Interleukin-2 Induces Peroxide Production by Primed Normodense Eosinophils of Patients with Asthma

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ABSTRACT

In this study we assessed, by flow cytometry, the effect of interleukin 2 (IL-2) on the oxidative burst of normodense eosinophils (Eos's) isolated from 15 patients with moderately severe extrinsic asthma and 17 controls. We found that IL-2 significantly induced peroxide (H_2O_2) production in normodense Eos's from patients with asthma on a time kinetics study. This rise was higher in patients with immunoglobulin E levels > 180 IU/mL versus normal immunoglobulin E values. The effect of IL-2 was partially blocked by using anti-Tac antibody. In contrast, IL-2 decreased H_2O_2 production in normodense Eos's from controls. Cell surface expression of CD25, CD122, CD132, and CD69 were also determined and no statistical differences were found between both groups. In conclusion, IL-2 is able to increase H_2O_2 production by normodense Eos's isolated from patients with asthma and it may contribute to bronchial epithelium damage and chronic inflammation. (Allergy and Asthma Proc 24:27–33, 2003)

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INTRODUCTION

Eosinophils (Eos's) play an important role in host immunity to parasitic infections and contribute to chronic inflammation in the pathogenesis of allergic diseases such as bronchial asthma, allergic rhinitis, and atopic dermatitis.[1] They are recognized as potent proinflammatory cells with the capacity to generate an array of inflammatory mediators, including toxic granule cationic proteins, enzymes, lipid metabolites,[2] cytokines,[3] and reactive oxygen metabolites.[4] A crucial step in the generation of oxygen metabolites is activation of the NADPH system, which is able to catalyze the one-electron reduction of molecular oxygen (O_2) to superoxide (O_2^-) in a so-called oxidative burst.[5]

Cytokines are important in the induction and/or modification of the effector function of eosinophils such as degranulation and superoxide production. Interleukin (IL) 5, IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce superoxide anion production by Eos's.[6]

On Eos's, IL-2 has been described as a potent Eos chemoatractant.[7] Also, *in vivo* infusion of IL-2 in the systemic circulation, as part of cancer chemotherapy, results in eosinophilia and change of Eos density that may, in turn, establish a subpopulation of cells with alterated metabolic activity.[8] Recently, Hoenstein *et al.*,[9] showed that IL-2 can activate Eos cultured on albumin-coated wells to release granule mediators (Eos peroxidase) and IL-6. The underlying mechanism is still unclear, but it has been proposed that IL-2 acts indirectly through an increased synthesis of IL-5, IL-3, and GM-CSF by activated T cells.[10] More recently, a

direct effect of IL-2 through its receptor on Eos has been proposed.[8]

The IL-2 receptor (IL-2R) is a multimeric molecule consisting of two obligate signaling subunits IL-2β (CD122) and -γ^c (CD132) and a variably expressed IL-2Rα subunit (CD25), which regulates affinity for IL-2.[11] Eos's express CD25 and CD132; however, no expression of CD122 was found by flow cytometry,[12,13] probably because of the intermediate level of detection for most flow cytometry apparatus (1000 molecules/cell) and the lack of high-affinity antibodies on cytokine receptors.[14] Moreover, using other approaches as functional cell responses, it is clear that Eos's express the IL-2R.[15] Eos's are able to transcribe messenger RNA (mRNA) for IL-2 synthesis and release and store this cytokine as a preformed mediator within the crystalloid core of the granules, which may be important as an autocrine and paracrine component of their effector function. This local IL-2 may serve as a reservoir for its rapid release in inflammatory reactions associated with eosinophilia.[3]

In this investigation, we have studied the effect of IL-2 oxidative burst (H_2O_2 production) of normodense Eos's isolated from patients with asthma on a time kinetic and compared it with homologous cells from healthy donors.

MATERIALS AND METHODS

Reagents

Monoclonal antibodies (MAb's) CD25-RD1(IL-2R1) and CD122-fluorescein isothiocyanate (FITC) (IL2R(p75)) and mouse isotype control antibodies immunoglobulin G1 [IgG1] FITC, IgG RD) were purchased from Coulter Immunology (Hialeah, FL). CD132-R-PE was obtained from Pharmigen (San Diego, CA). CD16b-FITC(Leu 11a) and CD69-PE(Leu-23) were acquired from Becton-Dickinson Immunocytometry Systems (Mountain View, CA). Percoll (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden), dextran, phorbol 12 myristate acetate (PMA; Sigma Chemical Co., St. Louis, MO), and 2′7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Portland, OR) were also used. Recombinant human IL-2 (rhIL-2) was kindly donated by Dr. Craig Reynolds, Biological Modifiers Program, National Cancer Institute, Frederick, MD.

Study Subjects

The patient group included 15 individuals with moderately severe extrinsic bronchial asthma (age range, 18–40 years) selected from the Allergic Diseases outpatient clinical unit of the Institute of Immunology, Central University Medical School. All patients were sensitive to at least one regional allergen (prick test), showed a reversibility of airway obstruction lower than 20% of forced expiratory volume in 1 second after the inhaled administration of 400 μg of albuterol, and experienced >7 attacks/year. None had received oral corticosteroid treatment for at least 3 months before the study and parasitic infection was ruled out in all of them by serial stool tests. Patients were in treatment with inhaled corticosteroids and bronchodilators, always in the recommended dosages. The control group consisted of 17 sex and age-matched nonatopic healthy subjects, selected from the laboratory staff of the Institute of Immunology and among voluntary blood donors of the Central University Hospital. The Institute Ethical Committee approved the study protocol.

Cell Separation

Eos were purified from ethylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral blood samples obtained from patients with bronchial asthma and controls, following the isolation method described by Gärtner.[16] Briefly, five parts of peripheral blood were mixed with one part of 6% dextran in 0.15 M of NaCl and kept at room temperature for 30 minutes. The leukocyte-rich suspension was collected and washed once with phosphate-buffered saline (PBS) gel (0.01 M of phosphate buffer, 2 mM of EDTA, 5 mM of glucose, and 0.1% gelatin) at 450 $\times$ *g* for 10 minutes at 4°C. The cells, adjusted at 25–30 $\times$ 10^6/mL, were resuspended in 1 mL of Percoll solution (density, 1.070 g/mL), layered over a discontinuous isotonic Percoll gradient and centrifuged at 1600 $\times$ *g* for 30 minutes. Percoll densities (1070, 1080, 1085, 1090, and 1100 g/mL) were obtained according to Day's protocol.[17] Normodense Eos from the 1.090- and 1.100-g/mL layers were collected and washed in PBS gel and contaminating red cells were lysed with 15 mL of NH_4Cl solution (150 mM of NH_4Cl, 10 mM of $NaHCO_3$, and 1 mM of EDTA). After mixing for 7 minutes at room temperature, the suspension was centrifuged at 450 $\times$ *g* for 10 minutes at 4°C and washed twice in PBS gel. Cell viability was estimated by trypan blue exclusion. The differential cell count was determined by examining 100 cells stained with eosin/methylene blue solution in methanol.

Flow Cytometric Analysis

Purified normodense Eos's were analyzed in an Epics Profile II flow cytometer (Coulter Electronics, Hialeath, FL), after previous alignment with DNA check fluorescent beads. Gain sets were adjusted for Eos's with large size and granularity on the forward angle light scatter versus side scatter. Flow cytometric analysis was performed on 5000 cells on the electronic map.

Eos Oxidative Burst

The H_2O_2 generated by the NADPH oxidase system was determined by the oxidation of DCFH-DA to DCF according to the procedure described by Davis.[18] In patients, Eos oxidative burst was assessed in the presence of different concentrations of rhIL-2 (250, 500, and 1000 U/mL). In controls, we used only rh IL-2 at 1000 U/mL, because the cell recovery was very low in this group of individuals. Nonstimulated (PBS gel) and PMA-stimulated

(100 ng/mL) conditions were included as controls.[19] Briefly, freshly isolated normodense Eos's were adjusted to 2×10^6 cell/mL and labeled with 100 μL of 0.2 mM of DCFH-DA for 15 minutes at 37°C, followed by a time kinetics study of H_2O_2 production. Flow cytometric analysis was performed using 488 nm of excitation with a green photomultiplier, using a 550 DL and 525 BP filter combination to quantify fluorescence emission. DCF formation, which is proportional to the hydrogen peroxide (H_2O_2) production was reported in logarithmic units of the mean green fluorescence intensity channel.

We investigated the role of p55 receptor (CD25) in IL-2–induced H_2O_2 production, incubating Eos's from patients with asthma with anti-Tac. Freshly isolated Eos's were incubated with 1-μg/mL of anti-Tac for 30 minutes at 4°C, washed with PBS gel, and then labeled with DCFH-DA. The H_2O_2 production was measured under nonstimulating conditions (control) and in the presence of 1000 U/mL of rhIL-2.

The specificity of the assay was assessed by incubating Eos's with 1 mM of maleimide before analysis of the oxidative burst in nonstimulated IL-2 and PMA-stimulated conditions as described previously.[20]

Antigen Surface Expression

CD16, CD25, CD122, CD132, and CD69 were determined by single- and dual-color flow cytometry on purified Eos preparations. Briefly, the cells were adjusted to 1×10^6/mL and incubated with the corresponding FITC or RD1 tagged MAb's for 30 minutes at 4°C. The cells were washed three times with PBS gel. MAb binding was quantified by flow cytometry and expressed as the percentage of positive stained cells. Nonspecific binding was determined using irrelevant mouse Ig isotypes IgG1-FITC and IgG-RD.

Total IgE Levels

Sera obtained from patients and controls were stored at −20°C until IgE analysis was performed. Total serum IgE levels were measured by the QuantiClone Total IgE radioimmunoassay (Kallestad Diagnostics, Inc., Chaska, MN). Mean level of total IgE in normal subjects was 57.6 ± 68.3 IU/mL (normal range given by the values of the commercial kit, 0–180 IU/mL).

Statistics Analysis

Measurements from control and patient Eos preparations were pooled for data analysis. Data for cell surface antigens are presented as the mean ± SD ($\bar{X} \pm$ SD) of the percentage of positive cells. Results for the oxidative burst are presented as the mean ± SE ($\bar{X} \pm$ SE) of the mean channel of fluorescence intensity. Statistical analysis was performed using Student's *t*-test for paired or unpaired data. The limit for significance was taken as $p < 0.05$ two tailed.

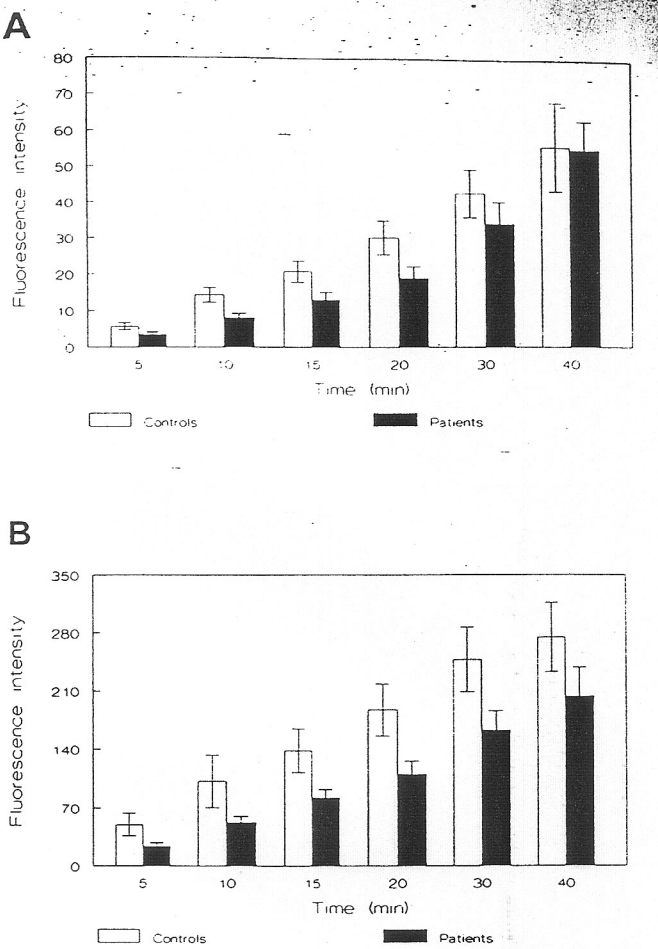

Figure 1. Kinetics of H_2O_2 production by normodense Eos's. Time-dependent kinetics of H_2O_2 production of (A) nonstimulated and (B) 100 ng/mL of PMA-stimulated Eos's isolated from 15 patients with asthma and 12 controls, determined by flow cytometry using DCFH-DA. Results are presented as $\bar{X} \pm$ SE of the mean channel fluorescence intensity, expressed in logarithmic units. By unpaired Student's t-test data, no significant differences were observed when H_2O_2 production from patients and controls were compared.

RESULTS

Oxidative Burst of Normodense Eos's

All Eos preparations showed (a) >97% viability as assessed by trypan blue dye exclusion; (b) Eos purity >90% as determined by eosin/methylene blue stain, and (c) <10% of CD16 expression assessed by flow cytometry.

The time course of the oxidative burst in normodense Eos isolated from patients with asthma and healthy donors was analyzed under nonstimulated and PMA-stimulated conditions. Normodense Eos's exhibited a spontaneous time-dependent production of H_2O_2 (Fig. 1 *A*). Although significant differences were not observed between both groups, normodense Eos's from patients produced lower H_2O_2 than controls. When Eos's were stimulated with PMA (100 ng/mL), the H_2O_2 production increased significantly ($p <$

0.01) in both groups as compared with nonstimulated cells at any time point. However, PMA-stimulated cells from patients with asthma showed a lower H_2O_2 production compared with controls, which was not statistically significant (Fig. 1 *B*). Despite the lower H_2O_2 production by patients, the oxidative burst index, which represents the ratio of the baseline to stimulated value of H_2O_2 production, did not show significant differences at any time point when patients and controls were compared.

To study the effect of IL-2 on Eos oxidative burst, cells were incubated with different concentrations of rhIL-2 (250, 500, and 1000 U/mL). In all cases, there was a time- and dose-dependent induction of H_2O_2 production by IL-2 in normodense Eos's isolated from patients with asthma. This IL-2 effect was significant at 1000 U/mL after 5 minutes of incubation ($p < 0.05$) as compared with nonstimulated cells (Fig. 2 *A*). On the other hand, the H_2O_2 production of normodense Eos's isolated from healthy donors was lower in the presence of 1000 U/mL of IL-2 when compared with nonstimulated Eos's (Fig. 2 *B*). This difference was significant ($p < 0.05$; $p < 0.01$) from 10 to 30 minutes of incubation.

Next, we investigated whether the use of anti-Tac was able to suppress IL-2 (1000 IU/mL)–induced H_2O_2 production by Eos's from patients with asthma. As illustrated in Fig. 3, 1 μg/mL of anti-Tac inhibited the IL-2–dependent increase of H_2O_2 production between 30 and 50%, which was significant ($p < 0.05$) at 20, 30, and 40 minutes of incubation.

To analyze whether a relationship may exist between total serum analyze IgE levels and Eos oxidative burst, patients with asthma were classified into normal (0–180 IU/mL) and elevated (>180 IU/mL) serum total IgE levels. Mean levels of total IgE were 745.6 ± 326 IU/mL and 103.5 ± 65.6 IU/mL, respectively. The analysis of H_2O_2 production by nonstimulated normodense Eos's did not show significant differences between both groups. Nonetheless, the differential increase in H_2O_2 production observed in IL-2– and PMA-stimulated normodense Eos's was higher on cells isolated from patients with asthma with elevated total IgE levels as compared with patients with normal total IgE levels under the same conditions at all time points studied. These increases were significant ($p < 0.05$) under IL-2–stimulated conditions at 10, 15, and 20 minutes (Fig. 4 *A*) and under PMA-stimulated conditions from 5 to 30 minutes (Fig. 4 *B*). Moreover, the differential increase in H_2O_2 production in PMA-stimulated Eos's from patients with asthma with elevated IgE levels was higher when compared with controls; this difference was not statistically significant ($p > 0.05$) at any time point.

Studying the effect of maleimide on DCF fluorescence in Eos's, we showed that maleimide (1 mM) is able to inhibit the oxidative burst of Eos's in nonstimulated conditions by 87.8 ± 7.3%. IL-2– and PMA-induced oxidative burst of Eos's were also inhibited with maleimide by 98.7 ± 0.2% and 99.2 ± 0.2%, respectively, in a time kinetics assay.

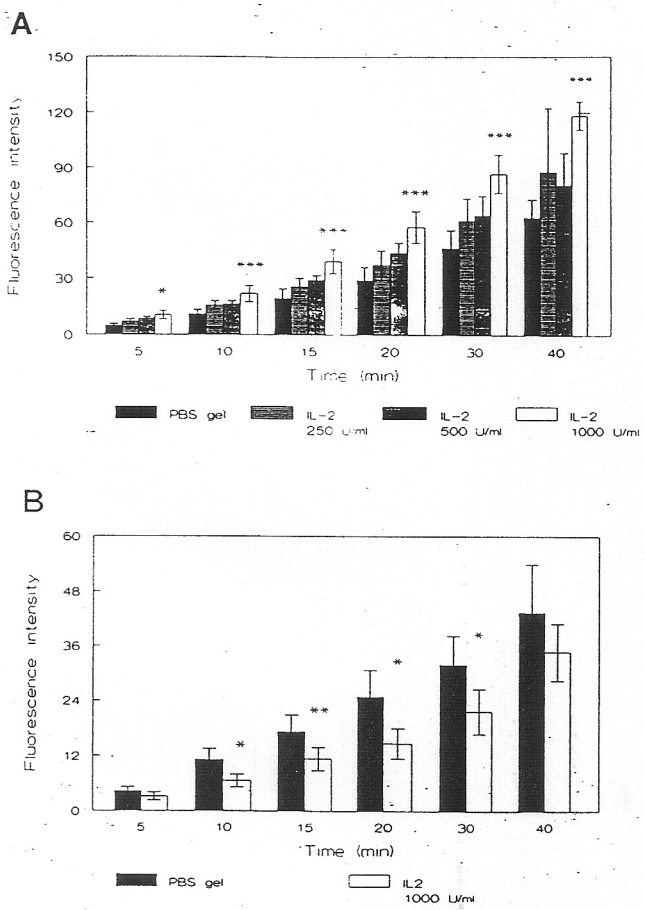

Figure 2. Effect of IL-2 on H_2O_2 production by normodense Eos's. (A) Freshly isolated Eos's from 15 patients with asthma were incubated with 0, 250, 500, and 1000 U/mL of rhIL-2 and the kinetics of H_2O_2 production were determined by flow cytometry using DCFH-DA. (B) The effect of 0 and 1000 U/mL of rhIL-2 on Eos's from 13 controls. Results are presented as $\bar{X} \pm SE$ of the mean channel fluorescence intensity, expressed in logarithmic units. By paired Student's t-test, significant differences ($p < 0.05$, **$p < 0.01$, and ***$p < 0.005$) were observed when PBS gel versus 1000 U/mL of IL-2 were compared.*

Eos Cell Surface Antigens

The IL-2Rα, IL-β, and -γ chains (CD25, CD122, and CD132, respectively) and the early activation marker CD69 were evaluated on freshly isolated normodense Eos's from 15 patients with asthma and 9 healthy donors (Table I). Our results showed that normodense Eos's from patients with asthma and controls express IL-2Rα and -γ chains, whereas the β-chain was not detectable. We did not find significant differences on the IL-2R expression when normodense Eos's from controls and patients were compared.

Normodense Eos's from controls and patients with asthma showed low levels of CD69 expression. However, normodense Eos's from patients with asthma showed a nonsignificant increase of CD69 expression as compared with controls.

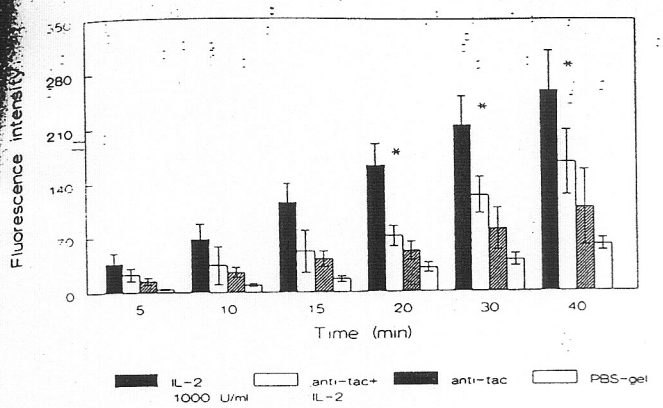

Figure 3. Effect of anti-Tac on IL-2–dependent H_2O_2 production. Freshly isolated Eos's from five patients with asthma were incubated with 1 μg/mL of anti-Tac and the subsequent H_2O_2 production determined in the absence or presence of IL-2 at 1000 U/mL by flow cytometry using DCFH-DA. Results are presented as $\bar{X} \pm$ SE of the mean channel fluorescence intensity, expressed in logarithmic units. H_2O_2 production by nonstimulated (PBS gel) and IL-2–stimulated Eos's was also performed. By paired Student's t-test significant differences ($p < 0.05$) were observed when H_2O_2 production induced by IL-2 in the presence or absence of anti-Tac were compared.*

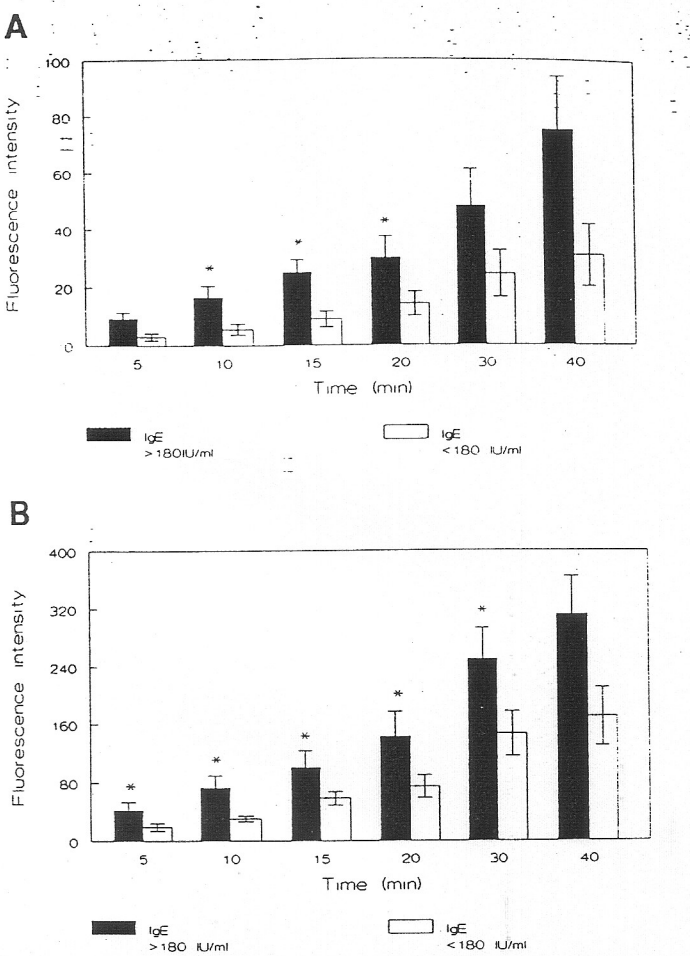

Figure 4. Relationship between H_2O_2 produced by normodense Eos's from patients with asthma and serum IgE levels. H_2O_2 production induced by (A) 1000 U/mL of IL-2 and (B) 100 ng/mL of PMA in normodense Eos's from 15 patients with asthma, 6 patients with normal total IgE levels, and 9 patients with elevated total IgE levels. Results are presented as $\bar{X} \pm$ SE of the difference of Eos H_2O_2 production with IL-2 or PMA minus basal production. By unpaired Student's t-test, significant differences ($p < 0.05$) were observed when patients with asthma with normal and elevated IgE levels were compared.*

DISCUSSION

IL-2 is a cytokine released mainly from activated T cells, which acts as a T cell growth factor. It also stimulates growth and differentiation of B cells and natural killer cells.[21] In addition, IL-2 has been suggested to be a chemoattractant factor for Eos's.[7] The main purpose of this report was to investigate the effect of rhIL-2 on the oxidative burst of highly purified normodense Eos's derived from patients with asthma and to compare it with homologous cells from healthy donors.

Our results showed that nonstimulated normodense Eos's from patients with asthma produced less H_2O_2 than controls, even if this difference was not significant. Also, Eos's from patients with asthma stimulated with PMA, which induces the oxidative metabolism by activating protein kinase C[22], produced lower H_2O_2 than control Eos's. These findings are in contrast with previous studies, which report an augmented Eos H_2O_2 production in patients who have asthma and allergies.[1,2] Nevertheless, Woschnagg[23] have reported that the initial rate of PMA-stimulated oxidative metabolism was higher in patients with allergic rhinitis than in controls during the pollen season, and the two groups showed no differences in oxidative metabolism preseason. Differences in the Eos subpopulations studied, in the methodology used to purify Eos's and in the assay used to evaluate H_2O_2 production, probably account for these discrepancies.

Because production of H_2O_2 by the NADPH oxidase system maybe regulated by different cytokines,[6] we assessed the effect of IL-2 in the regulation of this enzymatic system. Our results showed that IL-2 produced an opposite effect on the oxidative burst of normodense Eos's isolated from patients with asthma and controls. IL-2 increased peroxide production by Eos's from patients with asthma, whereas it inhibited oxidative burst responses by Eos's from control subjects. Moreover, anti-Tac (anti-CD25) was partially able to block the IL-2–dependent H_2O_2 production by normodense Eos's from patients with asthma. These findings suggest that IL-2 induces an oxidative burst in asthmatic Eos's *via* surface CD25. Results like ours have been reported previously on IL-2–dependent chemotaxis assays on human Eos's.[15]

We also looked for a possible relationship between total IgE serum levels and the Eos oxidative burst. We found an increased H_2O_2 production on both IL-2– and PMA-stimu-

TABLE I

Surface Antigen Expression on Normodense Eos's Obtained from 9 Controls and 15 Patients with Asthma (*P*), 6 Patients with Normal Total IgE Levels, and 9 Patients with Elevated Total IgE Levels

	Stained Cells (%)			
	CD25	CD122	CD132	CD69
Controls	10.2 ± 2.6	1.8 ± 0.5	21.6 ± 12.2	1.6 ± 1.4
Patients	13.2 ± 8	0.8 ± 1.5	23 ± 8.1	3.3 ± 2.9
P (IgE < 180 IU/mL)	11.1 ± 7.1	0.3 ± 0.2	24.5 ± 2.8	2.6 ± 3.6
P (IgE > 180 IU/mL)	14.3 ± 10.1	1.1 ± 1.8	24.2 ± 10.6	3.7 ± 2.8

Results are expressed as $\bar{X} \pm SD$ *of the percentage (%) of positive cells. No significant differences were observed in CD25, CD122, CD132, and CD69 expressions on Eos's isolated from controls and patients.*

lated Eos's from those patients with asthma with elevated total serum IgE as compared with those with normal IgE levels and controls. Although FcεRI and CD23 expression has not been detected by flow cytometry in Eos's from patients who are allergic, the inhibitory effects of both polyclonal and MAb's cross-reacting with FcεRI/CD23 on IgE-mediated functions of Eos's led us to suggest that Eos's express these receptors.[24] Thus, we suggest that an increase in serum total IgE on patients with asthma leads to a higher FcεR occupancy and, consequently, produce an IgE concentration–dependent priming of normodense Eos's and also may modulate the IL-2– and PMA-dependent H_2O_2 production. Inflammatory mediators such as leukotrienes and superoxide anions also are produced on IgE-dependent activation.[24]

This priming condition has been described previously for neutrophils and has been individualized as a step distinct from activation on the basis that it does not induce O_2^- generation but reduces the lag before the onset of O_2^- generation and amplifies the production of O_2^- in response to the agonist.[5] The precise mechanism of priming is unknown. However, there are a number of reports that point out that the major effect of priming agents on neutrophils is the regulation of cytosolic levels of free Ca^{2+}.[25] Our findings suggest that normodense Eos's of patients with asthma might be associated with cell priming. Cells in this category are said to be primed because they respond to stimuli that ordinarily do not produce responses on normodense Eos's.

The IL-2R occurs in three forms that exhibit different affinities for IL-2: the low-affinity monomeric IL-2Rα, the intermediate-affinity dimeric IL-2Rβγ, and the high-affinity trimeric IL-2Rαβγ.[11] Because differences in the expression of IL-2R chains may affect the ability of the cell to respond to IL-2, we examined the expression of IL-2R subunits on normodense Eos's from patients with asthma and controls. Our results showed that these cells express CD25 and CD132, whereas no expression or undetectable levels of the IL-2Rβ chain were found when analyzed by flow cytometry. These findings support recent reports showing that CD25 is expressed constitutively in Eos's, whereas CD122 is an inducible subunit of the IL-2R.[1,13] Other reports show that normal unstimulated Eos's express low amounts of CD25; however, CD25 is expressed on Eos's from patients with hypereosinophilic syndrome or normal Eos's stimulated with GM-CSF and IL-3.[12,26] Eos's express low levels of CD122, which are not detectable by flow cytometry, although a functional receptor may be detected by other techniques.[12,15] On the other hand, CD25 and CD132 are able to bind each other without the presence of CD122 and constitute the low-affinity IL-2R with intracellular signal transduction.[11] Our results suggest that normodense Eos's express this low-affinity IL-2R because it may be blocked substantially with anti-Tac, and it may explain, in part, why high concentrations of IL-2 are required to obtain significant effects on peroxide production by these cells.

The possibility that such a preactivation state would be present in normodense Eos's from patients with asthma is supported by the observation that these cells express marginally more CD69, an early activation marker, which is found only on activated Eos's.[26] More recently, ligation of CD69 was shown to induce apoptosis in human Eos's cultured with GM-CSF and IL-13. Therefore, this antigen might play an important role in Eos removal.[27]

In conclusion, our results show that normodense Eos's isolated from patients with asthma, although having the same density of the normodense Eos's from normal individuals, exhibited functional differences. Also, it is evident that IL-2, a Th1 cytokine, may play an important role in the activation and effector functions of Eos's isolated from patients with asthma. Whether the main action of IL-2 on eosinophil function is direct or indirect through other cells and mediators still has to be established. However, the direct effect of IL-2 on the oxidative burst of Eos's seems to be dual, *i.e.*, an increase in peroxide production in normodense Eos's from patients with asthma and inhibition in normodense Eos's from controls. The fact that Eos's can express and secrete IL-2 suggests that it may act as an autocrine regulatory pathway on Eos's, and it may explain the local Eos recruitment, the induction of the respiratory burst, and the production of large amounts of oxygen metabolites including superoxide (O_2^-) and hydrogen peroxide.

which have cytotoxic activity for host cells, contributing to the chronic state of cell activation in inflamed tissues.

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