

## **Granulocyte/Macrophage Colony-stimulating Factor and Interleukin 3 Release from Human Peripheral Blood Eosinophils and Neutrophils**

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### **Summary**

Human peripheral blood eosinophils released eosinophil survival-enhancing activity when stimulated with the calcium ionophore, ionomycin. The release of activity was detected as early as 3 h after stimulation and was inhibited by an immunomodulating agent, cyclosporin A. The survival-enhancing activity was completely abolished by treatment with anti-interleukin 3 (IL-3) and anti-granulocyte/macrophage colony-stimulating factor (GM-CSF) monoclonal antibodies. Moreover, IL-3 and GM-CSF were measurable in ionomycin-stimulated eosinophil supernatants by immunoassay. Eosinophils produced approximately one-half as much IL-3 and one-fifth as much GM-CSF as ionomycin-stimulated mononuclear cells. Neutrophils also produced IL-3 and GM-CSF, but the amounts were less than those produced by eosinophils. These observations suggest a novel role for eosinophils in pathophysiology of allergic inflammation and host defense mechanisms.

Eosinophils are blood leukocytes associated with helminth infections and allergic diseases, especially bronchial asthma, and may mediate immunity and tissue damage (1). Several cytokines, including IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF), induce eosinophilopoiesis and activate mature peripheral blood eosinophils in vitro (reviewed in reference 2). Recently, mRNA for these cytokines was detected in allergen-induced late-phase cutaneous reactions in atopic patients, suggesting that these cytokines play an important role in allergic inflammation (3).

Because human peripheral blood eosinophils, despite their high degree of specialization, retain the ability to synthesize cytokines such as TGF- $\alpha$  and IL-1 (4, 5), we investigated whether they can produce IL-3, IL-5, and GM-CSF. We found that ionomycin-stimulated eosinophils produce GM-CSF and IL-3 as shown by enhanced eosinophil survival and by immunochemical measurement.

### **Materials and Methods**

**Cells and Culture Conditions.** Eosinophils, neutrophils, and mononuclear cells were isolated from peripheral blood of healthy volunteers or patients with mild hay fever by discontinuous Percoll density gradient as previously described (6). Purities of eosinophils and neutrophils were  $\geq 91\%$  and  $\geq 99\%$ , respectively. Contaminating cells in the eosinophil preparations were only neutrophils and those in the neutrophil preparations were only eosinophils. Cell viabilities were  $>98\%$ . Cells were incubated ( $2 \times 10^5$  cells per 0.2 ml per well) in Hybri-Care medium (American Type Cul-

ture Collection, Rockville, MD) supplemented with 50  $\mu\text{g}/\text{ml}$  gentamicin and 10% defined calf serum in 96-well flat-bottomed microtiter plates (Falcon Labware, Lincoln Park, NJ) with ionomycin (Calbiochem Corp., San Diego, CA) in the presence or absence of PMA (500  $\text{pg}/\text{ml}$ ; Calbiochem Corp.). After 24 h at 37°C, cell-free supernatants were harvested and frozen until assayed.

**Eosinophil Survival Assay.** To measure eosinophil survival enhancing activity (7) in supernatants, eosinophils were freshly isolated from different donors and were cultured ( $2.5 \times 10^4$  cells per 0.2 ml per well) in Hybri-Care medium in the presence (25%; vol/vol) of supernatants as described above. After 96 h, cell viability and recovery were counted by staining cells with fluorescein diacetate and propidium iodide using a hemacytometer and an epifluorescent microscope. Specific inhibition of eosinophil survival enhancing activity was tested by incubating supernatants with rat mAbs against each of the cytokines (8). Supernatants were treated with antibodies for 1 h at 4°C and then cultured (12.5%; vol/vol) with freshly isolated eosinophils. Preliminary study showed that these antibodies inhibited eosinophil survival induced by the corresponding cytokine in a dose-dependent manner. Each of the antibodies specifically neutralized its respective cytokine and showed no crossreactivity with the other cytokines.

**Immunochemical Measurement of Cytokines.** The amounts of cytokines in the supernatants were measured by immunoenzymetric assay using rat monoclonal capture antibody followed by nitroiodophenyl (NIP)-derivatized detecting mAb. Bound NIP mAb was detected by rat monoclonal anti-NIP immunoperoxidase conjugate and developed by peroxidase chromogenic substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO) plus  $\text{H}_2\text{O}_2$  (8). The threshold sensitivities of assays were IL-3, 40  $\text{pg}/\text{ml}$ ; IL-5, 30  $\text{pg}/\text{ml}$ ; and GM-CSF, 20  $\text{pg}/\text{ml}$ .

Each assay was specific for its respective cytokine and showed no crossreactivity with other cytokines.

**RIA for Eosinophil-derived Neurotoxin (EDN).** The EDN levels in supernatants were measured by RIA to quantitate eosinophil degranulation, as described previously (9).

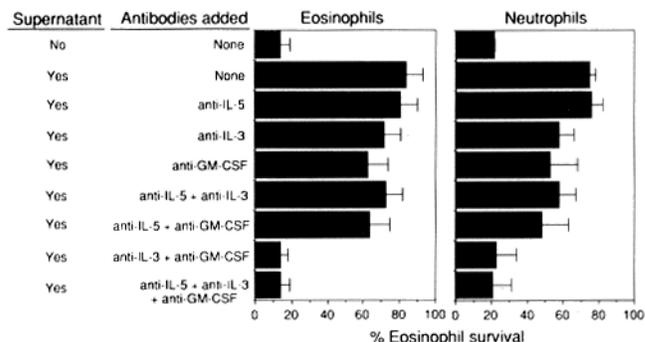
**Statistical Analysis.** The data were compared using paired or unpaired Student's *t* test.

## Results and Discussion

Human peripheral blood eosinophils die within 96 h in culture without specific cytokines, such as IL-3, IL-5, or GM-CSF (10). Treatment with the calcium ionophore, ionomycin, led to a striking enhancement of eosinophil survival in the absence of exogenous cytokines (Fig. 1 *a*). The response to ionomycin was dose dependent and was abolished by high concentrations of ionomycin. Neutrophil survival was not enhanced by ionomycin.

To determine whether ionomycin directly enhanced eosinophil survival or whether it stimulated eosinophils to produce cytokines, supernatants from eosinophils exposed to ionomycin for 24 h were added to freshly isolated eosinophils. Supernatants from eosinophils stimulated by ionomycin enhanced the survival of fresh eosinophils, and the production of factors that enhanced eosinophil survival was dose-dependent (Fig. 1 *b*). Although PMA (500 pg/ml) alone did not induce production of eosinophil survival-enhancing factor from eosinophils, it lowered the ionomycin concentrations required for stimulation. Supernatants from ionomycin-stimulated neutrophils also enhanced eosinophil survival (Fig. 1 *b*), although to a lesser degree than eosinophils ( $p < 0.01$  at 1  $\mu$ M ionomycin).

The possibility that neutrophils contaminating the eosinophil preparations were the source of the survival-enhancing factors was examined. First, 99% pure eosinophils from one donor responded to ionomycin virtually identically to those shown in Fig. 1 *b*. Second, eosinophils isolated by Percoll gradient centrifugation were further purified by culture with rhIL-5 (1 ng/ml) for 7 d; these conditions eliminated neutrophils. Eosinophils were  $\geq 99.8\%$  pure and  $>85\%$  viable and produced survival-enhancing activity with a dose-response to ionomycin similar to that shown in Fig. 1 *b*. Thus, eo-

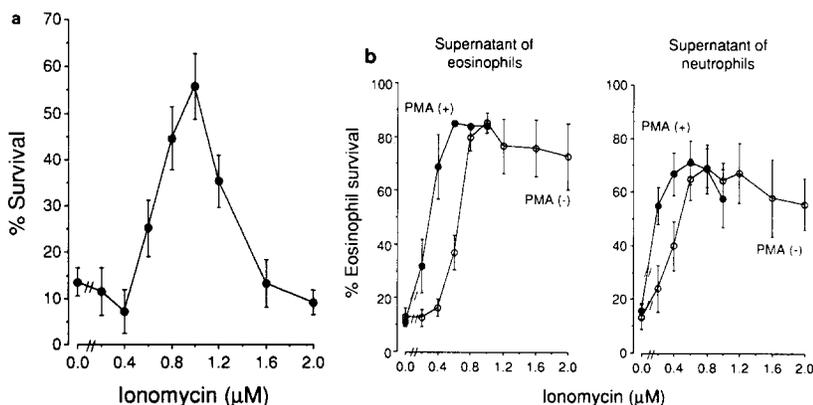


**Figure 2.** Inhibition of eosinophil survival-enhancing activity by mAbs to cytokines. Supernatants of eosinophils and neutrophils stimulated with ionomycin (1  $\mu$ M) were treated with antibodies for 1 h at 4°C and cultured with freshly isolated eosinophils. Data are presented as the mean  $\pm$  SEM from three separate experiments.

sophils themselves produce eosinophil survival-enhancing factors.

To determine whether eosinophil survival-enhancing activity was due to cytokines, supernatants were treated with neutralizing antibodies to IL-3, IL-5, or GM-CSF. Anti-IL-3 and anti-GM-CSF each partially inhibited eosinophil survival-enhancing activity, and, when used together, anti-IL-3 and anti-GM-CSF completely abolished survival-enhancing activity (Fig. 2). These findings indicate that eosinophil survival-enhancing activity from ionomycin-stimulated eosinophils and neutrophils is due to IL-3 and GM-CSF. Moreover, IL-3 and GM-CSF were measurable in the eosinophil supernatants (Table 1). Eosinophils produced about one-half as much IL-3 and one-fifth as much GM-CSF as mononuclear cells. IL-5 was not detected in supernatants of eosinophils or mononuclear cells. Neither IL-3, IL-5, nor GM-CSF were measurable in supernatants from neutrophils, suggesting that neutrophils produce smaller amounts of cytokines than eosinophils.

Comparison of eosinophil cytokine secretion and eosinophil degranulation showed that GM-CSF and IL-3 release begins 3 h after ionomycin stimulation and reaches high levels by 6 h (Fig. 3 *a*). However, no significant release of an eosin-



**Figure 1.** Ionomycin-induced survival of eosinophils (*a*) and release of eosinophil survival-enhancing activity from eosinophils and neutrophils (*b*). (*a*) Eosinophils were incubated with ionomycin. After 96 h, cell viability and recovery were determined. (*b*) Eosinophils and neutrophils were incubated with ionomycin alone (*open circles*) or with ionomycin and PMA (*closed circles*) for 24 h. Supernatants were collected and cultured with eosinophils freshly isolated from different donors. Eosinophil viability was determined after 96 h. Control supernatants from wells with stimulants, but without cells, were inactive. Data are presented as mean  $\pm$  SEM from four (*a*) and six (*b*) separate experiments.

**Table 1. Release of Cytokines by Leukocytes**

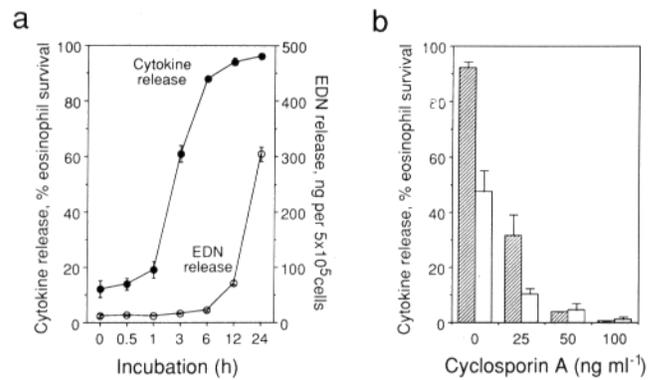
Cell	Stimulus	Cytokine released	
		IL-3	GM-CSF
		<i>pg/ml</i>	
Buffy coat	Ionomycin	87 ± 29 (2/4)*	104 ± 32 (3/4)
	Ionomycin + PMA	52 (1/4)	92 ± 11 (4/4)
Eosinophils	Ionomycin	134 ± 36 (5/6)	45 ± 20 (5/6)
	Ionomycin + PMA	212 ± 124 (3/4)	179 ± 75 (3/4)
Mononuclear cells	Ionomycin	171 ± 75 (2/4)	220 ± 47 (4/4)
	Ionomycin + PMA	425 ± 148 (4/4)	839 ± 289 (4/4)

Isolated eosinophils, neutrophils, mononuclear cells, and buffy coat cells ( $10^6$  cells/ml) were stimulated with ionomycin ( $1 \mu\text{M}$ ) in the presence or absence of PMA (500 pg/ml) for 24 h. Cell-free supernatants were assayed for IL-3, IL-5, and GM-CSF by immunochemical measurement as described in Materials and Methods. In the absence of ionomycin, IL-3 and GM-CSF production by any of the cell preparations were not measurable. Results (mean ± SEM) from four or six separate experiments are shown.

\* Experiments in which the cytokine was detected/total number of experiments.

ophil granule protein, EDN, occurred during the 6-h exposure of cells to  $1 \mu\text{M}$  ionomycin (<1.5% release of total EDN release). By 12 h and clearly at 24 h, appreciable amounts of EDN were released. These results suggest that eosinophil granule protein release and cytokine release are different events.

Cyclosporin A is a powerful inhibitor of cytokine synthesis from lymphocytes (11). Here, cyclosporin A completely abolished cytokine release from both eosinophils and neutrophils (Fig. 3 *b*). Cyclosporin A had no toxic effect on cells, as assessed by cell viability, and had no stimulatory or inhibitory



**Figure 3.** Ionomycin-stimulated cytokine and EDN release from eosinophils (*a*) and inhibition of cytokine release by cyclosporin A (*b*). Isolated eosinophils were incubated in the presence of  $1 \mu\text{M}$  ionomycin for up to 24 h. Supernatants were sequentially removed and frozen until assayed for released cytokine and for EDN. Total EDN content of eosinophils lysed in 0.5% NP-40 was 1,890 ng per  $5 \times 10^5$  cells. (*b*) Eosinophils (hatched bars) or neutrophils (open bars) were incubated with  $1 \mu\text{M}$  ionomycin and cyclosporin A for 24 h, and the supernatants were assayed for cytokine utilizing eosinophil survival. Data are presented as the mean ± SEM from four separate experiments.

effects on eosinophil survival induced by IL-3, IL-5, or GM-CSF. Therefore, in response to this immunomodulating agent, cytokine release from eosinophils and neutrophils is similar to lymphocytes.

Myeloid cells, including myeloid cell lines (12) and freshly isolated mast cells (13), also produce GM-CSF and IL-3. Our results suggest that eosinophils and neutrophils do likewise. Moqbel et al. (14) show expression of GM-CSF mRNA and immunochemical localization of GM-CSF to eosinophils stimulated with calcium ionophore or IFN- $\gamma$ , which supports our observations. GM-CSF and IL-3 are hemopoietic growth factors, causing proliferation, differentiation, and activation of eosinophils and/or neutrophils. Therefore, GM-CSF and IL-3 production by eosinophils and neutrophils may potentiate their roles in host defense and in inflammatory diseases.

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