

Cytokines directly induce degranulation and superoxide production from human eosinophils

Shiro Horie, MD, Gerald J. Gleich, MD, and Hirohito Kita, MD

Rochester, Minn.

Background: Cytokines are implicated in allergic diseases and can modulate effector functions of eosinophils stimulated by another agonist. However, little is known about the capacity of cytokines to directly trigger eosinophil degranulation.

Objectives: We attempted to determine whether cytokines can directly induce degranulation and superoxide production from eosinophils.

Methods: Eosinophils from normal donors were incubated with various cytokines in albumin-coated tissue culture plates for 4 hours. To quantitate degranulation, the amounts of eosinophil-derived neurotoxin in supernatants were measured by radioimmunoassay. In addition, superoxide production was measured by superoxide dismutase-inhibitable reduction of cytochrome c.

Results: IL-5, IL-3, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor- α , and RANTES all induced eosinophil degranulation. Granulocyte-macrophage colony-stimulating factor was the most potent and induced eosinophil-derived neurotoxin release comparable to that induced by secretory IgA beads, one of the most potent secretagogues for eosinophils. In addition, IL-5 and tumor necrosis factor- α were synergistic in their induction of eosinophil degranulation. In contrast, IL-1, IL-8, interferon- γ , and macrophage inflammatory protein-1 α did not induce degranulation. Finally, IL-5, IL-3, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor- α , but not RANTES, also induced superoxide production from eosinophils.

Conclusions: Certain cytokines directly induce eosinophil degranulation and superoxide production in vitro. Therefore these cytokines may be important in the release of toxic granule proteins from eosinophils in allergic diseases. (*J Allergy Clin Immunol* 1996;98:371-81.)

Key words: Eosinophils, cytokine, degranulation, superoxide production

Eosinophils play important roles in host defense against parasites, diseases associated with eosinophilia, and allergic diseases, such as bronchial asthma and atopic dermatitis.¹ Although eosinophils produce a number of inflammatory mediators, current information implicates toxic cationic proteins from the granules in the development of eosinophil-associated inflammation and tissue injury.¹ For example, eosinophil infiltration and dep-

osition of granule proteins were observed in the bronchial tissue specimens and bronchoalveolar lavage (BAL) fluids obtained from patients with bronchial asthma.^{2,3} Evidence from in vitro and in vivo studies shows that eosinophil granule proteins, such as major basic protein and eosinophil cationic protein, damage respiratory epithelium.^{4,5} Furthermore, the concentrations of granule proteins in BAL fluids correlate with the severity of disease in patients with symptomatic asthma.^{3,6} Despite this information involving eosinophils and granule proteins in the pathophysiology of the diseases, little is known about the mechanism or mechanisms that trigger the release of granule proteins.

Among various mediators involved in diseases associated with eosinophils, cytokines are likely one of the critical factors. For example, a T_{H2}-like cytokine message has been detected in BAL cells

From the Departments of Immunology and Internal Medicine, Mayo Clinic and Mayo Foundation, Rochester, Minn.

Supported by grants from the National Institutes of Health (AI 15231, AI 34577, AI 34486) and by the Mayo Foundation.

Received for publication June 8, 1995; revised Oct. 17, 1995; accepted for publication Nov. 17, 1995.

Reprint requests: Hirohito Kita, MD, Department of Immunology, Mayo Clinic, Rochester, MN 55905.

Copyright © 1996 by Mosby-Year Book, Inc.

0091-6749/96 \$5.00 + 0 1/1/70871

Abbreviations used

BAL:	Bronchoalveolar lavage
DCS:	Defined calf serum
EDN:	Eosinophil-derived neurotoxin
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
HEPES:	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HSA:	Human serum albumin
ICAM:	Intracellular adhesion molecule-1
IFN- γ :	Interferon- γ
MIP:	Macrophage inflammatory protein
PAF:	Platelet activating factor
Pipes:	Piperazine-N,N'-bis[2-ethanesulfonic acid]
PMA:	Phorbol myristate acetate
TNF:	Tumor necrosis factor

and bronchial biopsy specimens from patients with asthma.^{7,8} In addition, various cytokines, such as IL-1 β , IL-2, IL-5, IL-6, tumor necrosis factor (TNF)- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF), were detected in BAL fluids from patients with symptomatic asthma or after segmental challenge with allergen.^{9,10} After allergen challenge, IL-1 β , IL-5, IL-6, and GM-CSF were present in the nasal secretions of patients with allergic rhinitis.¹¹ Also, elevated levels of IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, TNF- α , GM-CSF, and/or interferon (IFN)- γ were detected in BAL fluids from patients with pulmonary eosinophilia of various causes.¹² Furthermore, levels of IL-5 in the nanogram per milliliter range were detected in sera from patients with episodic angioedema associated with eosinophilia¹³ and in patients with cancer treated with IL-2.¹⁴ Thus cytokines are markedly elevated in various diseases associated with eosinophils, and these cytokines may play crucial roles in eosinophilic inflammation.

Several *in vitro* studies suggest that cytokines are important in induction and/or modification of effector functions of eosinophils. In general, these cytokines can be divided into three categories depending on their biologic activities. The first class is eosinophil growth factors, such as IL-3, IL-5, and GM-CSF; they induce proliferation and differentiation of eosinophil lineage, enhance survival of mature eosinophils, and upregulate various functions of the cells.^{1,15} The second class is proinflammatory cytokines, such as IFN- γ and TNF- α ; they support *in vitro* survival and enhance certain functions of the cells, such as expression of Fc

receptors and adhesion molecules.^{1,15} The third class belongs to the newly discovered chemokine superfamily with strong chemotactic activity and rather restricted target cell specificity.¹⁶ Among cytokines in this class, IL-8, RANTES, macrophage inflammatory protein (MIP)-1 α , and monocyte chemoattractant protein-3 are known chemoattractants for human eosinophils and induce effector functions of the cells, such as degranulation.¹⁷ However, despite this rapidly increasing knowledge, no detailed studies have examined whether cytokines induce effector functions of eosinophils.

Previously, we found that cytokines, such as IL-5, GM-CSF, and IL-3, enhance eosinophil degranulation induced by Sepharose beads (Pharmacia, Piscataway, N.J.) coated with secretory IgA (sIgA) or IgG.¹⁸ Recently, we also found that GM-CSF can induce eosinophil degranulation in a β_2 -integrin-dependent manner.¹⁹ Therefore in this study, we tested the abilities of various cytokines (including eosinophil growth factors, proinflammatory cytokines, and chemokines) to directly induce effector functions of human eosinophils, such as degranulation and superoxide production.

METHODS**Cytokines and other soluble stimuli**

Human GM-CSF, IL-1 β , IL-3, TNF- α , MIP-1 α , RANTES, and IFN- γ were purchased from Genzyme Corp. (Cambridge, Mass.). Human IL-5 was a generous gift from Schering-Plough Research Institute (Kenilworth, N.J.). Human IL-8 was purchased from R&D Systems (Minneapolis, Minn.). Cytokines were diluted with phosphate-buffered saline containing 0.1% bovine serum albumin to 0.5 to 100 μ g/ml or 1000 U/ml (IFN- γ) and were stored at -70° C. Platelet activating factor (PAF) and phorbol myristate acetate (PMA) were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, Pa.) and Calbiochem (La Jolla, Calif.), respectively. PAF was dissolved in absolute ethanol at a concentration of 40 mmol/L and stored at -20° C. PMA was dissolved with dimethyl sulfoxide at a concentration of 5 mg/ml and stored at -20° C. All the stimuli were diluted in reaction medium immediately before the experiments. Solvent controls, containing ethanol or dimethyl sulfoxide diluted in medium ($\leq 0.025\%$), had no detectable effects on the eosinophil degranulation response and superoxide production. Immunoglobulins coupled to cyanogen bromide-activated Sepharose 4B beads (Pharmacia Inc.) were also used to stimulate eosinophil degranulation. The beads were coupled to sIgA (Accurate Chemical Scientific Corp., Westbury, N.Y.) or purified human serum IgG (Organon Teknica-Cappel, Malvern, Pa.) at a concentration of 5 mg of

protein per milliliter of packed beads, as described previously.²⁰

Eosinophil isolation

Eosinophil isolation was performed with minor modifications of the method described by Hansel et al²¹ by using a magnetic cell separation system (MACS; Becton Dickinson, San Jose, Calif.). Briefly, venous blood anticoagulated with 50 U/ml heparin was obtained from normal volunteers and diluted with phosphate-buffered saline at a 1:1 ratio. Diluted blood was overlaid on isotonic Percoll solution (density, 1.082 gm/ml; Sigma Chemical Co., St. Louis, Mo.) and centrifuged at 1000 g for 30 minutes at 4° C. The supernatant and mononuclear cells at the interface were carefully removed, and erythrocytes in sediment were lysed by two cycles of hypotonic water lysis. Isolated granulocytes were washed twice with piperazine-N,N'-bis[2-ethanesulfonic acid] (Pipes) buffer (25 mmol/L Pipes, 50 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L NaOH, 5.4 mmol/L glucose, pH 7.4) with 1% defined calf serum (DCS) (HyClone Laboratories, Logan, Utah), and an approximately equal volume of anti-CD16 antibody conjugated with magnetic particles (Miltenyi Biotec Inc., Auburn, Calif.) was added to the cell pellet. After 60 minutes of incubation on ice, 5 ml of Pipes with 1% DCS was added to the cell-antibody mixture. Resuspended cells were loaded onto the separation column positioned in the strong magnetic field of the MACS. Cells were eluted three times with 5 ml of Pipes buffer with 1% DCS. The purity of eosinophils counted by Randolph's stain was greater than 94%. Purified eosinophils were washed twice in Pipes buffer with 1% DCS and suspended in reaction medium.

Eosinophil degranulation

With the exception of the experiments shown in Fig. 1, eosinophil degranulation was induced by various stimuli in polystyrene tissue culture plates coated with human serum albumin (HSA). Briefly, to coat plates with HSA, 96-well flat-bottom tissue culture plates (Falcon no. 3072, Becton Dickinson) were coated with 30 μ l of 2.5% HSA (Sigma, A3782) dissolved in phosphate-buffered saline (pH 7.4) and incubated at 37° C for 2 hours. The purity of the HSA was certified by the manufacturer, and the product was globulin-free. After incubation, wells were washed three times with 0.9% NaCl and used immediately for the experiments. Eosinophils were washed twice with RPMI-1640 medium (Celox Inc., Hopkins, Minn.) with 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and resuspended in the same medium at 5×10^5 cells/ml. The soluble stimuli (i.e., cytokines) were also diluted in the same medium. Subsequently, 100 μ l aliquots of cell suspension were added to each HSA-coated well, and degranulation was initiated by adding 100 μ l of each stimulus. When Ig-coated Sepharose 4B beads were used as stimuli, 2.5×10^4 of beads suspended in 100 μ l

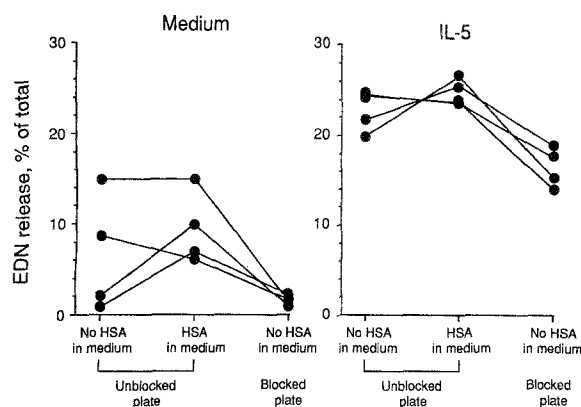


FIG. 1. Effects of HSA on spontaneous (left panel) and IL-5-stimulated eosinophil degranulation (right panel). Ninety-six-well tissue culture plates were coated with HSA for 2 hours or left untreated. Eosinophils suspended in medium with or without 0.1% HSA were added to the plate and incubated with or without 100 ng/ml IL-5 for 4 hours. Results are presented as mean values of four independent experiments performed in duplicate. Each dot represents one experiment; the results for a given eosinophil donor are connected by lines between dots.

of medium were added to the cell suspension. This ratio of eosinophils to Ig-coated beads was previously shown to stimulate maximal cellular degranulation responses.²⁰ After incubation at 37° C in 5% CO₂ for the indicated period, the supernatants from wells were carefully collected and frozen at -20° C until assayed for eosinophil-derived neurotoxin (EDN) as described below. All experiments were carried out in duplicate. In experiments shown in Fig. 1, the effects of HSA, either added to the incubation medium or coated onto the plates for eosinophil degranulation, were studied. In these experiments, 96-well flat-bottom tissue culture plates (Falcon no. 3072, Becton Dickinson) were either coated as described previously with 2.5% HSA or left untreated. Eosinophils were suspended in RPMI-1640 medium with 10 mmol/L HEPES in the presence or absence 0.1% HSA, added to the plates, and stimulated, as described above. In the experiments shown in Fig. 2, eosinophils were stimulated with or without 100 ng/ml IL-5 at intervals of up to 6 hours in HSA-coated plates. In the experiments shown in Fig. 3, eosinophils were preincubated for 30 minutes at 37° C with various concentrations of GM-CSF before they were stimulated by IgG-coated Sepharose beads.

EDN assay

To quantitate eosinophil degranulation, concentrations of EDN in the sample supernatants were measured by radioimmunoassay. The radioimmunoassay is a double-antibody competition assay in which radioiodinated EDN, rabbit anti-EDN antibody, and burro anti-rabbit IgG are used as reported elsewhere.²⁰ To measure the total content of EDN, eosinophils were lysed with 0.5% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) and 0.01 mol/L HCl. All assays were carried out in duplicate.

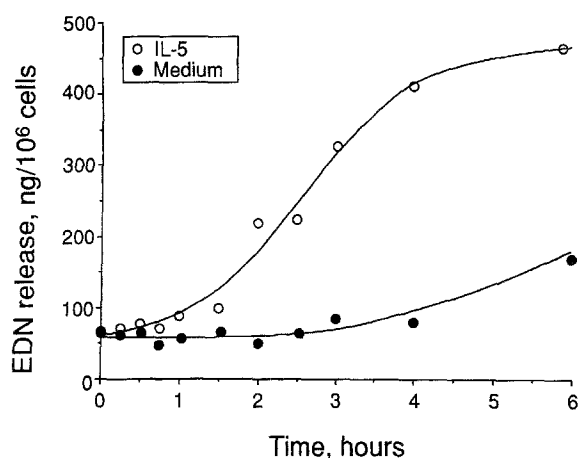


FIG. 2. Kinetics of eosinophil degranulation induced by IL-5. Freshly isolated eosinophils were incubated with 100 ng/ml IL-5 (*open circles*) or medium alone (*closed circles*) in the HSA-coated tissue culture plates for the indicated times, up to 6 hours. Data are presented as mean values of a representative experiment performed in duplicate.

Superoxide anion production

Generation of superoxide by eosinophils was measured by superoxide dismutase-inhibitable reduction of cytochrome *c*, as described elsewhere,²² with slight modifications. We again used 96-well flat-bottom tissue culture plates (Falcon no. 3072, Becton Dickinson) coated with 2.5% HSA, as described previously. Freshly isolated eosinophils were suspended in Hanks' balanced salt solution with 10 mmol/L HEPES and mixed with 100 μ mol/L cytochrome *c* (Sigma) at 1×10^6 cells/ml. One hundred microliters of cell suspension was dispensed onto plates, and the reactions were initiated by adding 100 μ l of stimulus diluted in Hanks' balanced salt solution with 10 mmol/L HEPES. Immediately after addition of stimuli, the reaction wells were measured for absorbance at 550 nm in a microplate autoreader (Thermomax, Molecular Devices, Menlo Park, Calif.), followed by repeated readings. Between absorbance measurements, the plate was incubated at 37°C. Each reaction was carried out in duplicate and compared with an identical control reaction that contained 20 μ g/ml superoxide dismutase (Sigma). Superoxide anion generation was calculated with an extinction coefficient of 21.1×10^3 L/mol $\cdot$ cm for reduced cytochrome *c* at 550 nm and was expressed as nanomoles of cytochrome *c* reduced per 1×10^6 cells.

Statistical analysis

Statistical significance of the differences among various treatment groups was assessed with paired Student's *t* test.

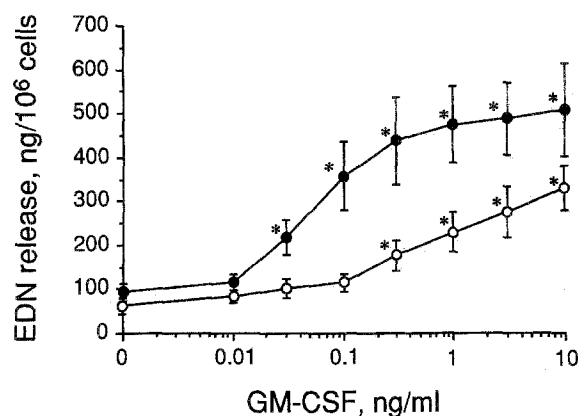


FIG. 3. Comparison of the effects of GM-CSF to directly induce eosinophil degranulation (*open circles*) and to enhance degranulation induced by IgG-coated beads (*closed circles*). To measure enhancement of IgG-induced degranulation by GM-CSF, eosinophils were preincubated for 1 hour with different concentrations of GM-CSF, and then IgG-coated beads were added to the eosinophils and incubated for 3 hours. Total incubation period was 4 hours. To measure GM-CSF direct induction of degranulation, eosinophils were incubated for 4 hours with various concentrations of GM-CSF. Results are presented as means $\pm$ SEM of three independent experiments performed in duplicate. *Significantly different ($p < 0.05$) from values obtained with eosinophils incubated with medium alone or with IgG beads alone.

RESULTS

Determination of experimental conditions

In a series of preliminary experiments, we noted that eosinophils spontaneously release EDN when they are incubated in uncoated "bare" tissue culture plates without any exogenous stimuli. Therefore to minimize spontaneous EDN release from unstimulated eosinophils, we examined the effects of proteins (i.e., HSA) included in the culture medium and coated onto the tissue culture plates (Fig. 1). As shown in the left panel, when eosinophils were incubated without HSA in the medium and in uncoated "bare" tissue culture plates, various degrees of spontaneous EDN release were observed (up to 15% of total EDN content). This nonspecific EDN release was not affected by adding 0.1% HSA to the incubation medium. In contrast, when nonspecific protein binding sites of the plates were blocked with 2.5% HSA before addition of eosinophils, nonspecific EDN release was markedly inhibited to less than 3% of total EDN. As shown in the right panel, eosinophils stimulated by 100 ng/ml IL-5 released EDN, regardless of whether HSA was in the medium or whether the plates were blocked with HSA. The

TABLE I. Eosinophil degranulation induced by various secretagogues

Stimulus	Cells (ng/10 ⁶)	EDN release*	
		Percent of total	No. of experiments
IL-1 (10 ng/ml)	124.1 ± 31.0	3.0 ± 0.6	5
IL-3 (10 ng/ml)	325.3 ± 52.1†	6.5 ± 1.1	9
IL-5 (10 ng/ml)	428.3 ± 70.0†	8.9 ± 1.6‡	9
GM-CSF (10 ng/ml)	697.3 ± 68.8†	13.2 ± 1.4§	9
IL-8 (100 ng/ml)	116.1 ± 27.5	2.4 ± 0.6	4
TNF-α (100 ng/ml)	173.2 ± 21.4†	4.0 ± 0.5	9
RANTES (100 ng/ml)	219.4 ± 23.1†	5.6 ± 1.1	4
MIP-1α (100 ng/ml)	131.2 ± 12.1	2.8 ± 0.9	3
IFN-γ (1,000 U/ml)	107.8 ± 26.1	2.7 ± 0.5	5
sIgA (beads)	701.6 ± 251.4†	14.5 ± 2.2	3
PAF (1 μmol/L)	550.4 ± 49.4†	16.4 ± 3.4	4
PMA (1 ng/ml)	780.4 ± 50.0†	21.5 ± 1.8	9
Medium	105.1 ± 10.8	2.3 ± 0.2	38
Total	4404.8 ± 276.3	100	38

The following cytokines were tested at 10 and 100 ng/ml: IL-1, IL-3, IL-5, IL-8, GM-CSF, TNF-α, RANTES, and MIP-1α. IFN-γ was examined at 100 and 1000 U/ml.

*The results listed are means ± SEM from the indicated numbers of experiments.

†*p* < 0.01 compared with values obtained with eosinophils incubated with medium alone.

‡*p* < 0.01 compared with IL-3 by paired *t* test in paired samples (*n* = 9).

§*p* < 0.01 compared with IL-5 by paired *t* test in paired samples (*n* = 9).

amount of EDN released from eosinophils in HSA-blocked plates was slightly less than that released from the unblocked plates. Therefore coating the tissue culture plates with HSA minimizes nonspecific eosinophil degranulation and maximizes specific release after stimulation by IL-5, resulting in a high (specific release)/(nonspecific release) ratio. Therefore all of the following experiments were performed in plates precoated with 2.5% HSA.

Next, to determine the appropriate incubation period for eosinophil degranulation, we studied the kinetics of EDN release after IL-5 stimulation. As shown in Fig. 2, in the absence of stimulus (medium alone), minimal amounts of EDN were released from eosinophils. When eosinophils were stimulated with IL-5, EDN release was detected at about 1 hour, increased in a time-dependent manner, and reached a plateau at 4 hours. Accordingly, we concluded that 4 hours of incubation was optimal for the study of eosinophil degranulation induced by cytokines.

Finally, we investigated the optimal concentrations of cytokines needed to induce eosinophil degranulation. Previously, we reported that GM-CSF enhances eosinophil degranulation induced by Sepharose beads coated with sIgA or IgG.¹⁸ In that report minimal but significant degranulation was observed with 500 U/ml GM-CSF alone with-

out Ig-coated beads.¹⁸ In this study, as shown in Fig. 3, 0.03 ng/ml GM-CSF significantly enhanced eosinophil degranulation induced by IgG-coated beads. Furthermore, this GM-CSF-enhanced degranulation was concentration-dependent, consistent with the previous report.¹⁸ In the absence of IgG beads, concentrations of GM-CSF at 0.3 ng/ml or higher were required to induce eosinophil degranulation. These findings suggest that at low concentrations, GM-CSF enhances eosinophil degranulation induced by other secretagogues and that by itself GM-CSF causes degranulation at relatively higher concentrations (i.e., nanogram per milliliter range of concentration). On the basis of these preliminary studies, we examined eosinophil degranulation by using nanogram per milliliter concentrations of various cytokines.

Effects of various cytokines on eosinophil degranulation

To investigate the potency of various cytokines and other physiologic stimuli to induce eosinophil degranulation, purified normal human eosinophils were incubated with each stimulus for 4 hours in HSA-blocked plates. As shown in Table I, 10 ng/ml IL-3, IL-5, and GM-CSF, as well as 100 ng/ml TNF-α and RANTES, induced substantial amounts of EDN release. When compared at 10 ng/ml, GM-CSF induced the most degranulation,

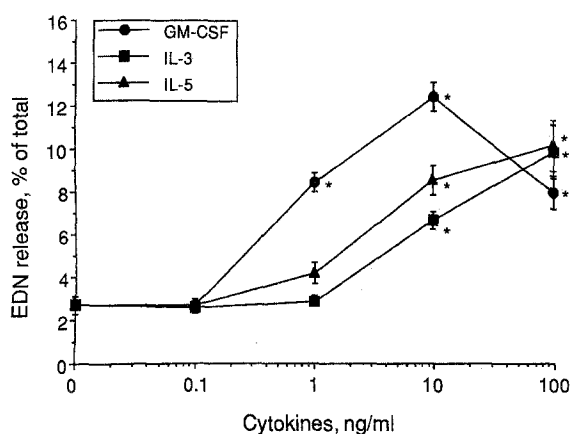


FIG. 4. Eosinophil degranulation induced by IL-3, IL-5, and GM-CSF. Freshly isolated eosinophils were incubated with various concentrations of GM-CSF (circles), IL-3 (squares), or IL-5 (triangles) in HSA-coated tissue culture plates for 4 hours. Results are presented as means $\pm$ SEM of four independent experiments. *Significantly different ($p < 0.05$) from values obtained with eosinophils incubated with medium alone.

IL-5 was intermediate, and IL-3 was the least potent. TNF- α and RANTES at 10 ng/ml did not induce release of EDN (less than 124 ng/10⁶ cells). IL-8 (10 and 100 ng/ml), IFN- γ (100 and 1000 U/ml), IL-1 β (10 ng/ml), and MIP-1 α (10 and 100 ng/ml) did not induce degranulation at any concentration tested. Consistent with previous reports, sIgA beads, PAF, and PMA (all known as potent secretagogues for eosinophil degranulation) were effective stimuli for EDN release.^{20, 23, 24} Interestingly, 10 ng/ml GM-CSF induced approximately 700 ng/10⁶ cells of EDN release; this amount is comparable to the EDN release induced by sIgA beads, one of the strongest physiologic secretagogues for eosinophils.²⁰

Comparison of IL-3, IL-5, and GM-CSF

IL-3, IL-5, and GM-CSF are eosinophil growth factors,^{1, 15} and they are known to share the β -chain of heterodimeric receptors.²⁵ Therefore we compared the potencies of these cytokines in inducing eosinophil degranulation. As shown in Fig. 4, GM-CSF induced significant EDN release at 1 ng/ml and showed maximal effect at 10 ng/ml. In contrast, IL-5 and IL-3 induced eosinophil degranulation at 10 ng/ml and even more degranulation at 100 ng/ml. The maximum amount of EDN release was not greatly different among these cytokines (10% to 12.5% of total EDN). On a mass basis, GM-CSF was about 10-fold more potent than IL-3 and IL-5.

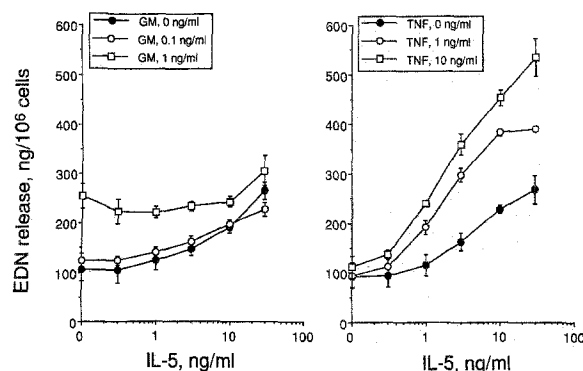


FIG. 5. Effects of GM-CSF (left panel) and TNF- α (right panel) on eosinophil degranulation induced by IL-5. Freshly isolated eosinophils were incubated with either various concentrations of IL-5 plus GM-CSF (no GM-CSF, closed circles; 0.1 ng/ml, open circles; 1 ng/ml, open squares) or various concentrations of IL-5 plus TNF- α (no TNF- α , closed circles; 1 ng/ml, open circles; 10 ng/ml, open squares) in HSA-coated tissue culture plates for 4 hours. Results are presented as means $\pm$ SEM of three experiments performed in duplicate.

Synergism between IL-5 and TNF- α

TNF- α and IL-5 act synergistically to induce expression of the intracellular adhesion molecule-1 (ICAM-1) and HLA-DR on eosinophils.²⁶ Also, TNF- α is found in BAL fluids from patients with symptomatic asthma and pulmonary eosinophilia.^{9, 12} Therefore we attempted to determine whether IL-5 and TNF- α synergistically induce eosinophil degranulation. Eosinophils were incubated with various concentrations of IL-5 in the presence of suboptimal concentrations of TNF- α . We also tested GM-CSF as a positive cytokine control. As shown in Fig. 5, left panel, the effects of the combination of IL-5 and GM-CSF were additive or less than additive; in contrast, as shown in Fig. 5, right panel, the combination of IL-5 and TNF- α was synergistic. For example, 1 ng/ml TNF- α by itself did not induce eosinophil degranulation. IL-5 (10 ng/ml) alone (no TNF) induced 218 ng/10⁶ cells of EDN release (138 ng/10⁶ cells of net EDN release) from eosinophils. When both 1 ng/ml TNF- α and 10 ng/ml IL-5 were added to the cells, eosinophils released 385 ng/10⁶ cells of EDN, resulting in 290 ng/10⁶ cells of net EDN release. Thus TNF- α and IL-5 synergistically induce degranulation of eosinophils, although TNF- α by itself is a weak secretagogue for eosinophils.

Kinetics of IL-5-induced superoxide anion production by eosinophils

Eosinophils are known to produce superoxide anion in response to PMA or PAF stimulation.^{22, 27}

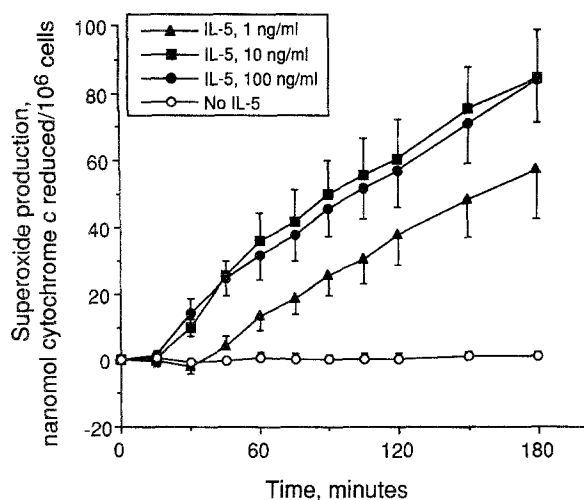


FIG. 6. Kinetics of superoxide production induced by IL-5. Eosinophils were incubated with various concentrations of IL-5 (no IL-5, open circles; 1 ng/ml, closed triangles; 10 ng/ml, closed squares; and 100 ng/ml, closed circles) as described in Methods. Results are presented as means $\pm$ SEM of three independent experiments performed in duplicate.

We have also shown that GM-CSF can induce superoxide production in a β_2 -integrin-dependent manner.¹⁹ Therefore we studied the effects of IL-5 on superoxide production by eosinophils. Eosinophils incubated in HSA-blocked plates were stimulated by IL-5, and the superoxide produced was quantitated. Fig. 6 shows that eosinophils stimulated with 10 or 100 ng/ml IL-5 produced superoxide, which was detectable after 30 minutes and increasing gradually thereafter. Eosinophils produced less superoxide when stimulated by 1 ng/ml IL-5 and no superoxide with medium alone. However, there were no differences in the amounts and kinetics of superoxide production after stimulation by 10 ng/ml and 100 ng/ml IL-5, suggesting that the effect of IL-5 reached a plateau at 10 ng/ml.

Superoxide production induced by various stimuli

Next, we attempted to determine whether cytokines other than IL-5 can induce superoxide production from eosinophils. Eosinophils were incubated at three concentrations (1, 10, 100 ng/ml or 10, 100, 1000 U/ml [IFN- γ]) of various cytokines for periods of up to 180 minutes. Among the cytokines that did induce superoxide production, all of the production was dependent on the cytokine concentration (data not shown). Table II summarizes the results and shows that at 10 ng/ml GM-CSF, IL-5, and IL-3 induced large amounts of

TABLE II. Superoxide production from eosinophils stimulated by various stimuli

Stimulus	Superoxide production (nmol cytochrome c reduced/ 10^6 cells)*	
	60 min	120 min
IL-1 β (10 ng/ml)	-2.0 ± 2.7	-1.8 ± 2.1
IL-3 (10 ng/ml)	$21.1 \pm 4.8^\dagger$	$76.8 \pm 7.4^\ddagger$
IL-5 (10 ng/ml)	$35.9 \pm 8.3^\dagger$	$60.5 \pm 12.1^\dagger$
GM-CSF (10 ng/ml)	$52.5 \pm 13.4^\ddagger$	$89.9 \pm 10.1^\ddagger$
IL-8 (100 ng/ml)	3.1 ± 2.7	-0.5 ± 1.8
TNF- α (100 ng/ml)	$12.7 \pm 3.6^\dagger$	$19.1 \pm 5.1^\dagger$
RANTES (100 ng/ml)	1.1 ± 2.0	0.8 ± 0.9
IFN- γ (1,000 U/ml)	4.2 ± 1.9	2.7 ± 2.2
sIgA (beads)	$35.8 \pm 4.6^\ddagger$	$58.6 \pm 7.4^\ddagger$
PAF (1 μ mol/L)	$52.6 \pm 4.6^\ddagger$	$79.3 \pm 9.3^\ddagger$
PMA (1 ng/ml)	$139.4 \pm 6.9^\ddagger$	$125.4 \pm 6.0^\ddagger$
Medium	1.4 ± 0.9	0.9 ± 0.7

Data are presented as means $\pm$ SEM of three experiments performed in duplicate.

$^\dagger p < 0.05$ compared with eosinophils incubated with medium alone.

$^\ddagger p < 0.01$ compared with eosinophils incubated with medium alone.

superoxide production. Consistent with the degranulation results (Table I), GM-CSF was the most potent stimulus among these three cytokines. In addition, the amounts of superoxide produced by eosinophils stimulated by GM-CSF were similar to those produced by eosinophils stimulated by sIgA beads or PAF, the strongest known physiologic secretagogues for eosinophils.^{20, 23} At 100 ng/ml, TNF- α also induced superoxide production, although less than that induced by 10 ng/ml GM-CSF, IL-3, or IL-5. In contrast, RANTES, IFN- γ , and IL-8 induced no superoxide production even at the highest concentration tested (100 ng/ml). IL-1 β at 10 ng/ml also did not induce superoxide production. With the exception of RANTES, the overall potencies of cytokines to induce superoxide production corresponded with those to induce eosinophil degranulation; in contrast, RANTES induced a moderate amount of EDN release but no superoxide production.

DISCUSSION

IL-5 and other eosinophil active cytokines, including IL-3 and GM-CSF, have been known to enhance effector functions of eosinophils, such as cytotoxicity, superoxide anion production, and degranulation.^{18, 28-32} Most of these studies investigated the effects of cytokines on eosinophils stimulated by another agonist, such as Ig-coated beads

or parasites. Previously, we found that eosinophils cultured with 400 pg/ml IL-5 for 7 days released their granule proteins, suggesting that IL-5 might induce eosinophil degranulation by itself.³³ However, the capacity of these cytokines to directly induce eosinophil effector functions has not been explored in detail. In this study, we demonstrated that by themselves GM-CSF, IL-5, and IL-3 induce appreciable degranulation and superoxide production from normal human eosinophils. Indeed, the extent of degranulation and superoxide anion induced by GM-CSF at 10 ng/ml was almost comparable to that induced by sIgA beads, one of the strongest secretagogues for eosinophils. Therefore certain cytokines may need to be recognized as another class of secretagogues for eosinophils.

By studying various cytokines for their eosinophil degranulation potencies, we made *four* observations. First, we found differences in degranulation potencies among the various cytokine classes. Compared with the potent effects of eosinophil growth factors, such as IL-3, IL-5, and GM-CSF, the potencies of chemokines, such as RANTES and MIP-1 α , and proinflammatory cytokines, such as IL-1 β , IFN- γ , and TNF- α , were weaker. RANTES and MIP-1 α have been recently recognized as powerful chemoattractants for eosinophils.^{34, 35} In addition, at relatively high concentrations RANTES can induce eosinophil cationic protein release (up to 10^{-7} mol/L = 900 ng/ml).^{34, 35} In our study 10 ng/ml RANTES did not induce significant degranulation, but 100 ng/ml RANTES did induce some EDN release, although less than that induced by 10 ng/ml GM-CSF, IL-3, or IL-5. Considering their strong chemotactic activity, it is surprising to find that RANTES and MIP-1 α are not strong secretagogues for eosinophils. Therefore the roles of chemokines and eosinophil growth factors in pathophysiology of eosinophilic inflammation may be different.

Second, we found differences in potency to induce degranulation among the eosinophil growth factors including IL-3, IL-5, and GM-CSF. The receptors for IL-3, IL-5, and GM-CSF are composed of two distinct subunits, α and β .²⁵ The α subunits are primary cytokine-binding proteins, and the β subunits are required for formation of high-affinity binding sites and for signal transduction. The GM-CSF, IL-3, and IL-5 receptors share the same β subunit,³⁶ and, therefore these cytokines share a number of common biologic activities, such as enhancement of survival of mature eosinophils³⁷ and degranulation.¹⁸ Although the possible differences in the biologic activities of

these cytokines have not been fully explored, we found that GM-CSF was approximately 10 times more potent than IL-3 and IL-5. Interestingly, this rank order of potency is similar to that for eosinophil survival. Prolonged eosinophil survival was detected at 0.1 pg/ml GM-CSF and 1.0 pg/ml IL-3 and IL-5 (Okubo et al. Unpublished data) and the 50% effective dose (ED₅₀) values for GM-CSF, IL-5, and IL-3 were 1.0, 10.0, and 10.0 pg/ml, respectively.³⁸ Although the reason for these differences is not clear, it may involve the affinity of receptors for these cytokines; the affinity of the GM-CSF receptor (dissociation constant [Kd] of 44 pmol/L³⁹) on human eosinophils is approximately 10 times higher than those for IL-3 (Kd = 470 pmol/L³⁹) and IL-5 (Kd = 518 pmol/L⁴⁰). Thus GM-CSF can be considered the most potent eosinophil-active cytokine.

Third, the action of TNF- α is also noteworthy. Although TNF- α by itself is a weak agonist for eosinophils to induce degranulation and superoxide production, we found that TNF- α is synergistic with IL-5 to induce eosinophil degranulation (Fig. 4). Previously, TNF- α was shown to work synergistically with IL-5 to induce expression of ICAM-1 and HLA-DR on eosinophils.²⁶ Our findings extend these observations and lead us to speculate that TNF- α may be an important cofactor for eosinophilic inflammation. TNF- α is known to be produced by human lung mast cells and pulmonary macrophages in sensitized lung tissues triggered by IgE.⁴¹ Furthermore, TNF- α enhances expression of adhesion molecules, such as ICAM-1 and vascular cell adhesion molecule-1, on the endothelial cells and airway epithelial cells.^{42, 43} Earlier in vitro studies showed that eosinophils adhere to and migrate through endothelial cells in an ICAM-1- and/or vascular cell adhesion molecule-1-dependent manner.⁴⁴⁻⁴⁶ Interestingly, TNF- α is detected in various biologic fluids from patients with asthma and eosinophilia.^{9, 12} Therefore TNF- α may be an important cytokine that accelerates the eosinophilic inflammation mediated by other cytokines, such as IL-5, and may play important roles in pathophysiologic mechanisms of allergic diseases and eosinophilia.

Finally, we found that relatively high concentrations of cytokines (nanogram per milliliter range) were required to directly induce eosinophil degranulation. It has been reported that GM-CSF enhanced eosinophil survival, starting at 1 pg/ml.⁴⁷ In this study we found that GM-CSF primed eosinophils for IgG-induced degranulation at 30 pg/ml and that it directly induced degranulation at

concentrations of 300 pg/ml or higher. Thus GM-CSF maintains eosinophil survival at low concentrations, primes them at moderate concentrations, and induces degranulation at the highest concentrations. These concentration-dependent, differential functions of cytokines may be important in the pathophysiology of diseases. Eosinophils may be maintained and prepared for activation by low concentrations of cytokines, which are encountered in the bloodstream, and they may be fully activated by high concentrations of cytokines, which are probably encountered in the midst of inflamed tissues.

We should be aware of the actual physiologic concentrations of cytokines. In recent reports cytokines, in particular IL-5, have been considered to be strongly implicated in the pathophysiology of bronchial asthma.³⁸ For example, messenger RNA of IL-5 was detected in cells of BAL fluids from patients with bronchial asthma.⁷ Indeed, BAL fluids collected from patients with allergic rhinitis 48 hours after antigen challenge contained 300 to 600 pg/ml IL-5.¹⁰ In addition, BAL fluid from a patient with chronic eosinophilic pneumonitis contained approximately 150 pg/ml IL-5.⁴⁸ Because the dilution factor of BAL fluid compared with alveolar fluid is estimated to be 100-fold,⁴⁹ the concentration of IL-5 in local tissue could be more than 15 ng/ml. Furthermore, the serum concentrations of IL-5 are more than 1 ng/ml in sera from patients with episodic angioedema associated with eosinophilia and IL-2 toxic syndrome.^{13, 14} Therefore it would not be unreasonable to speculate that the concentration of IL-5 in the inflammatory lesion could be as much as or more than 10 ng/ml, namely the concentration that induced eosinophil degranulation in vitro (Table I and Fig. 4). In addition, 1 ng/ml IL-5 was enough to induce eosinophil degranulation when combined with TNF- α (Fig. 4). Therefore we can confidently speculate that the concentrations of cytokines used in our study can be achieved in vivo, allowing cytokines to directly stimulate degranulation of eosinophils in various diseases associated with eosinophils.

Even though this report clearly demonstrates that certain cytokines by themselves, such as IL-5, IL-3, GM-CSF, can be potent secretagogues for eosinophils, other factors are involved in eosinophil functions in vivo. For example, transgenic mice expressing IL-5 are macroscopically normal apart from marked eosinophilia,⁵⁰ suggesting that either more than IL-5 is needed for initiation of disease or the presence of inhibitor factor(s) for eosinophil degranulation in vivo. Previously, we

found that cellular adhesion dependent on a $\beta 2$ integrin, $\alpha_M\beta_2$ (Mac-1), was required for eosinophil degranulation induced by GM-CSF.¹⁹ We have confirmed that this mechanism is also required for eosinophil degranulation induced by IL-5 (Kita et al. Unpublished data). Recently, we found that extracellular matrix proteins, fibronectin and laminin, coated onto polystyrene tissue culture plates markedly attenuated eosinophil degranulation induced by IL-5 or PAF by reducing $\beta 2$ -integrin-dependent cellular adhesion.⁵¹ Therefore some physiologic factors may regulate degranulation even in the presence of high concentrations of IL-5 in vivo. It would be reasonable to speculate that a complex local milieu is constructed in the inflammatory lesions, and it modulates degranulation of eosinophils exposed to the cytokines. Further studies, based on the fact that cytokines can directly induce eosinophil degranulation, and studies that mimic the local inflammatory environment will benefit the understanding of mechanism(s) of eosinophilic inflammation in a variety of human diseases.

We thank Mrs. Cheryl R. Adolphson for editorial assistance and Mrs. Linda H. Arneson for secretarial help.

REFERENCES

1. Gleich GJ, Kita H, Adolphson CR. Eosinophils. In: Frank SM, Austen KF, Claman HN, Unanue ER, editors. *Samter's immunological diseases*. 5th edition. Boston: Little Brown and Company, 1994:205-45.
2. Filley WV, Holley KE, Kephart GM, Gleich GJ. Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. *Lancet* 1982;2:11-6.
3. Broide DH, Gleich GJ, Cuomo AJ, et al. Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. *J Allergy Clin Immunol* 1991;88: 637-48.
4. Frigas E, Loegering DA, Gleich GJ. Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium. *Lab Invest* 1980;42:35-43.
5. Motojima S, Frigas E, Loegering DA, Gleich GJ. Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium in vitro. *Am Rev Respir Dis* 1989;139:801-5.
6. Bousquet J, Chané P, Lacoste JY, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033-9.
7. Robinson DS, Hamid Q, Ying S, et al. Predominant T_{H2}-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298-304.
8. Hamid Q, Azzawi M, Ying S, et al. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J Clin Invest* 1991;87:1541-6.
9. Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* 1992;89:958-67.

10. Sedgwick JB, Calhoun WJ, Gleich GJ, et al. Immediate and late airway response of allergic rhinitis patients to segmental antigen challenge: characterization of eosinophil and mast cell mediators. *Am Rev Respir Dis* 1991;144:1274-81.
11. Sim TC, Grant JA, Hilsmeier KA, Fukuda Y, Alam R. Proinflammatory cytokines in nasal secretions of allergic subjects after antigen challenge. *Am J Respir Crit Care Med* 1994;149:339-44.
12. Walker C, Bauer W, Braun RK, et al. Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. *Am J Respir Crit Care Med* 1994;150:1038-48.
13. Butterfield JH, Leiferman KM, Abrams J, et al. Elevated serum levels of interleukin-5 in patients with the syndrome of episodic angioedema and eosinophilia. *Blood* 1992;79:688-92.
14. van Haelst Pisani C, Kovach JS, Kita H, et al. Administration of interleukin-2 (IL-2) results in increased plasma concentrations of IL-5 and eosinophilia in patients with cancer. *Blood* 1991;78:1538-44.
15. Weller PF. The immunobiology of eosinophils [review]. *N Engl J Med* 1991;324:1110-8.
16. Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene "intercrine" cytokine family [review]. *Annu Rev Immunol* 1991;9:617-48.
17. Baggiolini M, Dahinden CA. CC chemokines in allergic inflammation. *Immunol Today* 1994;15:127-33.
18. Fujisawa T, Abu-Ghazaleh RI, Kita H, Sanderson CJ, Gleich GJ. Regulatory effect of cytokines on eosinophil degranulation. *J Immunol* 1990;144:642-6.
19. Horie S, Kita H. CD11b/CD18 (Mac-1) is required for degranulation of human eosinophils induced by human recombinant granulocyte-macrophage colony-stimulating factor and platelet-activating factor. *J Immunol* 1994;152:5457-67.
20. Abu-Ghazaleh RI, Fujisawa T, Mestecky J, Kyle RA, Gleich GJ. IgA-induced eosinophil degranulation. *J Immunol* 1989;142:2393-400.
21. Hansel TT, De Vries IJ, Iff MT, et al. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Methods* 1991;145:105-10.
22. Busse W, Randlev B, Sedgwick J. The effect of azelastine on neutrophil and eosinophil generation of superoxide. *J Allergy Clin Immunol* 1989;83:400-5.
23. Kroegel C, Yukawa T, Dent G, Venge P, Chung KF, Barnes PJ. Stimulation of degranulation from human eosinophils by platelet-activating factor. *J Immunol* 1989;142:3518-26.
24. Kita H, Abu-Ghazaleh RI, Gleich GJ, Abraham RT. Role of pertussis toxin-sensitive G protein in stimulus-dependent human eosinophil degranulation. *J Immunol* 1991;147:3466-73.
25. Miyajima A, Kitamura T, Harada N, Yokota T, Arai K. Cytokine receptors and signal transduction [review]. *Ann Rev Immunol* 1992;10:295-331.
26. Hansel TT, De Vries IJ, Carballido JM, et al. Induction and function of eosinophil intercellular adhesion molecule-1 and HLA-DR. *J Immunol* 1992;149:2130-6.
27. Zoratti EM, Sedgwick JB, Vrtis RR, Busse WW. The effect of platelet activating factor on the generation of superoxide anion in human eosinophils and neutrophils. *J Allergy Clin Immunol* 1991;88:749-58.
28. Rothenberg ME, Owen WF Jr, Silberstein DS, et al. Human eosinophils have prolonged survival, enhanced functional properties, and become hypodense when exposed to human interleukin 3. *J Clin Invest* 1988;81:1986-92.
29. Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG, Vadas MA. Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J Exp Med* 1988;167:219-24.
30. Yamaguchi Y, Hayashi Y, Sugama Y, et al. Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 is an eosinophil chemotactic factor. *J Exp Med* 1988;167:1737-42.
31. Silberstein DS, Owen WF, Gasson JC, et al. Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by biosynthetic (recombinant) granulocyte-macrophage colony-stimulating factor. *J Immunol* 1986;137:3290-4.
32. Lopez AF, Williamson DJ, Gamble JR, et al. Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. *J Clin Invest* 1986;78:1220-8.
33. Kita H, Weiler DA, Abu-Ghazaleh RI, Sanderson CJ, Gleich GJ. Release of granule proteins from eosinophils cultured with IL-5. *J Immunol* 1992;149:629-35.
34. Rot A, Krieger M, Brunner T, Bischoff SC, Schall TJ, Dahinden CA. RANTES and macrophage inflammatory protein 1 α induce the migration and activation of normal human eosinophil granulocytes. *J Exp Med* 1992;176:1489-95.
35. Alam R, Stafford S, Forsythe P, et al. RANTES is a chemotactic and activating factor for human eosinophils. *J Immunol* 1993;150:3442-8.
36. Lopez AF, Vadas MA, Woodcock JM, et al. Interleukin-5, interleukin-3, and granulocyte-macrophage colony-stimulating factor cross-compete for binding to cell surface receptors on human eosinophils. *J Biol Chem* 1991;266:24741-7.
37. Wallen N, Kita H, Weiler D, Gleich GJ. Glucocorticoids inhibit cytokine-mediated eosinophil survival. *J Immunol* 1991;147:3490-5.
38. Ohnishi T, Kita H, Weiler D, et al. IL-5 is the predominant eosinophil-active cytokine in the antigen-induced pulmonary late-phase reaction. *Am Rev Respir Dis* 1993;147:901-7.
39. Lopez AF, Eglinton JM, Gillis D, Park LS, Clark S, Vadas MA. Reciprocal inhibition of binding between interleukin 3 and granulocyte-macrophage colony-stimulating factor to human eosinophils. *Proc Natl Acad Sci USA* 1989;86:7022-6.
40. Chihara J, Plumas J, Gruart V, et al. Characterization of a receptor for interleukin 5 on human eosinophils: variable expression and induction by granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1990;172:1347-51.
41. Ohkawara Y, Yamauchi K, Tanno Y, et al. Human lung mast cells and pulmonary macrophages produce tumor necrosis factor- α in sensitized lung tissue after IgE receptor triggering. *Am J Respir Cell Mol Biol* 1992;7:385-92.
42. Wegner GD, Gundel RH, Reilly P, Haynes N, Letts LG, Rothlein R. Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. *Science* 1990;247:456-9.
43. Tosi MF, Stark JM, Smith CW, Hamedani A, Gruenert AC, Infeld MD. Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: effects on neutrophil-epithelial cell adhesion. *Am J Respir Cell Mol Biol* 1992;7:214-21.

44. Ebisawa M, Bochner BS, Georas SN, Schleimer RP. Eosinophil transendothelial migration induced by cytokines. I. Role of endothelial and eosinophil adhesion molecules in IL-1 β induced transendothelial migration. *J Immunol* 1992;149:4021-8.
45. Kyan-Aung U, Haskard DO, Poston RN, Thornhill MH, Lee TH. Endothelial leukocyte adhesion molecule-1 and intercellular adhesion molecule-1 mediate the adhesion of eosinophils to endothelial cells in vitro and are expressed by endothelium in allergic cutaneous inflammation in vivo. *J Immunol* 1991;146:521-8.
46. Walsh GM, Mermod JJ, Hartnell A, Kay AB, Wardlaw AJ. Human eosinophil, but not neutrophil, adherence to IL-1-stimulated human umbilical vascular endothelial cells is $\alpha_4\beta_1$ (very late antigen-4) dependent. *J Immunol* 1991;146:3419-23.
47. Ohnishi T, Kita H, Weiler D, et al. IL-5 is the predominant eosinophil-active cytokine in the antigen-induced pulmonary late-phase reaction. *Am Rev Respir Dis* 1993;147:901-7.
48. Kita H, Sur S, Hunt LW, et al. Cytokine production at the site of disease in chronic eosinophilic pneumonitis. *Am J Respir Crit Care Med* (in press).
49. Von Wichert P, Joseph K, Muller B, Franck WM. Bronchoalveolar lavage: Question of intraalveolar fluid? *Am Rev Respir Dis* 1993;147:148-52.
50. Dent LA, Strath M, Mellor AL, Sanderson CJ. Eosinophilia in transgenic mice expressing interleukin-5. *J Exp Med* 1990;172:1425-31.
51. Kita H, Horie S, Gleich GJ. Extracellular matrix proteins attenuate activation and degranulation of stimulated eosinophils. *J Immunol* 1996;156:1174-81.