

# Release of granule proteins by eosinophils from allergic and nonallergic patients with eosinophilia on immunoglobulin-dependent activation

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*The release of eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP) was evaluated after incubation of eosinophils (EOSs) from allergic subjects with the specific allergen or with anti-IgE monoclonal antibodies (MAbs). High levels of EPO could be released after addition of the specific allergen (and not unrelated ones) or anti-IgE MAb. Moreover, EPO release with the two stimuli was significantly correlated both in allergic and in nonallergic patients. In the same supernatants, another granule protein, ECP, could not be detected, suggesting a lack of correlation between EPO and ECP release after IgE-dependent stimulation. However, when EOSs with surface-IgA antibodies were incubated with anti-IgA MAb, both EPO and ECP were released. In contrast, incubation of EOSs with anti-IgG MAb induced mainly the release of ECP and not EPO. These results indicate that pharmacologically active mediators can be released by EOSs from allergic and nonallergic patients on immunoglobulin-dependent activation. The results also confirm the hypothesis of a selective release of the various granule proteins and raise the question of transduction signals delivered by the three Fc receptors (Fc<sub>γ</sub>R, FC<sub>α</sub>R, and FC<sub>ε</sub>R) present on human EOSs. (J ALLERGY CLIN IMMUNOL 1991;88:365-75)*

**Key words:** Eosinophils, Fc receptors, immunoglobulin isotypes, eosinophil peroxidase, eosinophil cationic protein

During the past 20 years, several lines of evidence have raised growing interest in the effector functions of EOSs regarding a variety of clinical states associated with hypereosinophilia, including parasitic and allergic diseases.<sup>1,2</sup> It has been clearly demonstrated that EOSs from hypereosinophilic patients are heterogeneous in their densities, functional activities, and biochemical features.<sup>3-13</sup> Sedimentation on metrizal-

## Abbreviations used

|                   |                               |
|-------------------|-------------------------------|
| FCM:              | Flow cytometry                |
| MAb:              | Monoclonal antibody           |
| HBSS:             | Hanks' balanced salt solution |
| MBP:              | Major basic protein           |
| ECP:              | Eosinophil cationic protein   |
| EPO:              | Eosinophil peroxidase         |
| EDN:              | Eosinophil-derived neurotoxin |
| MPO:              | Myeloperoxidase               |
| CL:               | Chemiluminescence             |
| EOS:              | Eosinophil                    |
| Ab:               | Antibody                      |
| FITC:             | Fluorescein isothiocyanate    |
| CL:               | Chemiluminescence             |
| LDH:              | Lactate dehydrogenase         |
| sIgG, sIgA, sIgE: | Surface IgG, IgA, and IgE     |

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mid gradients allows the separation of "hypodense" (density, <1.130 gm/L) and "normodense" EOSs (density, >1.135).<sup>14</sup> Hypodense EOSs are increased in the blood of patients with eosinophilia of different

**TABLE I.** Classification of patients with eosinophilia

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|                                                   |
|---------------------------------------------------|
| A. Patients with increase of serum IgE            |
| I. Allergic diseases (N = 7)                      |
| II. Parasitic infections (N = 8)                  |
| Taeniasis (1)                                     |
| Filariasis (5)                                    |
| Schistosomiasis (2)                               |
| B. Patients with or without increase of serum IgE |
| III. Hypereosinophilic syndrome (N = 5)           |
| IV. Miscellaneous (N = 9)                         |
| Aspergillosis (2)                                 |
| Churg-Strauss disease (4)                         |
| Fasciitis with eosinophilia (1)                   |
| Idiopathic edema (1)                              |
| PIE syndrome (1)                                  |

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etiology<sup>15</sup> as well as in the affected tissues, particularly in the lung.<sup>16</sup> Blood and tissue hypodense cells demonstrate an increased expression of Fc $\epsilon$ R II, the low-affinity receptor for IgE,<sup>16,17</sup> and capability of killing IgE-coated parasite targets.<sup>18</sup> Moreover, EPO, one of the highly cytotoxic basic proteins, is released by hypodense EOSs of patients with filarial infections, in the presence of specific antigens or antihuman IgE Abs.<sup>19</sup> It was also demonstrated that EOSs can bear cytophilically bound IgE, in relation to the levels of circulating IgE.<sup>16</sup> In regard to allergic diseases, one of the hallmarks of the late-phase reaction is a tissue infiltration of inflammatory cells, characterized by a significant increase of EOSs, which are considered to play a pivotal role in the course of late allergic inflammation.<sup>20-23</sup>

Effector functions of EOSs against parasites, as well as against host cells, appear to be mainly mediated by the release of cationic proteins, such as MBP, ECP, EDN, and EPO. All these preformed mediators display a strong cytotoxic potential.<sup>1</sup> The precise mechanisms leading to EOS degranulation, however, are still poorly understood. It is not yet clear, in particular, whether EOSs isolated from allergic patients could degranulate on exposure to allergens. Moreover, studies on parasite-infected patients have demonstrated that not all the granule proteins were simultaneously released after the same stimulus of activation, suggesting a differential release of EPO and ECP.<sup>19,24,25</sup> To answer these questions, we have evaluated the release of EPO and ECP by EOSs of allergic subjects, after the addition of the specific allergen or antihuman IgE MAbs, in comparison to EOSs from patients with eosinophilia of nonallergic origin. In addition, to investigate further the recently de-

scribed interactions between IgA and human EOS,<sup>25,26</sup> we have monitored the release of granule proteins after exposure of purified EOSs to antihuman IgE, antihuman IgA, and antihuman IgG MAbs and provided evidence that these three isotypes can be involved, although they are involved in different ways, in the Ab-dependent mechanisms of EOS activation.

## MATERIAL AND METHODS

### Reagents

Material was obtained as follows: Dextran T500, PRIST, and RAST from Pharmacia, Uppsala, Sweden; metrizamide from Nyegaard Co., Oslo, Norway; HBSS from GIBCO Ltd., Paisley, Scotland; fast Giemsa:Coffret RAL 555 from Société Chimique Pointet Girard, Clichy, France; MAbs (MAb to human IgE, IgA, and IgG, all mouse IgG1 isotype) from Immunotech, Marseille-Luminy, France; MAb anti-*Toxoplasma* (purified mouse IgG1) was a generous gift of Dr. Haleh Charif, Institut Pasteur, Lille, France; partially purified allergen extracts from Laboratoires des Staller-gènes, Fresnes, France; D-luciferin from Boehringer, Mannheim, Germany; Luminol from SERVA, Heidelberg, Germany; FITC-labeled F(ab')<sub>2</sub> fragment of antihuman IgE sheep Abs from Cappel Laboratories, Cochranville, Pa.; FITC-labeled F(ab')<sub>2</sub> fragment of antihuman IgA sheep Abs from TAGO Laboratories, Burlingame, Calif.; and FITC-labeled F(ab')<sub>2</sub> fragment of antihuman IgG Abs from Cappel Laboratories.

### Patients

EOSs were separated from the peripheral blood of 29 patients with eosinophilia (Table I). The degree of eosinophilia in allergic patients was mild to moderate (mean, 12.7%; range, 5% to 20%), and the total white cell counts were in the normal range. All patients presented symptoms of bronchial asthma; in two cases, symptoms of allergic rhinitis were associated. Skin prick test and RAST were positive toward the allergen used for the in vitro EOS stimulation, that is, *Dermatophagoides pteronyssinus* (five cases), *Dactylis glomerata* (two cases), or both (one patient). None of these subjects was receiving any specific immunotherapy or had been treated with corticosteroids. At the time of this study, one patient was receiving oral theophylline (300 mg twice daily).

In the 23 nonallergic patients, EOSs represented 8% to 85% (mean, 42%) of the circulating leukocytes. None of these patients was receiving systemic corticosteroids.

### EOS separation

Venous peripheral blood was drawn, after informed consent, into preservative-free heparin, and leukocytes were collected after dextran sedimentation for 45 minutes at room temperature. The leukocyte-rich plasma (buffy coat) was removed and washed in Tyrode's buffer. After lysis of contaminating red blood cells, leukocytes were separated by centrifugation on discontinuous metrizamide gradients ac-

cording to the technique of Vadas et al.,<sup>5</sup> adapted as previously described.<sup>6</sup> After this step, cell fractions were collected from each density layer and washed in HBSS. The degree of purity and the morphology of the cells were evaluated on cytocentrifuge preparations stained with Giemsa. Cell viability, assessed by the trypan blue exclusion test, was superior to 90%. Only cell fractions containing >85% EOSs, recovered at the interface between 22% and 23% metrizamide solutions (intermediate density EOSs) ( $1.125 < \text{density}, < 1.135$ ), were tested for functional activity, both for allergic and nonallergic donors. Functional tests were performed just after leukocyte separation in all cases.

### EOS activation

For the in vitro activation of purified EOSs, antihuman IgE, IgA, and IgG MAbs or specific allergens were added at various final concentrations (1 to 10  $\mu\text{g}/\text{ml}$  for Abs and 0.9 to 90  $\text{ng}/\text{ml}$  for allergens) to 0.2 ml samples of the EOS suspensions ( $2.5 \times 10^6$  cells per milliliter in HBSS). Controls were EOSs incubated with medium alone. An irrelevant MAb of the same isotype (mouse IgG1 anti-*Toxoplasma*) was tested together with antihuman MAbs on three separate experiments; no cell activation was detected (EPO index mean  $\pm$  SEM,  $0.77 \pm 0.72$ ).

After incubation for 60 minutes ( $37^\circ\text{C}$ ; 5%  $\text{CO}_2$ ), all tubes were immediately centrifuged at 400 g for 10 minutes at  $44^\circ\text{C}$ . The supernatants were recovered and kept overnight at  $+4^\circ\text{C}$  before performing the EPO release assay. Samples of each supernatant were stored at  $-20^\circ\text{C}$  until they were assayed for ECP.

### EPO release assay

The determination of released EPO was performed by a CL assay, adapted from the method according to Whitehead et al.<sup>27</sup> as previously described.<sup>19</sup> For the assay, small amounts (0.03 ml) of each sample were transferred into plastic tubes containing 0.03 ml of D-luciferin (160  $\mu\text{mol}/\text{L}$  of Tris HCl buffer, 0.01  $\text{mol}/\text{L}$ , pH 6.00), 0.03 ml of luminol (250  $\mu\text{g}/\text{ml}$  in Tris HCl), and 0.03 ml of  $\text{H}_2\text{O}_2$  (1.35  $\text{mmol}/\text{L}$  in Tris HCl). The enzymatic reaction was started by adding the cell supernatant, and light emission was monitored by a photometer (Nucleotimetre 107, Interbio CIV, Paris, France) in standard conditions (integration for 10 minutes at  $30^\circ\text{C}$ , manual reading), every 10 seconds for 1 minute. In these conditions, no cross-reactivity with neutrophil myeloperoxidase was detected.<sup>28</sup> However, to avoid any possible contamination, only cell suspensions with 85% or more EOSs were tested for the EPO release assay. In addition, the interference of an EPO-independent CL reaction, caused by the presence of labile oxygen products, was ruled out by performing CL assay after an overnight stay of the samples at  $+4^\circ\text{C}$ . Results of CL assays are usually expressed as percentage of the "total" EPO release (100%) obtained by cell lysis with Triton X100 or other detergents. In our study, detergents as well as sonication were poor means to extract EPO from EOSs. There-

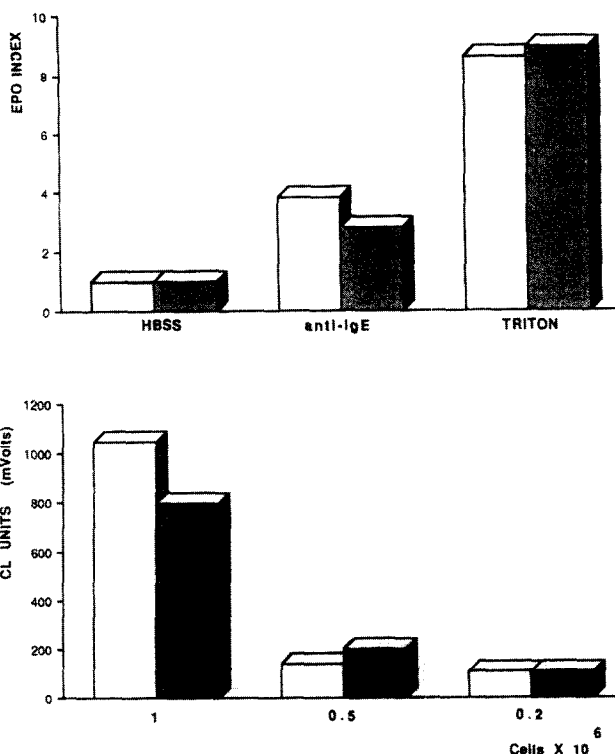


**FIG. 1.** Reproducibility of the CL assay. Experiments performed with cells from the same EOS donors on two occasions. **Top,** Allergic donor. Cells are incubated with medium (HBSS), or anti-IgE, or broken with detergent. Results are expressed as EPO index. **Bottom,** Nonallergic donor with eosinophilia. Different aliquots of cells are broken down with Triton X100. Results are expressed as CL units.

fore, to avoid an overestimate of the degree of EPO release, results were arbitrarily expressed as "index" of EPO release, calculated as follows:

$$\text{EPO index} = \frac{\text{CL units (Eos + stimulus)} - \text{CL units (stimulus alone)}}{\text{CL units (Eos + medium)}}$$

The test is considered positive for index values  $\geq 2$ .

The CL assay appears to be reproducible, as revealed by experiments performed with cells isolated from the same EOS donors on two different occasions (Fig. 1). To evaluate the variability of the two methods of expressing data (i.e., percent of total EPO release or EPO index), we performed 24 experiments including cell aliquots broken down with Triton X100. By expressing data as percent of total EPO release, mean results  $\pm$  SEM were the following: resting cells (HBSS),  $26.3\% \pm 4.5\%$ ; cells stimulated by anti-IgE,  $69.8\% \pm 16.3\%$ ; and cells broken with Triton, 100%. By expressing the same results as EPO index, mean values  $\pm$  SEM were resting cells, 1; cells incubated with anti-IgE,  $3.6 \pm 0.65$ ; and cells broken with Triton,  $8.5 \pm 2.01$ .

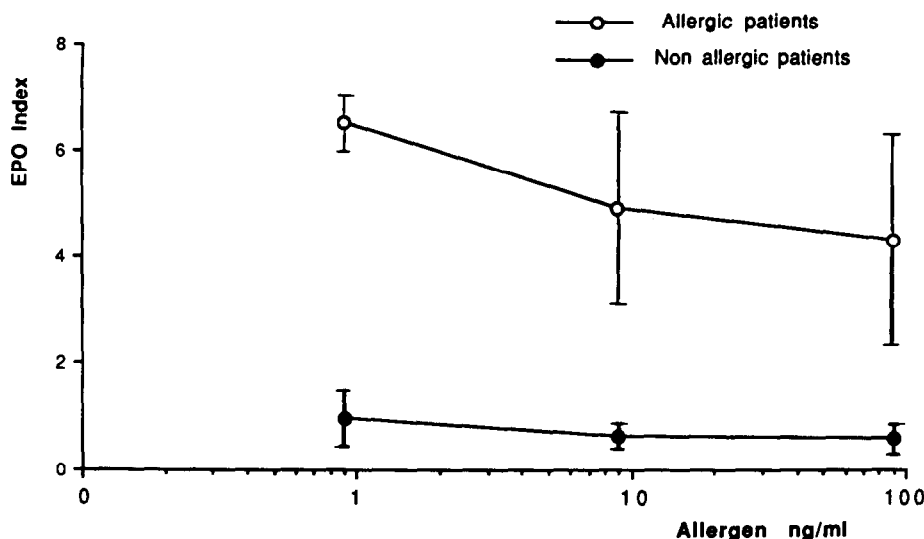


**FIG. 2.** Dose-response studies with allergen (0.9 to 90 ng/ml) in allergic (N = 5) and nonallergic patients (N = 7). Results are expressed as mean values  $\pm$  SEM. The response to allergen is not dose dependent. There is a significant difference of the response in the two groups of patients at each allergen concentration tested ( $p < 0.001$ ).

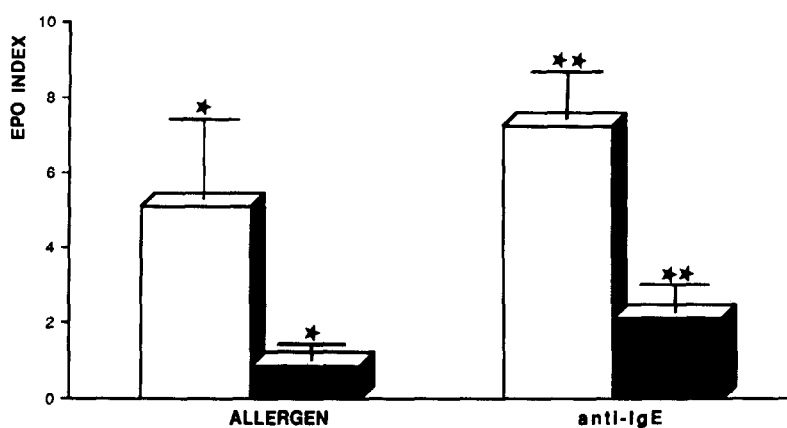


**FIG. 3.** EPO release (mean  $\pm$  SEM) in allergic (□) (N = 7) and non allergic (■) (N = 13) patients after incubation with allergen or anti-IgE Abs. The differences between allergic and non-allergic patients are significant both for allergen (\*) and anti-IgE (\*\*) ( $p < 0.001$ ).

In both cases, results indicate that relatively high amounts of EPO might be secreted on the interaction with the specific stimulus. Therefore, EPO index has a lower variability, as indicated by the standard errors of the mean. In fact, in this way, the high individual variability of basal EPO release can be ruled out. Thus, this method allows a better comparison between results obtained in different patients. EPO index does not reveal quantitative information regarding basal levels of EPO release in each patient; in contrast, it appears a suitable method for detecting eventual variation of the response to specific stimuli. Finally, if the hypothesis that EPO could be stored, like other cationic proteins (MBP, for instance) in an inactive form, we could not compare

results of mechanical breakdown with results obtained with stimuli that induce nonlytic cellular secretion.

### ECP assay

ECP was measured by a sandwich immunoradiometric assay,<sup>29</sup> which requires two different MAb antihuman ECP, EG1 and EG2.<sup>30</sup> Briefly, 50  $\mu$ l of each sample supernatant was added to polyvinylchloride microtiter plates, previously coated with anti-ECP Ab EG1. After 1 hour at 37° C, the amount of bound ECP was assessed by adding <sup>125</sup>I-labeled EG2. Results are usually expressed as micrograms per liter on the basis of a standard curve obtained by measurement of purified ECP. However, to allow comparison with data

of EPO release assay obtained in different aliquots of the same supernatants, results were arbitrarily expressed as ECP index, calculated by the following formula:

$$\text{ECP index} = \frac{\mu\text{g/L ECP (EOS + stimulus)}}{\mu\text{g/L ECP (EOS + medium)}}$$

The different stimuli never contained ECP that is highly specific of EOSs. These values were not subtracted as in the EPO measurement.

### FCM analysis

To evaluate the presence of sIgE, sIgA, or sIgG on purified EOSs, FCM analysis was performed as previously described.<sup>16, 25</sup> Briefly, volumes of 0.5 ml of the cell suspensions ( $1 \times 10^6$  cells per milliliter of HBBS) were directly incubated with FITC-labeled F(ab')<sub>2</sub> fragment of antihuman IgE, antihuman IgA, or antihuman IgG sheep Abs, at a final dilution of 1/10 (30 minutes at +4° C). After two washings, cells were fixed with paraformaldehyde. Stained cell suspensions were then analyzed for surface fluorescence with an Orthocytofluorograph 50H (Ortho Diagnostic Systems, Westwood, Mass.). Results are expressed as percent of cells with fluorescence intensity above the threshold (percent positive cells in the peak or percent fluorescent cells).<sup>31</sup>

### LDH assay

To exclude a possible cell lysis during EOS activation, the presence of the cytoplasmic enzyme, LDH, was monitored in the cell supernatants. The test was performed as previously described.<sup>19</sup> No evidence of cell death was observed in the samples.

## RESULTS

### Allergen and anti-IgE Abs induce EPO release by EOSs from allergic patients

In preliminary experiments performed on a limited number of patients, EPO release, monitored by a CL assay after incubation of EOSs with different doses of allergen, was demonstrated to be higher for cells from allergic patients than for cells from nonallergic patients, for all the concentrations tested (Fig. 2). Therefore, EOSs isolated from seven allergic patients were incubated with a final dilution of 0.9 ng/ml of the specific allergen (*D. glomerata* or *D. pteronyssinus*). EOSs from 13 nonallergic patients with hypereosinophilia were tested in the same conditions, and the release of EPO was evaluated. As illustrated in Fig. 3, only EOSs isolated from allergic patients released significant amounts of EPO after stimulation with the specific allergen (EPO index range, 2.3 to 12.7 for allergic patients and 0.4 to 1.34 for nonallergic patients).

To investigate the specificity of allergen-induced EPO release, EOSs from allergic patients were in-

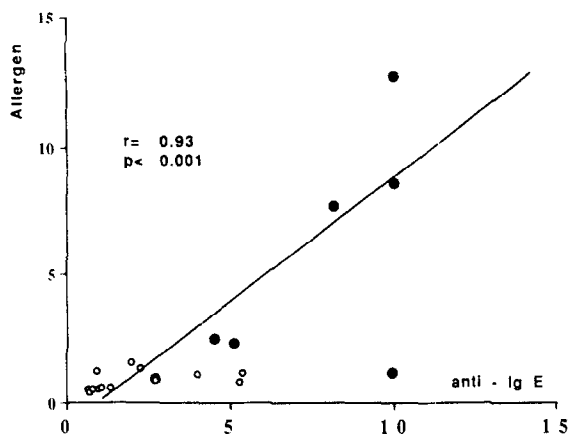


FIG. 4. Correlation between EPO release induced by allergen or anti-IgE in allergic (●) and nonallergic (○) patients ( $r = 0.93$ ;  $p < 0.001$ ;  $N = 20$ ).

cubated with unrelated allergen in five cases (*Polistes* venom, or *D. pteronyssinus* or *D. glomerata*); no EPO release was observed in such experiments (data not presented). The release of EPO was not due to cell death, as demonstrated by the absence of cytoplasmic LDH in the supernatants.

In the same group of patients (Fig. 3), EPO release was measured after the addition of antihuman IgE Abs at a final dilution of 5  $\mu\text{g/ml}$ , chosen on the basis of previously performed dose-response studies. Similar to stimulation with allergen, EOSs from allergic patients released higher levels of EPO than EOSs from nonallergic patients. Moreover, there was a significant correlation between EPO release induced by allergen and by antihuman IgE MABs ( $r = 0.93$ ;  $p < 0.001$ ), suggesting the participation of sIgE in this mechanism of release (Fig. 4).

### EPO release in function of serum IgE levels

EOSs from the seven allergic patients and from 16 nonallergic subjects with eosinophilia, with or without elevated serum IgE levels, were incubated with antihuman IgE MABs at a final concentration of 5  $\mu\text{g/ml}$  (Table II). All the allergic patients under study had increased serum levels of total IgE, as assessed by PRIST (range, 126 to 1060 kU/L; median, 419 kU/L; mean, 552 kU/L). All EOSs purified from allergic patients responded to stimulation with anti-IgE MABs in releasing EPO. In nonallergic patients, elevated IgE levels were found in 11 cases (range, 154 to 4700 kU/L; median 949 kU/L; mean, 1300 kU/L). Interestingly, the statistical analysis performed by Student's *t* test for unpaired samples revealed a significant difference in IgE-induced EPO

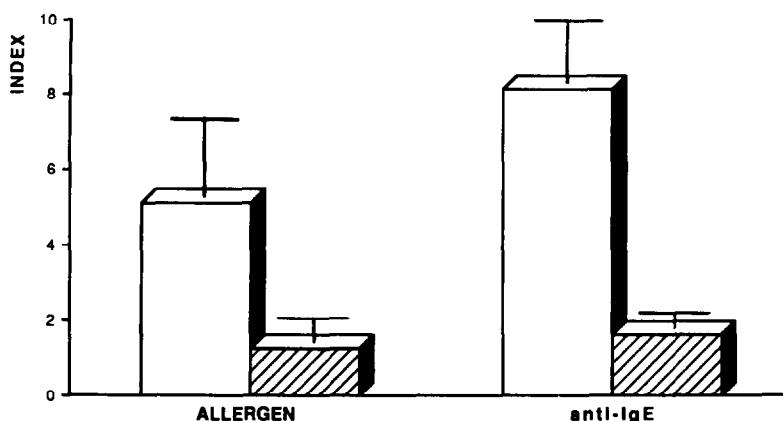


FIG. 5. Comparison of EPO (□) and ECP (▨) release by EOSs from six allergic patients in the presence of allergen or anti-IgE (mean ± SEM); low levels of ECP and high EPO levels.

TABLE II. EPO release after IgE-dependent stimulation of EOSs

|                          | Allergic patients (group I) |            | Nonallergic patients (groups II, III, IV) |            |
|--------------------------|-----------------------------|------------|-------------------------------------------|------------|
|                          | Hyper IgE                   | Normal IgE | Hyper IgE                                 | Normal IgE |
| No. of patients          | 7                           | —          | 11                                        | 5          |
| EPO index (mean ± SD)    | 7.2 ± 3.8                   | —          | 2.4 ± 1.9                                 | 0.9 ± 0.8  |
| No. of positive patients | 7                           | —          | 5                                         | 1*         |
| EPO index (mean ± SD)    | 7.2 ± 3.8                   | —          | 3.4 ± 2.3                                 | 2.27*      |

\*Only one positive patient.

release among the 11 patients with elevated IgE levels and the five subjects presenting normal serum IgE ( $t = 3.2$ ;  $p < 0.01$ ). Nevertheless, not all patients with high IgE levels demonstrated an EPO release on in vitro stimulation with anti-IgE.

#### Absence of ECP release after IgE-dependent stimulation of EOSs from allergic or nonallergic patients

ECP was measured by a radioimmunoassay in aliquots of the same samples assayed for EPO. The comparison between EPO and ECP release by EOSs from six allergic patients is illustrated in Fig. 5. In none of the six allergic patients did the addition of the specific allergen or antihuman IgE induce a significant release of ECP. When results were expressed in absolute values (micrograms per liter), no difference was found between resting cells (mean value, 2.5  $\mu\text{g/l}$ ) or cells incubated with anti-IgE (1.8  $\mu\text{g/l}$ ) or with allergen (2.3  $\mu\text{g/l}$ ). A similar comparison between EPO and ECP release after stimulation with anti-IgE Abs in a total of 16 patients revealed the absence of correlation between EPO and ECP release (Fig. 6).

#### Both EPO and ECP are released after stimulation by antihuman IgA

To investigate the IgA-dependent EOS activation, the release of EPO and ECP was evaluated after incubation of EOSs with antihuman IgA MAbs for 1 hour at 37° C. The release of EPO and ECP was compared in 14 patients, and the results are illustrated in Fig. 7. Six of the 14 patients released significant levels of EPO after stimulation with antihuman IgA, whereas nine patients released significant amounts of ECP. Very interestingly, the individual results of EPO and ECP release were significantly correlated, as illustrated in Fig. 7 ( $r = 0.6$ ;  $p < 0.02$ ).

#### Antihuman IgG induce ECP but low EPO release

Similarly, the release of EPO and ECP was compared on different aliquots of the same supernatants of EOSs purified from 11 patients after incubation with antihuman IgG MAbs for 1 hour at 37° (Fig. 8). Except for one patient revealing high levels of both EPO and ECP, all the patients' EOSs releasing significantly high levels of ECP (index range, 4 to up to 12) did not release significant amounts of EPO.

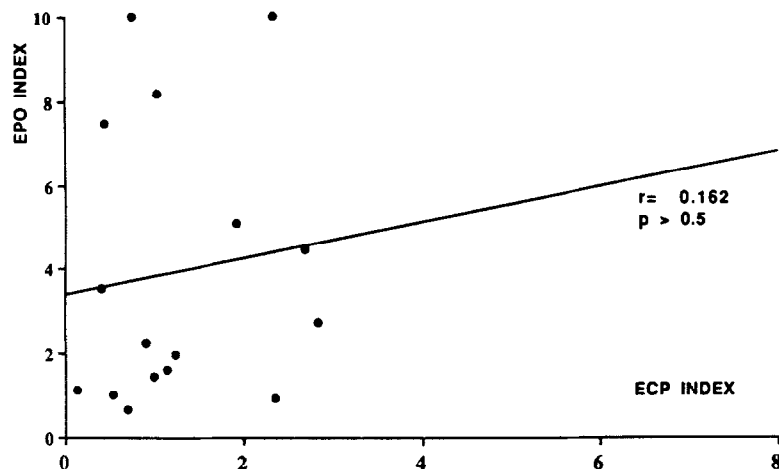


**FIG. 6.** Lack of correlation between EPO and ECP release in the total group of patients tested for anti-IgE (N = 16); high levels of EPO and low ECP levels.

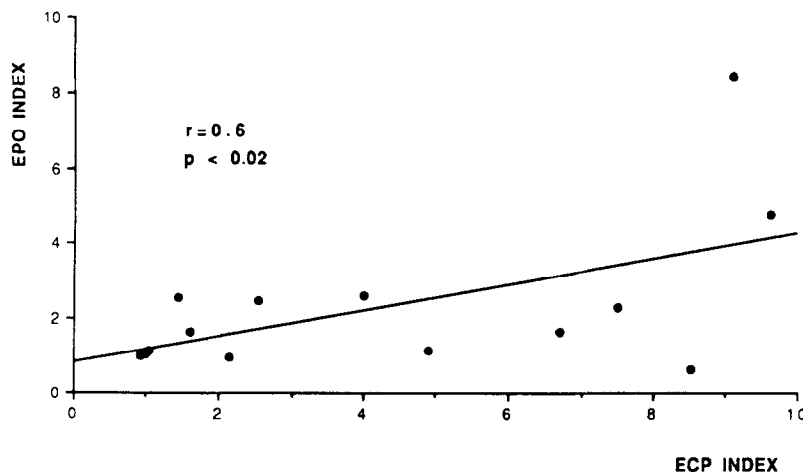


**FIG. 7.** Significant correlation between EPO and ECP release in the supernatants of EOSs from 14 eosinophilic patients after incubation with anti-IgA Abs.

### Presence of cytophilic IgE and IgA by FCM

FCM was performed on EOSs purified from the peripheral blood of 12 patients with eosinophilia, after staining with FITC-labeled  $F(ab')_2$  fragment of anti-human IgE sheep Abs to detect membrane-bound IgE; sIgE-bearing EOSs could be detected (range, 5.5% to 27.8% of positive cells). The percentage of positive cells was increased in 10 patients with elevated IgE levels (mean positive cells,  $18\% \pm 9.6\%$ ), compared with values obtained in two patients with normal IgE levels (individual results, 10.3 and 6.8). However, because of the low number of patients in this second group, statistical analysis of variance could not be performed.

In regard to FCM performed to detect sIgA, 16

subjects were studied, nine patients in group A (mean positive cells,  $15.5 \pm 12$ ) and seven patients in group B (mean positive cells,  $8.1 \pm 4.9$ ). There was no significant difference in the percentage of positive cells between the two groups. It appeared, in general, that the same patients were positive for both sIgE and sIgA. Moreover, statistical analysis performed with McNemar's test did not reveal a significant difference in the presence of sIgE and sIgA, which implies that the same EOS populations might express both sIgE and sIgA. However, additional experiments, specially with double labeling, are required to confirm this hypothesis. Because of the limited amount of EOSs and because we had previously demonstrated that EOSs from patients with eosinophilia did not express high

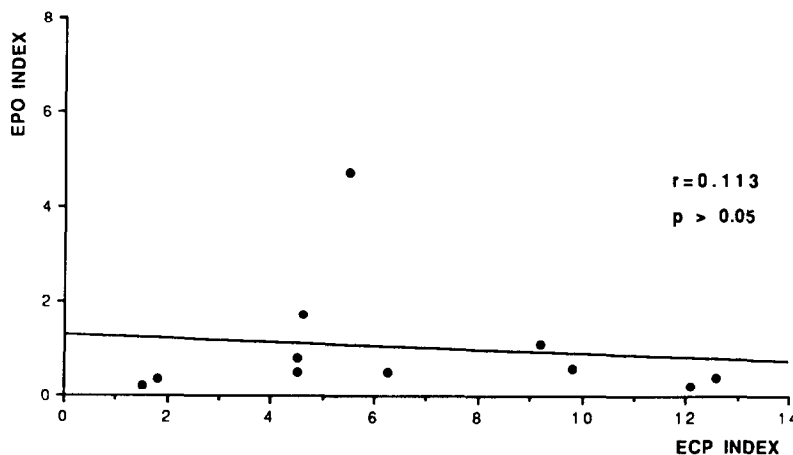


FIG. 8. Lack of correlation between EPO and ECP released by EOSs from 11 patients after incubation with anti-IgG Abs. High levels of ECP and low levels of EPO.

amounts of cytophilic IgG,<sup>16</sup> only a few experiments were performed by FCM with antitotal IgG MABs. These experiments confirmed the low levels of sIgG (mean positive cells,  $6.5 \pm 2.2$ ).

## DISCUSSION

The involvement of EOSs in the pathogenesis of allergic diseases is suggested by the presence of increased numbers of hypodense EOSs in patients with bronchial asthma<sup>32</sup> or allergic rhinitis.<sup>33</sup> The morphology of hypodense EOSs, as well as the raised blood levels of granule proteins observed in hyper-eosinophilic situations, suggest that these EOSs have at least partly degranulated.<sup>34-36</sup> However, there have been very few studies dealing with the stimuli inducing EOS degranulation and the subsequent release of granule mediators. Based on previous data obtained with parasite-infected patients,<sup>19</sup> the purpose of the present work was to investigate the release of two potent cytolytic proteins, EPO and ECP, by EOSs from allergic patients stimulated *ex vivo* by allergen. In parallel, their stimulation with anti-IgE, anti-IgA, or anti-IgG MABs allowed us to evaluate the participation of surface immunoglobulin bound to EOSs in the exocytosis of these two granule proteins.

The results presented here indicate that highly purified blood EOSs from allergic patients released EPO under *in vitro* exposure to specific allergens. Interestingly, only the allergens related to the patient symptoms and eliciting positive skin tests and RAST induced extracellular EPO release. The possible involvement of cytophilic IgE is suggested by the significant correlation between EPO released by allergen and by anti-IgE MABs for individual patients. Our data also suggest that EOSs from patients sensitized by a given allergen can bear cytophilically bound specific IgE Abs and, by releasing

EPO on interaction with this allergen, might therefore directly participate to the pathology of allergic diseases.

The recent demonstration of sIgE and sIgA on intestinal EOSs from parasite-infected mice,<sup>37</sup> as well as on human EOSs,<sup>24</sup> prompted us to investigate the role of sIgA in the release of mediators by EOSs. EPO could be released after direct incubation with anti-human IgA MABs. Interestingly, among all the patients under study, the percentage of patients responding to anti-IgA (6/14) or to anti-IgE (13/23) were quite similar, and there was no significant difference between the degree of activation in both cases. FCM analysis revealed, in addition, that the proportion of EOSs presenting sIgE or sIgA was not different, whereas EOSs isolated from the same patients appeared generally positive for both surface immunoglobulin. The possibility of a coexpression and/or an association between the two Fc receptors for IgE and for IgA on the same EOS subpopulation is presently under investigation. An alternative hypothesis could be that the regulation of the two Fc receptors might be controlled by the same or by linked factors (such as interleukins, for instance). There was no direct correlation between the percentage of surface immunoglobulin-bearing EOSs detected by FCM and their degree of *in vitro* functional reactivity. These results might imply that the presence of cytophilic immunoglobulin is required but probably not sufficient to induce EOS degranulation. The state of activation of EOSs, the presence of other surface markers, such as CR3 demonstrated to be involved in IgE-dependent cytotoxicity,<sup>38</sup> might influence the degree of releasability.

Since EPO is not the only cytolytic molecule involved in EOS-mediated pathology and also because our earlier studies have suggested a differential release

of EPO and ECP after IgE-dependent stimulation,<sup>24</sup> the presence of ECP was evaluated in the same aliquots as those assayed for EPO. As already demonstrated for EOSs from parasite-infected patients,<sup>19</sup> ECP was never detected after *ex vivo* stimulation with the allergen or with anti-IgE Abs, confirming our hypothesis of selective release of mediators. It appears unlikely that these negative results might be due to the assay used for ECP titrations, since very significant ECP levels could be detected after stimulation with anti-IgA Abs or with anti-IgG Abs. The absence of ECP release is presumably not due to the absence of IgE receptors, since sIgE was detected in the group of patients releasing EPO and not ECP. The inverse response to anti-IgG Abs was also very surprising, specially since very few cytophilic IgG could be detected. The antitotal IgG Abs that were used might have resulted in an underestimation of the presence of minor IgG subclass, such as IgG4. In this context, it was previously demonstrated in the rat experimental model that not all the IgG subclasses could be involved in EPO release.<sup>28</sup> These results, associated to recently published results,<sup>39</sup> suggest that IgA and IgG Abs are potent stimuli for the release of ECP and EDN but not IgE Abs. The difference in EOS response to IgE, IgA, and IgG Abs might be linked to different signal transduction pathways delivered by the three Fc receptors (Fc $\epsilon$ R, Fc $\alpha$ R, and Fc $\gamma$ R). In fact, other studies have demonstrated a variability in EOS response to different activators.

The first level of variability could be related to human EOS heterogeneity.<sup>3-19</sup> EOSs with the "hypodense" phenotype are believed to be more activated than normodense EOSs. In addition, they express IgE Fc receptors with higher affinity as well as increased IgE-dependent cytotoxicity.<sup>18</sup> It is therefore not surprising that hypodense EOSs demonstrated enhanced mediator release in response to IgE-dependent stimulation. Not only EPO but also newly generated mediators, such as platelet-activating factor acether<sup>40</sup> or leukotriene C<sub>4</sub><sup>41</sup> have been demonstrated to be preferentially released by hypodense EOSs on activation through IgE Abs, whereas normodense EOSs responded to IgG-dependent activation.<sup>41</sup> Hypodense EOSs represent most tissue EOSs as well as the highest proportion of blood EOSs from hypereosinophilic patients, indicating therefore their potential role as effector inflammatory cells in IgE-dependent mechanisms.<sup>40</sup> The second factor potentially involved in the differential release of mediators could concern the stimulus to which the cells are exposed. For instance, Winqvist et al.<sup>42</sup> have demonstrated that an optimal ECP release was obtained when human EOSs were exposed to insoluble stimuli, such as opsonized particles. In this study, ECP and EDN release was com-

pletely parallel, in contrast to the negligible release of EPO,<sup>42</sup> confirming thus our hypothesis of selective release. However, this factor is probably not the only factor involved, since it has been recently demonstrated that normodense EOSs released EDN in response to IgG- or IgA-coated Sepharose beads but not to IgE-coated Sepharose beads.<sup>26</sup> Apart from EOS heterogeneity and the nature of the stimulus, the observed differences in EPO and ECP release on *in vitro* activation might be due also to the different kinetics in the release of the various granule mediators, as previously suggested.<sup>43</sup> In the present study, EOS activation was followed for 1 hour, a period of time that could be insufficient to detect a later ECP release. However, this would indicate that IgE-dependent activation induced a delayed ECP release, since high levels of ECP could be detected after IgA- or IgG-dependent activation only for 1 hour.

Taken all together, these results suggest that EPO is not released from human EOSs in the same conditions of activation as ECP and EDN and raises the very interesting question of the mechanism of exocytosis of these granule proteins. We have no definitive arguments to answer this question, but recent electron microscopy studies performed on tissue EOSs by immunogold labeling suggest that the granule proteins might leave the granule through the cytoplasm by a vesicular transport mechanism similar to the mechanism described for delayed-type cutaneous basophil hypersensitivity.<sup>40</sup> Additional experiments are needed to elucidate this intriguing phenomenon and specially to know the transduction signal delivered by activation of the various Fc receptors. These results suggest that EOS activation is a complex phenomenon depending on the stimulus used but also to the state of activation or differentiation of cells.

Perhaps the most novel and interesting data are those concerning IgA-mediated EOS activation; sIgA Abs appeared to represent a potent stimulus, since both EPO and ECP could be released. These results confirm our previous findings on the interactions between IgA and EOSs<sup>25</sup> and are in total agreement with recent studies on IgA-mediated release of EDN.<sup>26, 39</sup> These findings might be of relevance to the pathogenesis of a variety of human diseases characterized by eosinophilia involving mucosal surfaces. It should be noted that EOSs are increased in the nasal mucosa of allergic patients during natural pollen exposure, and there is a relationship between EOS activation and allergen-induced local inflammation.<sup>44</sup> However, there is a lack of correlation between the variable ECP levels in nasal lavages and the local hyperresponsiveness observed after nasal allergen challenge.<sup>45</sup> In contrast, elevated blood levels of MBP, ECP, and EDN have been detected in patients with allergic asthma after

bronchial challenge, whereas EPO levels were reduced.<sup>36</sup> The involvement of EOSs, secretory IgA Abs, and probably ECP has also been suspected in coeliac disease.<sup>46</sup> All these data point to the selective release of EOS mediators in vivo. However, in vivo pathways of EOS secretion might differ from the in vitro situation, depending, for instance, on exposure to a particular local microenvironment. In this respect, the influence of cell-cell interactions, mediated by intercellular adhesion molecules<sup>47</sup> or the role of soluble factors, such as cytokines,<sup>48</sup> have been recently suggested.

We would like to specially dedicate this work to the memory of Dr. Po Chun Tai.

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