

Expression of mRNA and Immunoreactivity for the Granulocyte/Macrophage Colony-stimulating Factor in Activated Human Eosinophils

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Summary

Using *in situ* hybridization, we have shown that activated human peripheral blood eosinophils express mRNA for granulocyte/macrophage colony-stimulating factor (GM-CSF). Between 15 and 27% of eosinophils gave positive hybridization signals for GM-CSF mRNA after stimulation with the calcium ionophore A23187 or interferon γ , and 4 and 6% after incubation with interleukin 3 (IL-3) or IL-5. Activated eosinophils also gave specific immunoreactivity with an anti-GM-CSF polyclonal antibody, suggesting translation of the mRNA. These data indicate that eosinophils may be an important source of GM-CSF at sites of allergic inflammation. Furthermore, the identification of GM-CSF production by human eosinophils suggests that the pro-inflammatory potential of this cell type may be substantially greater than hitherto recognized.

Cytokines of the IL-3, IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF) gene cluster may be important in the pathogenesis of atopic allergic inflammation in man (1). The production of these cytokines by activated T lymphocytes (reviewed in reference 2) and mast cells (3) has been well documented. The contribution of eosinophils, prominent cells in the inflammatory infiltrate in allergic disease, to cytokine release is not yet fully realised. The description of transforming growth factor α (TGF- α) and IL-1 α production by human and murine eosinophils, respectively (4, 5), lead us to explore the potential of human eosinophils to elaborate pro-inflammatory cytokines in allergy.

Materials and Methods

Eosinophils. These were obtained from the peripheral blood of subjects with eosinophils of >8% (range, 8–70%) in association with atopy, helminthic infection, or the hypereosinophilic syndrome. The mean percentage eosinophil purity (as determined by Kimura stain and confirmed by differential counts using May-Grunwald-Giemsa) using discontinuous metrizamide gradients (6), was 95.4% \pm 1.1 (range, 90.0–99.8%). Neutrophils were the major contaminating cells.

Cell Stimulation. Eosinophils were incubated under sterile conditions with either culture medium alone, A23187 (10^{-6} M), r-IFN- γ (500 or 1,000 U/ml), rhIL-3, or rhIL-5 (both at 300 U/ml) (kind gifts from Dr. Paul Winter, Biogen Research Corp., Cambridge, MA; Bio-Trans Inc., Los Angeles, CA; and Dr. Colin Sanderson, National Institute for Medical research, Mill Hill, UK, respectively). The culture medium consisted of RPMI 1640 sup-

plemented with 10% FCS, 50 μ M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml gentamycin. A23187 stimulation was continued either for 60 min or 24 h (i.e., 30-min incubation with A23187, after which cells were washed once and incubated in fresh culture medium for 24 h). IL-3, IL-5, and IFN- γ stimulation was for 24 or 48 h in culture medium. Ribonucleotide vanadyl complex (20 mM; Sigma Chemical Co., Poole, UK) was added to the cell suspension before preparing cytopsins to inactivate eosinophil ribonuclease (RNase). Cytopsins were prepared on poly-L-lysine-coated slides, fixed in 4% paraformaldehyde in PBS, and washed twice in 15% sucrose in PBS.

In Situ Hybridization. *In situ* hybridization was performed as previously described (7, 8). Briefly, cDNA for GM-CSF was inserted into a PGEM-1 vector, linearized, and transcribed in the presence of 35 S-UTP and T7 and SP6 RNA polymerases for antisense (complementary RNA) and sense (having identical sequence to mRNA) riboprobes, respectively. Cells were permeabilized with proteinase K, and nonspecific binding of 35 S was inhibited by 0.1 M triethanolamine, acetic anhydride, iodoacetamide, and *N*-ethylmaleimide. Antisense or sense probes ($1\text{--}1.5 \times 10^6$ cpm/slide) in hybridization buffer containing dithiothreitol (100 mM) were heated to 60°C for 1 h before hybridization (16 h at 40°C). During high stringency ($4\text{--}0.1 \times$ SSC at 45°–50°C) post-hybridization washings, unhybridized single-stranded RNA was removed by RNase-A. Autoradiography was followed by counterstaining with either haematoxylin, May-Grunwald Giemsa, or carbol chromotrope 2R. PHA-stimulated (5 μ g/ml) human mononuclear cells were used as controls for GM-CSF mRNA⁺ cells. Negative controls included the GM-CSF sense probe and cytopsins treated with RNase-A solution before hybridizing with the antisense probe. As a further control, cells were incubated with cold antisense probe before adding the 35 S-labeled antisense cRNA probe to compete for hybridiza-

tion with GM-CSF mRNA. This resulted in a substantial reduction in the number of GM-CSF mRNA⁺ cells. The percentage of positive cells was determined by "blind" counting >1,000 cells/cytospin, using an eyepiece graticule, as described (1).

Immunocytochemistry. This was performed using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method as previously described (9). A goat IgG anti-GM-CSF antibody (α -GM-CSF; British Bio-technology, Oxford, UK) was selected for its ability to neutralize the bioactivity of both recombinant and natural human GM-CSF with no crossreactivity with IL-1 α or β , IL-2, IL-3, IL-4, IL-6, TNF- α , TNF- β , or G-CSF. Cytospins were washed in Tris-buffered saline (TBS), and α -GM-CSF was added at a final concentration of 100 μ g/ml and allowed to incubate overnight (4°C). Slides were briefly washed in TBS, APAAP-conjugated rabbit anti-goat was added at 1:40 dilution, and developed with Fast Red. PHA-stimulated mononuclear cells were used as a positive control. An antigen absorption test resulted in a marked reduction in immunoreactivity of α -GM-CSF in both IFN- γ -activated eosinophils and PHA-stimulated mononuclear cells in the presence of 10 μ g/ml of rhGM-CSF, indicating the specificity of the antibody.

Results and Discussion

Eosinophils incubated either with A23187 or recombinant IFN- γ , IL-3, or IL-5 (cytokines that upregulate eosinophil effector function) (10–12) gave clear hybridization signals with the GM-CSF antisense probe (Table 1). Negative signals were obtained with the sense probe in all preparations examined. After a 60-min incubation with A23187, 15.1% of the cells were GM-CSF mRNA⁺. This percentage increased to 27.3% after a 24-h incubation. With medium alone, only 0.5–1% were GM-CSF mRNA⁺. Both IL-3 and IL-5 increased the number of GM-CSF mRNA⁺ from 1% to 6.1% and 4.6%, respectively. After incubation with IFN- γ (500 U/ml), 16.5% (24 h) and 18.7% (48 h) of the eosinophils

Table 1. *In Situ Hybridization of Human Eosinophils with Riboprobes for Human GM-CSF*

Treatment	Time	n	Percentage mRNA ⁺ cells GM-CSF probes (mean \pm SEM)	
			Antisense	Sense
Medium alone	60 min	4	0.5% \pm 0.2	0
A23187 (10 ⁻⁶ M)	60 min	6	15.1% \pm 2.9	0
A23187 (10 ⁻⁶ M)	24 h	3	27.3% \pm 6.4	0
Medium alone	24 h*	6	1.0% \pm 0.14	0
IFN- γ (500 U/ml)	24 h	6	16.5% \pm 2.5	0
IFN- γ (500 U/ml)	48 h	4	18.7% \pm 1.1	0
IFN- γ (1,000 U/ml)	48 h	3	23.3% \pm 3.5	0
IL-5 (300 U/ml)	24 h†	3	4.6% \pm 1.2	0
IL-3 (300/ml)	24 h†	3	6.1% \pm 1.4	0

* Two out of six experiments were 48-h incubation.

† One out of three experiments was 48-h incubation.

Table 2. *Detection of Eosinophil-associated GM-CSF by Immunostaining*

Treatment	Anti-GM-CSF
Medium alone	2.3 \pm 1.8
A23187 (60 min)	26.6 \pm 7.7
A23187 (24 h)	30.3 \pm 10.5
IFN- γ (48 h)	29.0 \pm 9.8

Immunoreactivity of human eosinophils with an anti-GM-CSF antibody using APAAP. The counts represent mean \pm SEM ($n = 3$) of percentage of positively staining cells.

expressed mRNA for GM-CSF. Increasing the concentration of IFN- γ to 1,000 U/ml over 48 h resulted in a further increase in the percentage of mRNA⁺ cells to 23.3%. The identification of mRNA for GM-CSF in activated, but not resting, eosinophils suggests that the protein is not constitutively expressed but induced after stimulation.

Both RNase pre-treatment of the eosinophil preparations and competition experiments (using cold and radiolabeled probes) resulted in a substantial reduction in the antisense GM-CSF mRNA signal. These observations, together with the negative hybridization observed with the sense probe, confirm the specificity of the signal.

Evidence for translation of GM-CSF mRNA in activated eosinophils was obtained by immunocytochemical staining with an α -human GM-CSF. Eosinophils incubated with A23187 or IFN- γ , but not medium alone, gave positive staining (Table 2).

The identification of picogram amounts of GM-CSF in supernatants of Ionomycin-stimulated human eosinophils, but not controls (13), provides important additional evidence to support the hypothesis that this cell type has the capacity to synthesize, store, and secrete GM-CSF.

We were particularly careful to ensure that the GM-CSF mRNA hybridization signals could not be attributable to the small number of contaminating leukocytes (Fig. 1 A). A combination of in situ hybridization with eosinophil-specific staining (carbol chromotrope 2R) confirmed the localization of GM-CSF mRNA to eosinophils (Fig. 1, C and D). Furthermore, the mean purity of the eosinophils used in this study was >95%, while the mean percentage of GM-CSF mRNA⁺ cells was up to fourfold higher than the maximum percentages of contaminating cells.

We recently demonstrated mRNA expression for the IL-3, IL-4, IL-5, and GM-CSF gene cluster in allergen-induced late-phase cutaneous reactions in atopic subjects (1). Although the T lymphocyte is considered to be a major source of these cytokines, our data raise the possibility that infiltrating eosinophils also contribute to the overall cytokine production in allergic tissue reactions. Bearing in mind the well-established association of eosinophils with asthma, the production of cytokines by eosinophils may also be important in this disease. Human eosinophils have been shown to express and re-

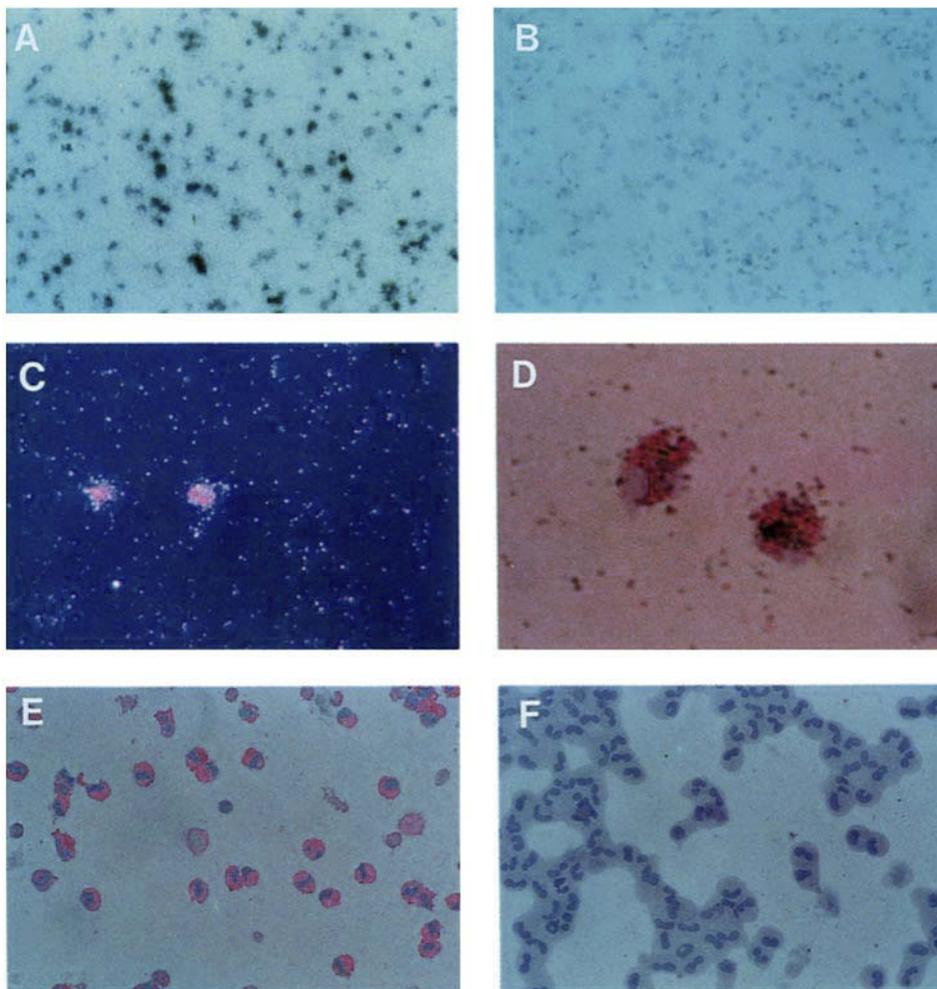


Figure 1. Detection of GM-CSF mRNA and GM-CSF immunoreactivity in activated human eosinophils. (A) Positive in situ hybridization with antisense GM-CSF riboprobe in eosinophils stimulated with IFN- γ (500 U/ml, 48 h). (B) Similar preparation, treated with a GM-CSF sense probe showing no positive hybridization signals. (C and D) Sub-cellular localization of GM-CSF mRNA in human eosinophil stimulated with IFN- γ , stained with carbol chromotrope 2R (60 min in 1% solution), and visualized by dark field illumination (C) or phase contrast microscopy (D). (E and F) Immunocytochemical staining of human eosinophils using an anti-GM-CSF polyclonal antibody. Positively stained cells were observed in preparations incubated with IFN- γ (1,000 U/ml, 48 h) (E), but not with medium alone (F).

lease transforming growth factor α (4), although its relevance to allergic inflammation is yet to be determined. GM-CSF, on the other hand, is a particularly versatile pro-inflammatory mediator that has a wide range of biological activities, including the differentiation of granulocytes and macrophage precursor cells as well as the proliferation of monocytes and endothelial cells (reviewed in reference 14). Together with IL-3, GM-CSF is required for the early growth and differen-

tiation of eosinophil precursors (15). GM-CSF also activates neutrophils, eosinophils, monocytes, and basophils (14). Therefore, in addition to basic proteins and lipid mediator release (16), GM-CSF may also contribute to eosinophil-mediated tissue injury. It also raises the possibility of an autocrine mechanism, where stimulated eosinophils may both release and respond to GM-CSF.

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