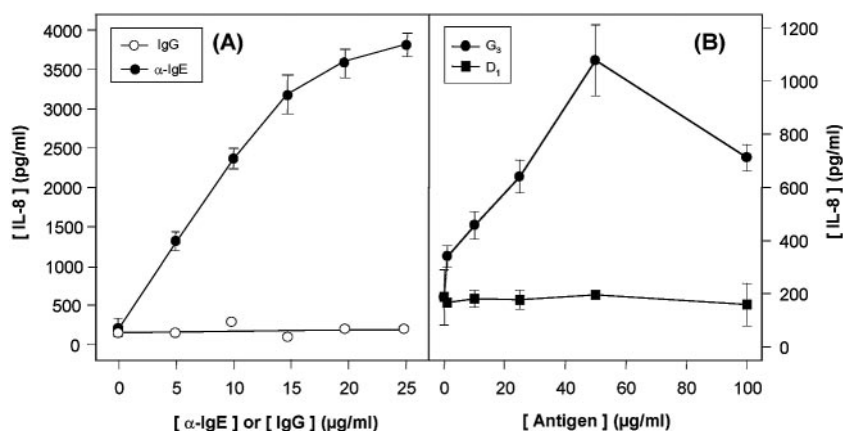
RNA extraction, reverse transcriptase (RT), and conventional PCR

As RT-PCR is a sensitive method, it was important to isolate RNA for extremely pure neutrophils to avoid false-positive results as a result of contamination with eosinophils. To this end, neutrophils for RT-PCR were purified by the micromagnetic beads system as described above, but the procedure was repeated three more times using 4 µg/ml CD9 Ab each time. Effective removal of eosinophils was evaluated by flow cytometry [fluorescein-activated cell sorter analysis (EPICS Elite, Coulter-IZASA)] with anti-CD9-RD1 Ab (Coulter-IZASA). The contaminating eosinophils were between 0.001% and 0.004% [39, 40]. Total RNA was isolated from 5 × 10⁶ neutrophils using the RNA isolation kit RNeasy (Qiagen, IZASA), following the manufacturer's protocols. Total RNA (1 µg) was reverse-transcribed to first-strand cDNA using random primers. The first-strand cDNA was amplified with primer sets for human IL-8 by conventional PCR [41]. Aliquots taken after 30 cycles (20 µl) were electrophoresed on 1.5% agarose gels. The IL-8 0.2-kb and β-actin 0.5-kb PCR products were visualized on ethidium bromide-stained agarose gels and photographed under UV light.

Real-time PCR

After total RNA extraction, 750 ng was subsequently used for cDNA synthesis as described above. Real-time PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Qiagen, IZASA, Portugal) and the thermocycler conditions recommended by the manufacturer. PCR reactions were performed in triplicate in a total volume of 25 µl containing 2 µl of the RT reaction. Each sample was analyzed for rRNA 28S to normalize for RNA input amounts and to perform relative quantifications. Primers were designed using the computer program Primer Express (Applied Biosystems). Primers (IL-8 forward: 5'-

Fig. 1. Dose-dependent effect of anti-IgE Ab and specific antigen on IL-8 production from human neutrophils. (A) Neutrophils (10^6 cells/0.5 ml) obtained from allergic patients were incubated with increased amounts of anti-IgE (α -IgE) for 18 h (●). The control goat IgG is shown (○). Human IL-8 content in the culture supernatants was determined using an ELISA assay. (B) Neutrophils (10^6 cells/0.5 ml) from allergic patients who were specifically sensitized to G_3 were incubated with G_3 (●) or D_1 (■), to which the patients were not sensitized. The values shown are the mean $\pm$ SEM from four independent assays in which each measurement was performed in duplicate for each group.



ATTAGCCACCATCTTACCTCACAGT-3'; IL-8 reverse: 5'-GTGCTTCCACATGTCCTCACA-3'; rRNA 28S forward: 5'-GTGACGCGCATGAATGGA-3'; rRNA 28S reverse: 5'-CCCTTGGCTGTGGTTTCG-3') were generated and used to amplify 50-bp and 100-bp fragments, respectively. Melting curve analysis showed a single sharp peak with the expected temperature melting (T_m) for all samples. For the relative quantification of gene expression, the comparative threshold cycle (C_T) method was used as described in User Bulletin 2 for ABI Prism 7700 sequence detection system. C_T represents the PCR cycle at which an increase in reporter fluorescence above a background signal can first be detected (10 times the standard deviation of the baseline). First, internal control (rRNA28S) C_T values were subtracted from the gene-of-interest C_T values to derive a ΔC_T value. The relative expression of the gene-of-interest was then evaluated using the expression $2^{-\Delta\Delta C_T}$, where the value for $\Delta\Delta C_T$ was obtained by subtracting the ΔC_T of the calibrator from each ΔC_T , using the mean of the control ($t=0$) as the calibrator.

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. A P value <0.05 was considered significant.

RESULTS

Induction of IL-8 production by antigens and anti-IgE Ab in human neutrophils

As polymorphonuclear leukocytes express receptors to IgE and are able to bind IgE molecules [29, 32], in the present work, we set out to analyze whether the challenge of human neutrophils with anti-IgE/specific antigens was able to regulate the inflammatory cytokine IL-8 production. In this regard, neutrophils from allergic patients were incubated with anti-IgE Ab, and the IL-8 levels in the supernatant were measured. As is shown in **Figure 1A**, anti-IgE Ab challenge released significant levels of IL-8. The negative-control goat IgG did not produce any appreciable levels of IL-8 (Fig. 1A). Figure 1B shows the specificity of the response to antigens. Neutrophils from patients who were specifically allergic to G_3 show a clear response when cells were incubated with this antigen. However, when cells were incubated with D_1 (to which the patients were not sensitized), no IL-8 release was detected. Antigens were ineffective on neutrophils from healthy donors.

The time-course of IL-8 production by neutrophils from allergic patients, stimulated with different agents, is shown in **Figure 2**. The accumulation of IL-8, which was already significantly elevated after 3 h for anti-IgE (15 μ g/ml) or fMLP

(100 nM), increased slightly until 6 h and then rose dramatically up to 18 h. However, 1 μ g/ml LPS-dependent IL-8 accumulation became significant only at 6 h of incubation and then continued to rise until 18 h. The maximum response reached was observed with LPS ($P<0.001$ vs. anti-IgE or fMLP treatment), and the treatment with anti-IgE Ab was higher than the fMLP-dependent effect ($P=0.002$).

IL-8 mRNA expression by anti-IgE stimulation in human neutrophils

To examine whether the IL-8 gene expression was parallel to the release of cytokine, the expression of IL-8 mRNA was analyzed by RT-PCR. To this end, neutrophils from allergic patients were incubated with anti-IgE Ab (15 μ g/ml) at different times (3, 6, and 18 h), and IL-8 mRNA was analyzed.

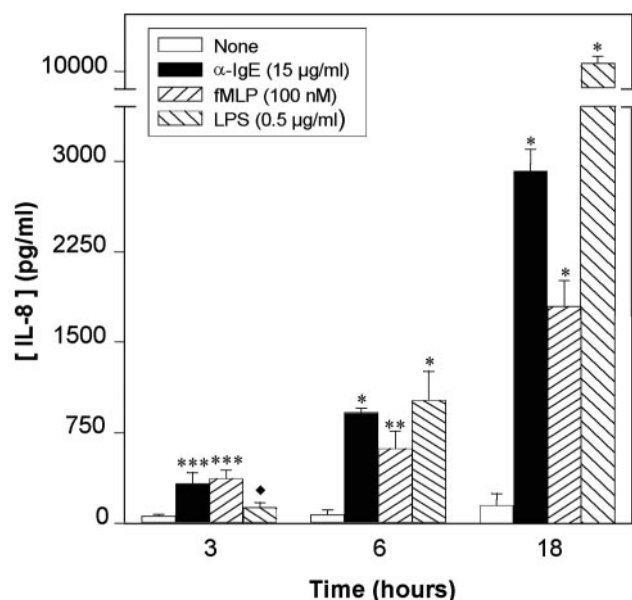


Fig. 2. Time-course of IL-8 release by neutrophils stimulated with anti-IgE Ab, fMLP, and LPS. Neutrophils isolated from allergic patients were incubated at 37°C with 15 μ g/ml anti-IgE (α -IgE), 100 nM fMLP, and 0.5 μ g/ml LPS. At indicated times, incubation medium was collected for IL-8 measurement. Plotted values are the mean $\pm$ SEM from five assays in which each measurement was performed in duplicate. Statistical significance (vs. control at each time): *, $P < 0.001$; **, $P = 0.006$; ***, $P = 0.007$; ♦, $P = 0.004$.

Conventional PCR analysis (**Fig. 3A**) shows that the induction appeared within 3 h of treatment and was maintained for 18 h. A small amount of mRNA appeared in unstimulated cells, in agreement with a previous report [42] describing a moderate transcription of IL-8 mRNA induced in human neutrophils by plastic adherence after 6 h of incubation. mRNA quantities were measured by real-time PCR. We observed that the treatment of cells with anti-IgE Ab induced an ~ 1.5 -fold accumulation of the IL-8 mRNA after 3 h of exposure, with a maximum effect at 18 h ($\sim$ fivefold induction).

Presence of specific IgE on the neutrophil surface from asthmatic patients

The presence of specific IgE from the neutrophil cell surface was examined in human neutrophils from asthmatic patients. Neutrophils from T_0 -sensitized patients were treated as indicated in Materials and Methods, and the supernatants obtained were used to analyze the reactivity against antigenic extracts separated by SDS-PAGE. Antigenic extracts are complex mix-

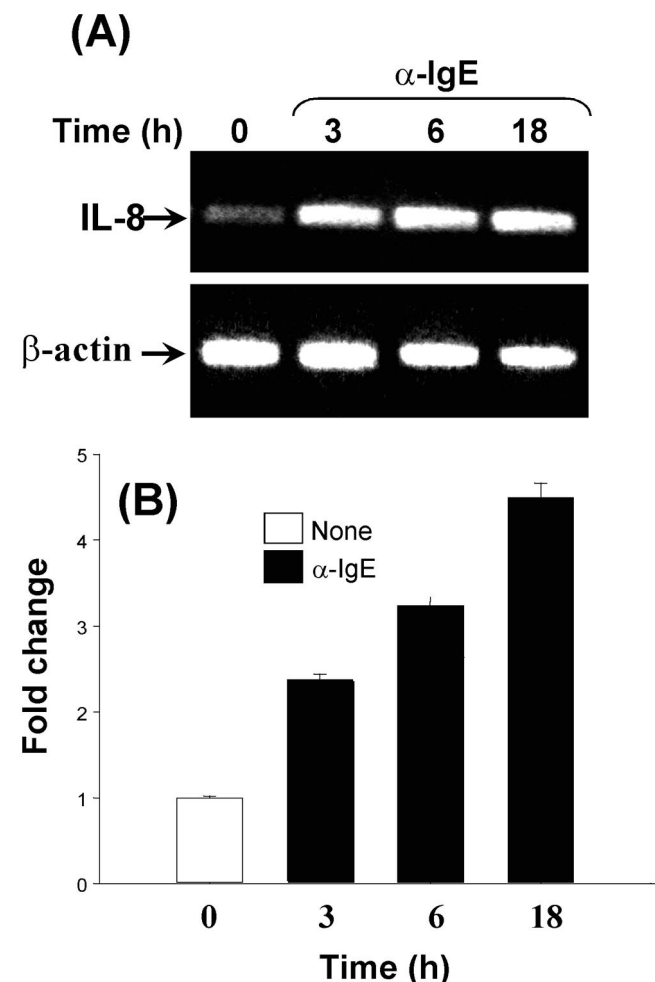


Fig. 3. IL-8 mRNA expression in anti-IgE Ab-stimulated human neutrophils from allergic patients. Neutrophils (5×10^6 cells/1 ml) were incubated in the presence or absence of 15 μ g/ml anti-IgE (α -IgE) for the indicated times. Total RNA was isolated and reverse-transcribed, and the resulting cDNA was amplified by conventional PCR (A) or by real-time PCR (B). In conventional PCR, β -actin was used to normalize the amount of RNA. For real-time PCR, we show the average fold induction $\pm$ SEM from two separate measurements.

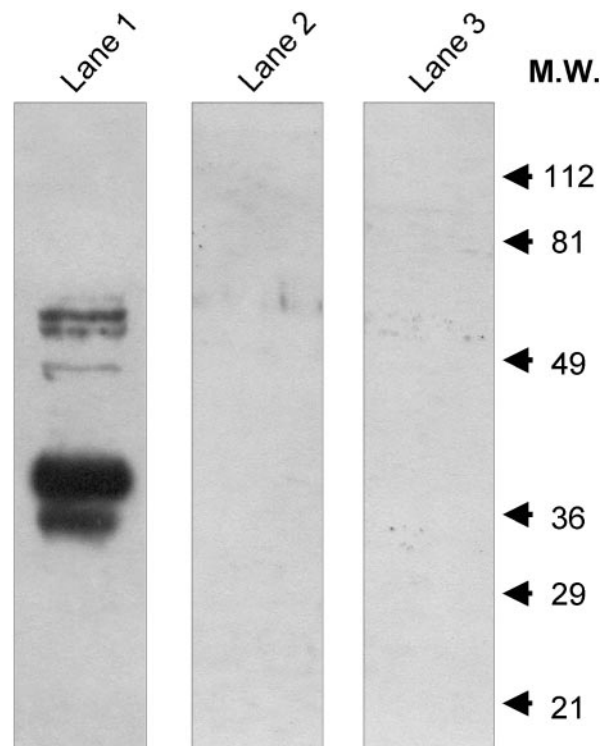


Fig. 4. Reactivity of specific IgE (eluted from the neutrophil cell surface) to allergenic extracts. The extracts of specific antigens were resolved by SDS-PAGE and transferred to PVDF membranes. Then, membranes were probed with different supernatants: Lane 1, T_0 extract was probed with supernatants from neutrophils of a patient with T_0 -specific IgE after the treatment to dissociate IgE from the cell surface; lane 2, T_0 extract was probed with supernatants from neutrophils of a patient with T_0 -specific IgE without the treatment to dissociate IgE from the cell surface; lane 3, D_1 extract was probed with supernatants from neutrophils of a patient with T_0 -specific IgE after the treatment to dissociate IgE from the cell surface. After washing, membranes were incubated with HRP-conjugated anti-human IgE and revealed with an ECL assay. Molecular weights (M.W.) are shown.

tures of proteins in which all components may be regarded as potential allergens. However, when these extracts are challenged with serum from allergic individuals, some constituents reveal higher IgE-binding prevalence than others. With regard to the T_0 pollen extracts, immunoblotting analysis has shown the presence of at least 20 protein bands with allergenic activity, and each individual serum is sensitive to one or more components [43, 44].

We have found (**Fig. 4**) immunoreactive bands only with T_0 antigen extracts (Lane 1), and no reactivity was detected when the eluted supernatants were incubated with other antigens extracts (i.e., D_1), to which the patients were not sensitized (Lane 3), or when the supernatants were from cells without treatment (Lane 2). Similar results were previously obtained for other antigens tested [32]. In the present case, the apparent molecular weight of the two major observed bands from T_0 extracts, which bind to IgE eluted from the neutrophil surface, were of ~ 36 and ~ 40 kDa. Other minor bands with molecular weight in a range of 49–60 kDa were also detected. The diversity of the size of these bands could represent different T_0 antigenic components recognized by neutrophils, with potential capacity to activate these cells (e.g., releasing IL-8 cytokine).

These results suggest the existence of specific IgE bound to the cellular membrane of human neutrophils from allergic patients.

Enhanced expression of IL-8 by anti-IgE Ab is dependent on a calcium- and ROS-activated pathway

As it has been reported that IL-8 expression is under the control of calcium [45, 46] and ROS production [47, 48], we analyzed the potential implication of these mediators in the IL-8 production stimulated by anti-IgE Ab. Data presented in **Table 1** indicate that treatment with trifluoperazine, an inhibitor of Ca^{2+} -dependent calmodulin (CaM) activity, decreased the IL-8 production generated by neutrophils exposed to anti-IgE Ab in a dose-dependent manner. In addition, preincubation with the calcium chelator EGTA abolished the release of IL-8 to the medium.

To study the role of ROS, we used several types of pharmacological inhibitor and free radical scavengers. The two inhibitors tested, HMAP, which alters reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity competing with NADPH for binding site on oxidase [49], and DPI, a reagent that blocks flavin adenine dinucleotide binding to a flavin site of the oxidase [50], effectively cancelled anti-IgE-induced IL-8 release (**Fig. 5A**) and synthesis of mRNA (**Fig. 5B**). Moreover, the scavengers assayed, PDTC, a reducing reagent that blocks the oxidative effect of O_2^-/ROS [51], and TEMPO, a well-known agent that cancels the oxidative effect of hydroxyl radicals and other oxygen-free radicals [52], drastically reduced the IL-8 production and synthesis of mRNA (**Fig. 5, A and B**).

CsA suppressed IgE-dependent IL-8 production by human neutrophils

One of the targets for calcium mobilization and intracellular redox status is calcineurin (phosphatase 2B) [53, 54]. The putative participation of calcineurin in anti-IgE-dependent IL-8 production was analyzed using CsA, an immunosuppressive agent that inhibits its phosphatase activity. The results presented in **Figure 6A** indicate that CsA interfered in a dose-dependent manner with the induction of IL-8 when neutrophils were stimulated with anti-IgE Ab. This inhibitory effect was also observed in a similar range in neutrophils from

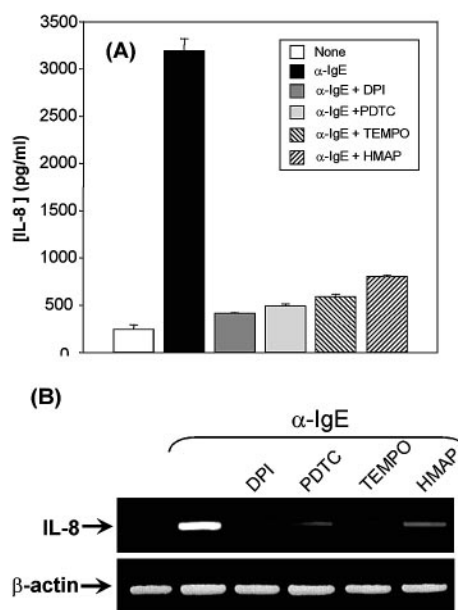


Fig. 5. ROS dependence of anti-IgE Ab-induced IL-8 production by human neutrophils. Cells from allergic patients were preincubated with the following agents: HMAP (500 μM), DPI (10 μM), or TEMPO (100 μM) for 30 min or PDTC (100 μM) for 60 min, as indicated. Then, anti-IgE (α -IgE; 15 $\mu\text{g}/\text{ml}$) was added over 20 h (A) or 3 h (B). IL-8 release (A) was assayed as indicated in Materials and Methods. IL-8 mRNA (B) was analyzed by conventional PCR. (A) Values shown are the mean $\pm$ SEM from three separate experiments, in which each measurement was performed in duplicate. (B) Data shown are representative of another three separate experiments.

G_3 -sensitized subjects challenged with this antigen. However, the stimulation of LPS-dependent IL-8 production was less sensitive to the effect of CsA than when anti-IgE Ab or G_3 were the stimuli (**Fig. 6B**).

To examine the possibility that the impairment of IL-8 production by CsA was a result of the inhibition of IL-8 mRNA expression, neutrophils from allergic donors were preincubated with the indicated concentration of CsA for 30 min before the addition of 15 $\mu\text{g}/\text{ml}$ anti-IgE Ab. **Figure 7** illustrates that the enhanced anti-IgE-dependent expression of IL-8 mRNA was blocked in neutrophils exposed to CsA. Figure 7 also presents the effect of treatment of cells with 2.5 mM EGTA. Thus, the suppression of extracellular calcium determined a partial reduction of IL-8 mRNA expression.

DISCUSSION

The results described here show for the first time that the IgE-dependent mechanism is an effective stimulus for IL-8 production by neutrophils. Our results indicate that in vitro activation of neutrophils was very specific for antigens. Thus, antigens other than those that produce clinical symptoms did not stimulate IL-8 production, and antigens were ineffective on neutrophils from healthy donors. Presumably, the mechanism of the antigen-dependent IL-8 release is through an IgE-dependent mechanism, as only patients with specific IgE on the neutrophil surface, positive to these antigens, demonstrated IL-8 release. Results similar to those obtained for antigens were reached when anti-IgE Ab were

TABLE 1. Ca^{2+} Dependence of IL-8 Release by α -IgE-Stimulated Human Neutrophils

Additions	IL-8 (ng/ml)
None	0.23 $\pm$ 0.05
α -IgE (15 $\mu\text{g}/\text{ml}$)	3.95 $\pm$ 0.54
α -IgE (15 $\mu\text{g}/\text{ml}$) + TFP (1 μM)	2.48 $\pm$ 0.10 ^a
α -IgE (15 $\mu\text{g}/\text{ml}$) + TFP (10 μM)	0.63 $\pm$ 0.10 ^b
α -IgE (15 $\mu\text{g}/\text{ml}$) + EGTA (2.5 mM)	0.07 $\pm$ 0.01 ^b

Human neutrophils from allergic patients were preincubated with TFP or EGTA, where indicated, for 1 h at 37°C. Then, after washing, anti-IgE was added to the suspended cells, as indicated, for another 20 h at 37°C. The IL-8 release was analyzed by ELISA in the culture supernatants after cell removal by centrifugation. Values are the mean $\pm$ SEM from three independent experiments performed in triplicate. ^a $P < 0.05$ versus anti-IgE alone. ^b $P < 0.001$ versus anti-IgE alone.

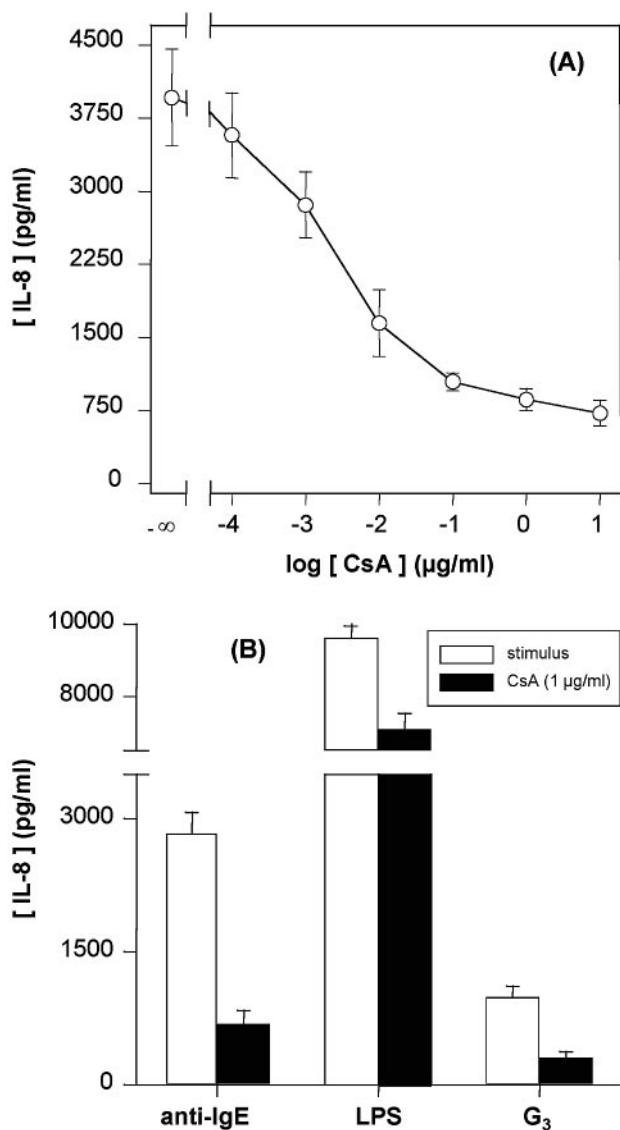


Fig. 6. CsA suppressed the IL-8 release from activated human neutrophils. (A) Neutrophils from allergic patients were preincubated for 30 min with the indicated concentration of CsA before the addition of 15 μ g/ml anti-IgE (α -IgE) and were then incubated for another 20 h. The amount of secreted IL-8 in the cell-free supernatants was analyzed by ELISA. (B) Neutrophils from G₃-sensitized patients were incubated in the presence or absence of 1 μ g/ml CsA for 30 min before the addition of stimulus: 15 μ g/ml anti-IgE, 0.5 μ g/ml LPS, or 50 μ g/ml G₃ for another 20 h. The values shown are the mean $\pm$ SEM from three separate experiments.

used as the stimulus in cells from allergic and healthy donors. At 3 h, the level of IL-8 produced by neutrophils from allergic patients, stimulated by anti-IgE Ab, was comparable with that stimulated by fMLP and higher than that produced by LPS. However, it was less potent than those stimuli at 18 h. Although neutrophils cultured with IL-8 induce only a modest, additional release of IL-8 [55], there is a possibility that the effect of anti-IgE/antigens on these cells is exaggerated by the released IL-8 itself, working as a stimulus to release more cytokine through an autocrine mechanism.

Real-time PCR analysis of RNA isolated from highly purified peripheral blood neutrophils demonstrated that the levels of IL-8 mRNA synthesized by allergic patients were low in

untreated neutrophils after 18 h of incubation, whereas treatment of cells with anti-IgE Ab induced a significant time-dependent increase in the level of specific mRNA, which was significant at 3 h of treatment and reached maximum levels at 18 h. In agreement with present data, purified eosinophil granule major basic protein increased IL-8 mRNA levels in neutrophils after 3 h of incubation [56]. Other agents, such as thapsigargin (an inhibitor of microsomal Ca²⁺ pumps), caused a rapid induction (within 5 min) of IL-8 mRNA [46].

IL-8 is a classical, adenosine-uridine element-rich mRNA [57]. These elements are believed to be associated with message instability, translational efficiency, and rapid turnover. In this regard, the increase in IL-8 mRNA level observed after IgE-dependent stimulation of human neutrophils could result from transcriptional and/or stabilization activities of the mRNA. Thus, IgE-dependent treatment of neutrophils stimulated IL-8 expression at mRNA and protein levels.

Previously, we have shown that in neutrophils from allergic patients, Ca²⁺ levels increased when the cells were challenged with the specific antigens or anti-IgE Ab [33]. Other studies demonstrated that although unstimulated peripheral blood neutrophils produced minimal levels of IL-8, the Ca²⁺ ionophore A23187 increased IL-8 to levels comparable with those found in exudative neutrophils isolated from an inflammatory site *in vivo* [45]. The data suggested that elevation of intracellular [Ca²⁺] may be an important physiologic signal regulating production of the inflammatory cytokine IL-8 by neutrophils. The studies presented in the current report extend those observations by demonstrating that incubation with antigen or anti-IgE Ab of freshly isolated peripheral blood neutrophils from allergic patients caused a dose-dependent increase in the production of the chemokine IL-8 to levels comparable with those previously observed in exudative neutrophils [45]. The secretion of synthesized IL-8 was inhibited by addition of EGTA, suggesting that the influx of Ca²⁺ was necessary not only for the synthesis of IL-8 but its secretion as well. These findings are similar to the case of thapsigargin-induced IL-8 production in human neutrophils [46]. TFP, a calmodulin (CaM) inhibitor, inhibits IgE-dependent liberation of IL-8 by neutrophils from allergic patients. CaM is involved in many fundamental signaling pathways, activating the function of various kinases (CaM kinases I and II, myosin light-chain kinase), phosphatase

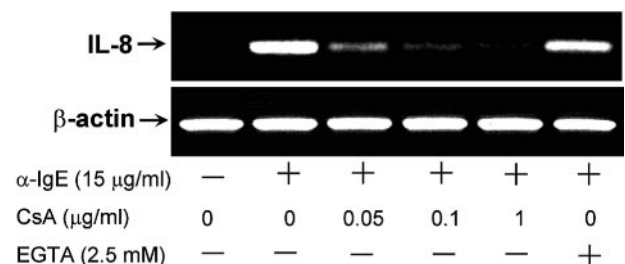


Fig. 7. Effect of increasing amounts of CsA on the expression of IL-8 mRNA. Neutrophils from allergic donors were incubated in the presence or absence of CsA for 30 min or 2.5 mM EGTA for 1 h, and further, 15 μ g/ml anti-IgE (α -IgE) was added. Total RNA was extracted after 3 h of incubation, reverse-transcribed, and amplified by conventional PCR. mRNAs of IL-8 and β -actin are shown. Data presented are representative of three experiments performed in similar conditions.

tases (calcineurin), ion channels (plasma membrane Ca^{2+} pump), and other cytosolic enzymes, such as phosphodiesterase, adenylate cyclase, and nitric oxide synthetase [58]. Thus, it is not surprising that inhibitors of CaM typically inhibit cell activation processes. CaM inhibitors have been shown to inhibit neutrophil oxidative burst, membrane-activated complex-1 up-regulation, secondary granule release, cell migration, cell motility [59, 60], and in our case, IgE-dependent IL-8 liberation. In this study, we demonstrate that TFP or EGTA decreased the IL-8 production in human neutrophils. Both data are in agreement with the role of calcium-dependent pathways in the IgE-dependent IL-8 liberation by neutrophils.

Another goal of this study concerned the presumptive role of the ROS in the IgE-dependent IL-8 production by human neutrophils. We previously reported an enhanced oxidative burst after stimulation with specific antigens or anti-IgE Ab in neutrophils from allergic patients [33, 34]. Furthermore, several lines of evidence have indicated the participation of oxidants in the IL-8 gene expression in different cell types, such as homocysteine-mediated IL-8 expression in human monocytes [47] or the H_2O_2 /TNF- α -induced IL-8 synthesis by human umbilical vein epithelial cells [48]. In agreement with these reports, our results indicate that the effects of anti-IgE Ab were primarily mediated by ROS through NADPH oxidase, as inhibitors of this enzyme, such as DPI and HMAP, and free radical scavengers, such as PDTC or TEMPO, abolished IL-8 synthesis and secretion.

Previously, we described a specific calcineurin activity in neutrophil lysates, which is dependent on Ca^{2+} and redox status [38, 54]. For this reason, in the current study, we also explored the potential role of this intracellular protein in the stimulatory effect of an IgE-dependent treatment toward IL-8 production in neutrophils.

Here, we present evidence that CsA, an immunosuppressive drug that binds to specific immunophilin and interferes with the activation of the Ca^{2+} /CaM-dependent serine/threonine phosphatase calcineurin (or protein phosphatase 2B) [61], inhibits, in a dose-dependent manner, the IgE-dependent induction of IL-8 mRNA and protein in neutrophils from allergic patients. Present data are in accord with earlier reports that IL-8 induction by the CD28 costimulatory pathway in Jurkat cells is sensitive to CsA [62] and that the thapsigargin-induced up-regulation of the IL-8 gene in neutrophils is inhibited by CsA [46].

Besides being a potent chemoattractant and stimulus for neutrophils [1, 7], IL-8 is also a chemoattractant for T lymphocytes [5] and for IL-5-primed eosinophils [63], as well as a stimulus for basophils primed by IL-3 [64]. Thus, IgE-induced IL-8 production by neutrophils could contribute to inflammatory events characteristic of chronic asthma. However, the effect of IgE on neutrophil IL-8 release may be even more relevant to the pathophysiology of acute asthma. In addition to promoting an abnormally high neutrophil influx, IL-8 release could prolong neutrophil activation in this pathological condition in an autocrine-like manner, and the resulting increased release of elastase and other mediators [65] could exacerbate changes such as formation of the mucus plugs observed in fatal asthma. Furthermore, levels of IL-8 were elevated during the acute exacerbation of bronchial asthma [66] or challenge with

bronchial antigens [67]. In summary, we show evidence that an IgE-dependent mechanism is able to release IL-8 and up-regulate IL-8 mRNA expression by neutrophils from allergic patients. Furthermore, these events are mediated through a Ca^{2+} , ROS, and the calcineurin-dependent pathway.

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