

# Effect of interleukin-5 and granulocyte-macrophage colony stimulating factor on in vitro eosinophil function: Comparison with airway eosinophils

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*Eosinophils are hypothesized to be crucial in the development of allergic airway inflammation; however, the actual mechanisms that determine their inflammatory activity are still largely undefined. To investigate the factors that regulate eosinophil function in allergic airway disease, we have previously used segmental bronchoprovocation with allergen to study ex vivo eosinophil function. To determine whether the functional changes associated with airway eosinophils obtained by bronchoalveolar lavage 48 hours after antigen challenge are caused by exposure to airway-generated cytokines, normodense blood eosinophils were cultured in vitro with recombinant human interleukin-5 (IL-5) or granulocyte-macrophage colony stimulating factor (GM-CSF). The effect of cytokine exposure was then evaluated on selected cell functions. In vitro incubation with these cytokines for 24 hours significantly increased eosinophil membrane expression of CD18 and CD11b compared with culture in medium alone or eosinophils obtained by bronchoalveolar lavage. N-formyl-methionyl-leucyl-phenylalanine-stimulated superoxide anion generation was slightly but significantly enhanced by incubation with IL-5 but not with GM-CSF. In addition, spontaneous adhesion to human umbilical vein endothelial cell monolayers was increased after exposure to both IL-5 and GM-CSF. However, activated adhesion was enhanced only by culture with IL-5 and stimulation with N-formyl-methionyl-leucyl-phenylalanine. The magnitude of functional changes after in vitro preincubation of eosinophils with these cytokines did not achieve levels of superoxide anion and adhesion noted with airway eosinophils obtained after segmental bronchoprovocation with allergen. These observations raise the possibility that the contribution of IL-5 and GM-CSF to phenotypic changes of airway eosinophils is principally to enhance survival and expression of adhesion proteins. These data also suggest that, in addition to the generation of proinflammatory cytokines, other factors contribute to phenotypic changes in eosinophils as they migrate from the blood to the airway. (J ALLERGY CLIN IMMUNOL 1995; 96:375-85.)*

**Key words:** Eosinophil, cytokine, interleukin-5, granulocyte-macrophage colony stimulating factor, allergy, superoxide anion, adhesion, integrins, airway inflammation

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The selective migration of eosinophils to the airways after allergen challenge and their biologic activity suggest that these cells are important effector cells in the allergic response.<sup>1</sup> Eosinophils exert pathologic effects through a variety of cellular functions such as adhesion, chemotaxis, degranulation of preformed products, and de novo generation of lipid mediators and oxygen metabolites.<sup>2-4</sup> Activation of these functions results in many of the histologic and physiologic features of asthma including epithelial damage, mucus production, edema, and bronchospasm.<sup>4-8</sup> To understand the mechanisms associated with allergic inflammation

*Abbreviations used*

|                               |                                                          |
|-------------------------------|----------------------------------------------------------|
| BAL:                          | Bronchoalveolar lavage                                   |
| CB:                           | Cytochalasin B                                           |
| EPO:                          | Eosinophil peroxidase                                    |
| FMLP:                         | <i>N</i> -formyl-methionyl-leucyl-phenylalanine          |
| GM-CSF:                       | Granulocyte-macrophage colony stimulating factor         |
| HBSS:                         | Hanks' balanced salt solution                            |
| HUVEC:                        | Human umbilical vein endothelial cell                    |
| IL-5:                         | Interleukin-5                                            |
| LTC <sub>4</sub> :            | Leukotriene C <sub>4</sub>                               |
| O <sub>2</sub> <sup>-</sup> : | Superoxide anion                                         |
| PAF:                          | Platelet activating factor                               |
| PMA:                          | Phorbol myristate acetate                                |
| PS:                           | Penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml) |
| SBP:                          | Segmental bronchoprovocation                             |
| SOD:                          | Superoxide dismutase                                     |

more fully and to establish the function of airway eosinophils, we have used the technique of segmental bronchoprovocation (SBP) with antigen.<sup>1</sup> With this approach we are able to evaluate an immediate (5-minute) and late (48-hour) response to airway antigen challenge. Moreover, at 48 hours large numbers of bronchoalveolar lavage (BAL) airway eosinophils can be retrieved. When the function of these airway eosinophils was compared with their circulating counterpart, significant increases in surface adhesion markers, adhesion, survival, and generation of superoxide anion (O<sub>2</sub><sup>-</sup>) were observed.<sup>9</sup>

Lavage fluids obtained 48 hours after SBP with antigen contain the eosinophil-reactive cytokines interleukin-5 (IL-5)<sup>1,10</sup> and granulocyte-macrophage colony stimulating factor (GM-CSF).<sup>11</sup> In addition, when tested in vitro, these cytokines have been reported to act as eosinophil functional agonists and/or primers.<sup>12-15</sup> Thus it is possible that the enhanced function of airway eosinophils obtained after SBP with allergen is caused by in vivo exposure to these cytokines and that these cells maintain these functional changes when evaluated ex vivo. To test the hypothesis that in vivo exposure to a specific cytokine contributes to phenotypic and functional changes, blood eosinophils were incubated in vitro with either IL-5 or GM-CSF. After cytokine treatment eosinophils were activated to determine whether this exposure modifies the function of blood cells as with airway eosinophils evaluated ex vivo 48 hours after allergen challenge. Although in vitro incubation with cytokines en-

hanced eosinophil surface markers, it did not functionally upregulate cells to the levels observed with airway eosinophils 48 hours after SBP with allergen.

## METHODS

### Reagents

Phorbol myristate acetate (PMA), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), platelet activating factor (PAF), cytochrome C, cytochalasin B (CB), gelatin, and superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (St. Louis, Mo.). RPMI, fetal calf serum (FCS), penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml) (PS), *o*-phenylenediamine HCl, newborn calf serum, collagen (type IV), and Hanks' balanced salt solution (HBSS) were purchased from Gibco BRL (Grand Island, N.Y.). Recombinant human IL-5 (10<sup>6</sup> to 10<sup>7</sup> U/mg) and GM-CSF (3 × 10<sup>7</sup> to 5 × 10<sup>7</sup> U/mg) were obtained from Genzyme Corp. (Cambridge, Mass.). Calcium ionophore A23187 was purchased from Calbiochem (La Jolla, Calif.) and Percoll from Pharmacia (Piscataway, N.J.). Antibodies for flow cytometry were obtained from Becton Dickinson (San Jose, Calif.) and included fluorescein isothiocyanate-labeled mouse anti-human CD18 (clone L130), mouse anti-human CD11a (G-25.2), and mouse anti-human CD11b (D12).

### Subjects

To facilitate eosinophil recovery and to be consistent with our prior studies of allergic subjects,<sup>1,9</sup> only patients with documented allergic rhinitis were used as blood donors. The subjects were nonsmokers, received no medication, had no history of asthma, and were studied when they had no clinical symptoms of their allergic disease. All subjects demonstrated at least one positive response to skin prick testing (wheal >3 mm after 10 minutes) from a panel of common allergens including ragweed, cat, and house dust mite. Each subject gave written informed consent as prescribed by the University of Wisconsin Human Subjects Committee.

### Eosinophil separation

Eosinophils were isolated from whole blood as previously reported.<sup>16</sup> Briefly, heparinized (10 U/ml) blood was density-separated by 4% dextran and Ficoll-Hypaque gradient sedimentation. The resulting granulocytes were then layered over multiple discontinuous-density Percoll gradients and centrifuged at 700g for 20 minutes at ambient temperature. The cell band at the 1.095 to 1.100 gm/ml interface was collected and yielded highly pure (>90%; contaminating cells were neutrophils) eosinophils for further study. Cell viability was greater than 95% by trypan blue exclusion.

### Eosinophil survival

As previously described,<sup>9</sup> 10<sup>5</sup> eosinophils per well were cultured in RPMI with 1% FCS and 1% PS, with added cytokine, in 96-well tissue culture plates (Corning

Glass Works, Corning, N.Y.). After incubation for 48 hours at 37° C in 5% carbon dioxide, cell viability was determined by trypan blue exclusion on a hemocytometer. Eosinophil survival rate was calculated as follows:

% Survival =

$$\left( \frac{\text{Viable cells per mm}^3 \text{ at 48 hours}}{\text{Viable cells per mm}^3 \text{ at baseline}} \right) \times 100$$

### Eosinophil-cytokine culture

Eosinophils were resuspended in RPMI–10% FCS–1% PS for long-term culture (24 and 48 hours) or HBSS–0.1% gelatin for short-term culture (1 hour). Eosinophils, 10<sup>6</sup> cells/ml, were incubated in 24-well tissue culture plates with medium alone, IL-5, or GM-CSF in a humidified incubator at 37° C, 5% carbon dioxide. Experimental cytokine concentrations were based on initial dose-response studies on in vitro cytokine-promoted eosinophil survival. As in the SBP studies, in which eosinophils were isolated from lavage fluid, the peripheral eosinophils were then washed with HBSS–0.1% gelatin, resuspended in reaction buffer, and assayed for functional activity and receptor expression. An in vitro incubation time of 48 hours or less was based on the time frame of eosinophil exposure to various factors in the airway when retrieved 48 hours after SBP with allergen.<sup>1</sup>

### Characterization of cytokine effects on eosinophils

Eosinophils were assessed before cell culture with or without cytokine to determine baseline function.

**Membrane receptors.** Eosinophil surface membrane integrin molecules (CD18, CD11a, and CD11b) were assessed by indirect receptor-antibody labeling and flow cytometry.<sup>9</sup> After cytokine exposure, washed eosinophils were incubated with an anti-integrin antibody or an equivalent amount of isotype control immunoglobulin (for nonspecific binding) for 30 minutes at 4° C. The cells were then washed three times and mixed with fluorescein isothiocyanate-labeled anti-isotype antibody, again for 30 minutes at 4° C. After a final wash, cells were scanned on a FACScan flow cytometer (Becton Dickinson). Dead cells were gated out with propidium iodide as a marker. The resulting data were converted from a log to a linear scale and isotype controls subtracted; results are expressed as mean relative fluorescence.

**Superoxide anion generation.** Superoxide anion (O<sub>2</sub><sup>•−</sup>) generation was assessed as SOD-inhibitable reduction of cytochrome C by 5 × 10<sup>5</sup> cells in 30 minutes as previously reported.<sup>17</sup> Eosinophils, 10<sup>5</sup> cells/well, were incubated in 96-well plates with 100 μmol/L cytochrome C and activator. The absorbance of the reaction was monitored at 550 nm for 60 minutes (37° C) and reached

a maximum by 30 minutes after exposure to individual activators. SOD was added to identical reaction mixtures and used as a control. Activators assessed included receptor-dependent (FMLP, PAF) and receptor-independent (PMA, A23187) compounds.<sup>9</sup> CB (5 μg/ml) was added to reactions when FMLP, PAF, or A23187 was used as an agonist to enhance O<sub>2</sub><sup>•−</sup> generation. This concentration of CB had no direct effect on the respiratory burst. Not all activators could be assayed in each experiment because of a limited number of eosinophils. Results are expressed in nanomoles O<sub>2</sub><sup>•−</sup> (equivalent to nanomoles cytochrome C reduction) minus spontaneous release and SOD controls.

**Adhesion.** Eosinophil adhesion to collagen or confluent human umbilical vein endothelial cell (HUVEC) (American Type Culture Collection, Rockville, Md.) monolayers was measured as eosinophil peroxidase (EPO) activity of adherent cells.<sup>9</sup> Ninety-six-well tissue culture plates were coated with collagen (50 μg/ml) for 1 hour at 37° C and washed twice with HBSS, and eosinophils (10<sup>4</sup> cells per 0.1 ml) were added. In other studies HUVEC were grown to confluence in collagen-coated tissue culture flasks and passaged into collagen-coated 96-well tissue culture plates with endothelial cell basal medium (EBM; Clonetics, San Diego, Calif.). After 24 hours of incubation at 37° C, the wells were washed twice with warm HBSS and 10<sup>4</sup> eosinophils per well were immediately added for adherence studies. The eosinophils were stimulated with activator at 37° C for 20 minutes. The reaction wells were washed three times with warm HBSS, and EPO substrate consisting of 1 mmol/L hydrogen peroxide, 0.1% Triton X-100 (Sigma) and 1 mmol/L *o*-phenylenediamine HCl in 0.05 mmol/L (hydroxymethyl) aminomethane tris buffer (pH = 8.0) was added. Eosinophils (10<sup>4</sup>) were used as a measure of total EPO activity. After 30 minutes at ambient temperature, 3 mol/L sulfuric acid was added to stop the reaction. Absorbance was measured at 490 nm, and percent adhesion was calculated as percent EPO activity remaining in the adherent eosinophils minus spontaneous adhesion. This assay is EPO specific and is not affected by myeloperoxidase from contaminating neutrophils.<sup>9</sup>

### Statistics

Eosinophil functional assessment was done in duplicate or quadruplicate (adhesion), and the replicates were averaged. Data are expressed as mean values ± SEM and were analyzed by analysis of variance. When analysis of variance indicated significant differences, a Scheffé test was used to compare differences between specific groups. A *p* value less than 0.05 was considered statistically significant.

## RESULTS

### Effect of IL-5 and GM-CSF on in vitro eosinophil survival

Both recombinant human IL-5 and GM-CSF promoted in vitro eosinophil survival in a dose-

**TABLE I.** Effect of IL-5 and GM-CSF on 48-hour eosinophil survival

| Dose (U/ml) | Eosinophil survival (%) |                   |
|-------------|-------------------------|-------------------|
|             | IL-5<br>(n = 4)         | GM-CSF<br>(n = 2) |
| 0           | 24.7 ± 2.8              | 14.3              |
| 0.1         | 19.8 ± 2.0              | 15.7              |
| 1           | 58 ± 7.2                | 40.8              |
| 10          | 72.1 ± 4.6              | 68.2              |
| 100         | 72.5 ± 4.4              | 86.2              |

Values are expressed as means ± SEM.

dependent fashion (Table I). These studies confirmed previous observations,<sup>10, 14, 15</sup> established the bioactivity of each cytokine, and determined relevant concentrations of cytokines for use in cell culture.

#### Effect of cytokines on eosinophil integrin molecules

Eosinophils were incubated with medium only or with cytokine (10 and 100 U/ml) for 24 hours. Baseline (preculture and medium-only cultures) integrin expression was different between the two cytokines because of a separate, unpaired series of experiments performed for each (Fig. 1). Inclusion of IL-5 in the eosinophil culture significantly enhanced expression of the CD18 cell surface marker over preculture and medium-only culture conditions (Fig. 1, A). Interestingly, when eosinophils were cultured in medium only for 24 hours, the level of CD11b expression significantly decreased. Addition of IL-5 to the eosinophil culture prevented this loss of surface marker. Although CD11a expression tended to increase after IL-5 incubation, these changes were not significant ( $p > 0.05$ ). Similarly, long-term (24-hour) incubation of eosinophils with GM-CSF resulted in significantly greater expression of CD18 and CD11b compared with eosinophils cultured with medium only (Fig. 1, B). Changes in CD11b occurred after as little as 1 hour of cytokine culture, whereas changes in CD18 required longer incubation periods (data not shown). Other concentrations of cytokines were not assessed.

#### Effect of in vitro cytokine incubation on $O_2^-$ generation

Isolated blood eosinophils were cultured with IL-5 (Fig. 2) or GM-CSF (Fig. 3) for 1 and 24 hours; after this exposure the cells were washed and their respiratory burst assessed. Cytokine by

itself did not initiate eosinophil  $O_2^-$  generation (data not shown). Furthermore, when compared with control cells (incubated with medium only), neither 10 nor 100 U/ml IL-5 exposure for 1 hour changed eosinophil  $O_2^-$  generation after activation by 0.1  $\mu$ mol/L FMLP (Fig. 2, A). In contrast, enhanced  $O_2^-$  generation stimulated by either 0.1  $\mu$ mol/L FMLP or 0.1  $\mu$ mol/L PAF, both in the presence of 5  $\mu$ g/ml CB, occurred when eosinophils were incubated with a higher dose of IL-5 (100 U/ml).

Eosinophils cultured 24 hours in medium only generated less FMLP-induced  $O_2^-$  when compared with preculture cells and cells cultured in either cytokine (Figs. 2, B, and 3, B). When 24-hour cytokine incubation times were assessed, only FMLP activation (without CB) caused a slight but significant enhancement of  $O_2^-$  generation after incubation with both doses of IL-5 (Fig. 2, B). Interestingly, IL-5 pretreatment for 24 hours tended to decrease  $O_2^-$  generation after PAF activation. Although this was not found to be a significant change, it was consistent and would likely become significant with the addition of more experiments. Longer incubation times (up to 48 hours) did not further promote  $O_2^-$  production (data not shown).

In a second series of experiments, neither short-term (Fig. 3, A) nor long-term (Fig. 3, B) incubation of eosinophils with either 10 or 100 U/ml GM-CSF significantly changed eosinophil  $O_2^-$  production to FMLP. PAF was not assessed in long-term GM-CSF studies.  $O_2^-$  generation stimulated by the more potent receptor-independent activators PMA or A23187 was not affected by either IL-5 or GM-CSF preincubation (data not shown).

#### Effect of in vitro cytokine incubation on eosinophil adhesion

In the following experiments, both spontaneous adhesion and adhesion after cell activation (spontaneous adhesion was subtracted to measure activation only) were evaluated. Activated eosinophil adherence (20 minutes) was similar between the two substrates; consequently, only eosinophil adhesion to HUVEC is shown. Neither IL-5 nor GM-CSF alone activated immediate (20-minute) eosinophil adhesion to HUVEC-coated culture plates (data not shown). However, long-term culture of isolated blood eosinophils with 10 U/ml of IL-5 for either 24 or 48 hours enhanced spontaneous adhesion, with significance achieved after 48 hours of incubation with 10 U/ml IL-5 (Fig. 4).

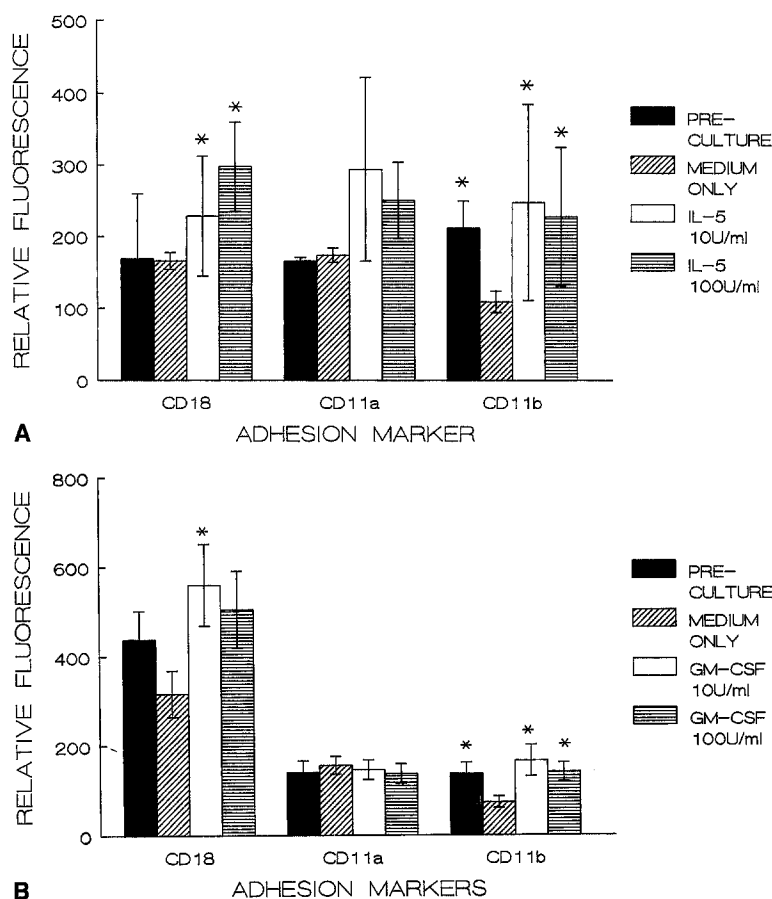


**FIG. 1.** Effect of pretreatment of blood eosinophils with IL-5 (**A**;  $n = 3$ ) or GM-CSF (**B**;  $n = 6$ ) on cell membrane adhesion marker expression. Eosinophils,  $10^6$  cells/ml, were assessed before culture, or after 24 hours of culture with medium only or with cytokine, by monoclonal antibodies to specific adhesion markers. Expression of membrane receptors was measured by flow cytometry and is expressed as relative mean fluorescence minus values for isotype controls. Asterisk,  $p < 0.05$  versus medium only. Values for  $p$  are determined by Scheffé constants when analysis of variance was significant for experimental groups.

However, when spontaneous adhesion was subtracted, a slight but significant ( $p = 0.022$ ) enhancement of adhesion was noted when eosinophils were activated by  $0.1 \mu\text{mol/L}$  FMLP, but only after 24 hours of exposure to IL-5. Interestingly, a 48-hour incubation of eosinophils with  $10 \text{ U/ml}$  IL-5 resulted in decreased PAF ( $1 \mu\text{mol/L}$ )-activated adhesion, a trend also observed with 24-hour IL-5 pretreatment and  $\text{O}_2^-$  generation (Fig. 2, B).

A similar study with GM-CSF also resulted in enhanced spontaneous adhesion after cytokine culture but no change in stimulated adhesion (data not shown). These results are based on three experiments, and additional studies may result in significance in certain conditions such as spontaneous adhesion after GM-CSF pretreatment.

### Comparison with airway eosinophils after SBP with allergen

To put our results into perspective, we compared the function of cytokine-treated eosinophils with responses of airway eosinophils obtained 48 hours after SBP with antigen. To estimate the relative enhancement in eosinophil function seen with either cytokine treatment or retrieval from the airway, the following comparative formula was used (Fig. 5):

$$\begin{aligned} & \% \text{ Change in eosinophil function} \\ &= \frac{\text{Mean function of airway eosinophils or} \\ & \quad \text{cytokine-incubated eosinophils}}{\text{Mean function of control blood eosinophils}} \\ & \quad \times 100 \end{aligned}$$

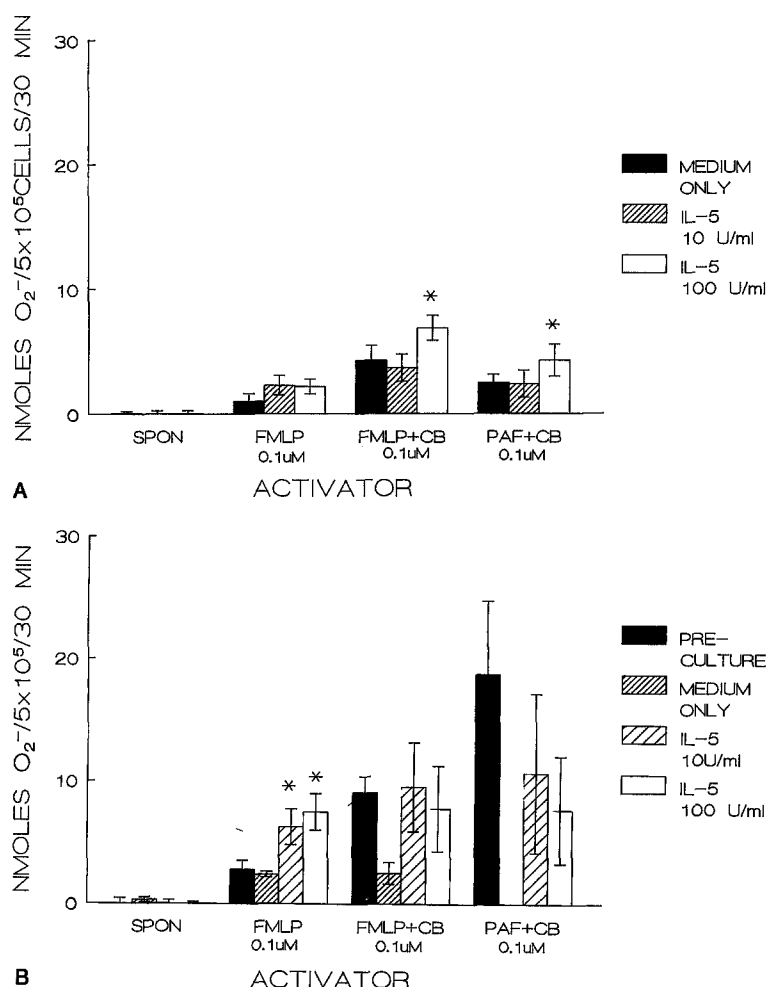


**FIG. 2.** Effect of blood eosinophil culture with IL-5 on subsequent  $O_2^-$  generation. Eosinophils,  $10^6$  cells/ml, were cultured for 1 hour (**A**;  $n = 6$ ) or 24 hours (**B**;  $n = 3$ ) with 10 or 100 U/ml IL-5 and then activated. Asterisk,  $p < 0.05$  versus medium only.

No significant functional differences were observed between control peripheral eosinophils used in this study and blood eosinophils collected 48 hours after SBP with allergen from our previous study.<sup>9</sup>

## DISCUSSION

We have found that in vitro exposure of blood eosinophils to recombinant IL-5 or GM-CSF had variable effects on the function of these cells. Of the functions evaluated, cytokine exposure primarily affected expression of integrins CD11b and CD18, and generation of  $O_2^-$  by FMLP. However, these two cytokines had a limited capacity to upregulate the activity of blood eosinophils when compared with similar assessment of airway eosinophils (Fig. 5).

Walker et al.<sup>18</sup> demonstrated increased eosinophil CD11b/CD18 expression after a 3-hour incu-

bation with either IL-5 or GM-CSF. Although the enhanced expression of CD11b/CD18 on eosinophils observed by Walker et al. was less than ours (<40% vs >90%), such differences may be due to the duration of cytokine exposure (3 vs 24 hours). Although we observed cytokine-dependent increased CD11b by 1 hour of incubation, CD18 was not yet affected. In a murine model Coëffier et al.<sup>19</sup> found that IL-5 but not GM-CSF primes eosinophil generation of  $O_2^-$  and intracellular calcium movement. We have also observed that GM-CSF primes neutrophil but not eosinophil  $O_2^-$  through the enhanced generation of leukotriene  $B_4$ .<sup>20</sup> Furthermore, long-term (3 to 7 days) culture of eosinophils with interleukin-3, IL-5, or GM-CSF enhances cell survival and leukotriene  $C_4$  ( $LTC_4$ ) generation and cytotoxicity after activation<sup>13, 21, 22</sup>; however, coculture with 3T3 fibroblasts was neces-

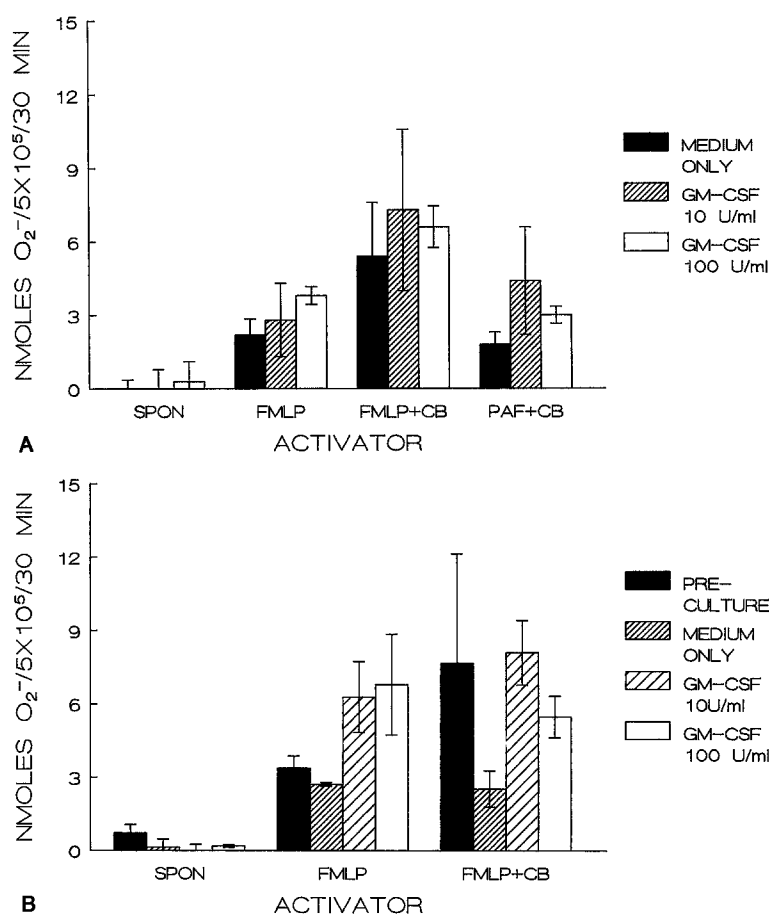


**FIG. 3.** Effect of blood eosinophil culture with GM-CSF on subsequent  $O_2^-$  generation. Eosinophils,  $10^6$  cells/ml, were cultured for 1 hour (**A**;  $n = 5$ ) or 24 hours (**B**;  $n = 3$ ) with 10 or 100 U/ml GM-CSF and then activated. Analysis of variance was not significant ( $p > 0.05$ ) for any conditions.

sary to achieve these changes. Others have reported that short-term (<2 hours) IL-5 exposure enhanced eosinophil degranulation,<sup>12,23</sup> oxygen metabolism production,<sup>24</sup> and LTC<sub>4</sub> generation.<sup>21,22</sup> These diverse observations suggest that the effects of these cytokines are regulated by many in vitro and in vivo factors.

In vitro incubation of eosinophils with cytokines increased the expression of integrin adhesion markers (Fig. 5, *A*); interestingly, the resulting enhancement to in vitro cytokine exposure was more pronounced than in vivo changes found with airway eosinophils obtained 48 hours after SBP. Both CD11b and CD18 expression were enhanced by long-term IL-5 and GM-CSF preincubation. In comparison, CD11b expression was elevated only slightly in the BAL eosinophils, and no significant change was observed for CD18.<sup>9</sup> These results are consistent with observations by Kroegel et al.<sup>25</sup> They reported that after SBP with antigen, BAL

eosinophils had elevated expression of CD11b, which is similar to the response of blood eosinophils after incubation with the agonists PAF or FMLP. They concluded that blood eosinophils become activated during their migration to the airways. Tomioka et al.<sup>26</sup> reported similar in vitro studies in which GM-CSF enhances peripheral eosinophil CD11b expression. They reported, and we observed, that CD11b expression decreases during long-term culture (days) and that this decrease is inhibited by the presence of GM-CSF in the culture medium. Both studies demonstrated that cytokine culture preserved CD11b expression over time.

We decided to focus our studies on FMLP activation because only FMLP demonstrated increased effects on the stimulation of the BAL eosinophil respiratory burst and adhesion.<sup>9</sup> In vitro cytokine culture also appears to enhance specifically FMLP-induced functions. Although in vitro

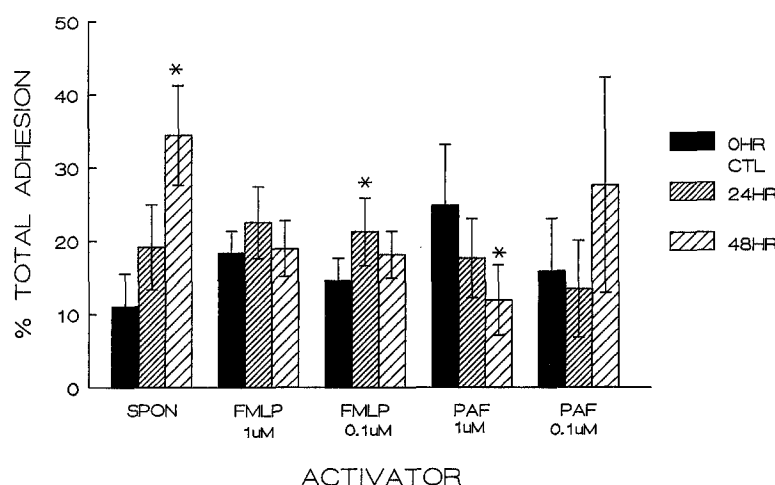


**FIG. 4.** Effect of cytokine culture of blood eosinophils on subsequent adhesion to HUVEC. Eosinophils,  $10^6$  cells/ml, were cultured with 10 U/ml of IL-5 ( $n = 5$ ) for 0 (preculture adhesion; 0HR CTL), 24, or 48 hours and then activated for 20 minutes. Adhesion was measured as percent EPO activity remaining in well after vigorous washing. Asterisk,  $p < 0.05$  versus preculture adhesions.

exposure to cytokines was found to enhance the eosinophil's inflammatory activity, the degree to which this change occurred was variable, and when compared with airway cells, which may have been exposed to cytokines and other factors *in vivo*, was often less. When the function of BAL eosinophils is compared further with *in vitro* cultured blood eosinophils, cytokine exposure alone does not appear to be sufficient for functional upregulation. FMLP activation of IL-5-cultured (but not GM-CSF-cultured) eosinophils resulted in an enhancement of  $O_2^-$  production but to a lesser degree than that noted in airway cells, and only when CB is absent from the reaction (Fig. 5, B). As observed for adhesion markers, 24-hour cytokine pretreatment appears to preserve rather than enhance  $O_2^-$  generation (Figs. 2, B, and 3, B). Eosinophil adhesion was not changed by either IL-5 or GM-CSF *in vitro* treatment (Fig. 5, C) and was much lower than airway eosinophils stimulated with FMLP.

Our studies to assign a role for these cytokines in determining eosinophil function have limitations. First, only selected periods of *in vitro* culture were assessed. However, we did incubate cells and cytokines up to 48 hours in an attempt to simulate conditions from the SBP protocol; in the SBP protocol eosinophils were retrieved 48 hours after antigen challenge. Second, only two concentrations of cytokines were studied because of a limited availability of blood eosinophils. Nonetheless, the tested concentrations of both IL-5 and GM-CSF prolonged eosinophil *in vitro* survival and in this regard were similar to that

achieved with BAL fluid obtained 48 hours after SBP with allergen.<sup>9, 10</sup>

Despite these constraints an *in vitro* exposure of eosinophils to a single cytokine did not reproduce the functional upregulation observed in eosinophils obtained from BAL fluid after SBP with allergen. Therefore it is very likely that a combination of soluble mediators (e.g., PAF,  $LTC_4$ , and other cytokines) generated by endothelial cells, epithelial cells, mast cells, fibroblasts, or infiltrating leukocytes are essential to achieve function similar to that of airway eosinophils. The complexity of such multiple-factor *in vitro* experiments is likely relevant to the *in vivo* situation but is a major hurdle for studies with isolated cells. However, when we incubated peripheral eosinophils in BAL fluid obtained 48 hours after antigen challenge, functional changes other than prolonged *in vitro* survival were not observed (data not shown). This suggests that changes in eosinophil phenotype occur before arrival in the airway or are not caused solely by soluble factors (i.e., cytokines). Alternatively, it is possible that relevant factors are transient and no longer present in the BAL fluid at 48 hours.

Eosinophils were cultured with IL-5 or GM-CSF in uncoated tissue culture flasks. It is possible that the surface on which the cells are cultured is crucial for resulting functional activity. *In vivo*, eosinophils are exposed to and adhere to endothelium, interstitial matrix, and epithelium during their migration to the airways. These substrates, in conjunction with soluble cytokines, may be the factors that prime eosinophil function and alter

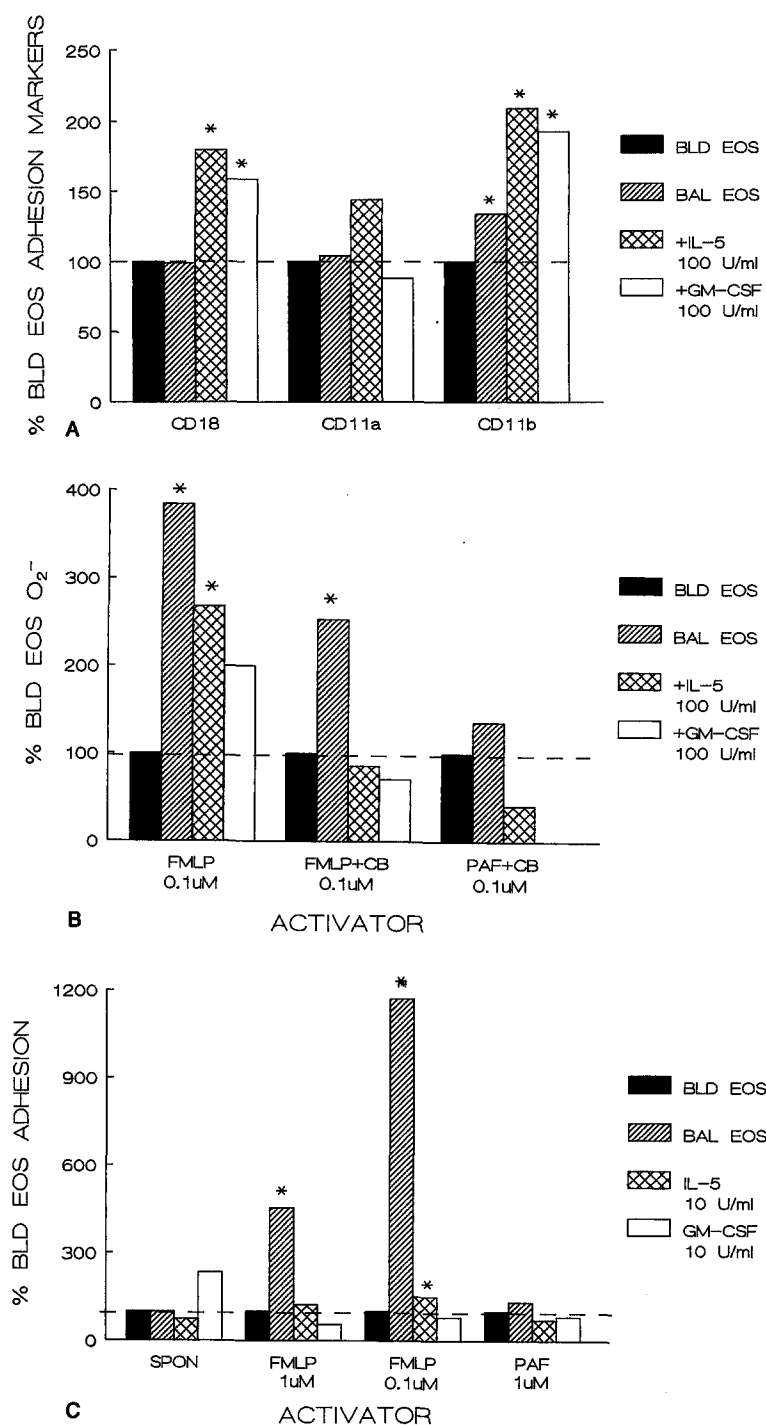


**FIG. 5.** Comparison of mean values of 24-hour in vitro-primed blood eosinophil (BLD EOS) ( $n = 2$  to  $6$ ) and in vivo-primed BAL eosinophil (BAL EOS) ( $n = 6$ ) function 48 hours after SBP with antigen for adhesion membrane markers, (A),  $O_2^-$  generation (B), and adhesion (C). Comparison is expressed as percent blood eosinophil baseline function: for BAL eosinophils control is function of corresponding blood cells, and for cytokine cultures it is function of blood eosinophils cultured in medium alone. Asterisk,  $p < 0.05$  versus blood eosinophils.

cell phenotype. Such surfaces have been observed to enhance eosinophil function in vitro.<sup>27-29</sup> Therefore a combination of cell adhesion and cytokine exposure may create an airway eosinophil.

The involvement of eosinophils in allergic inflammation and asthma is a complex process, and cytokine participation is likely a critical element. We believe that our observations add

insight as to the specific role for IL-5 and GM-CSF in eosinophil-associated allergic inflammation. Our data suggest that a principal activity of IL-5 and GM-CSF may be limited to the promotion of cell survival and expression of some adhesion proteins, with only modest effect on other cell functions. Although in vitro culture of eosinophils with these cytokines affected selected cell functions, the resulting changes did not always achieve features observed with airway eosinophils. Therefore determinants of the airway eosinophil phenotype likely consist of a complex interplay of numerous inflammatory promoting factors, including cytokines, singly or in combination, adhesion proteins, and other lung-derived factors. Defining more specifically how each of these elements acts on the eosinophil is essential to assessing this cell's participation in the pathogenesis of asthma.

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