Interleukin-5 Induces CD34⁺ Eosinophil Progenitor Mobilization and Eosinophil CCR3 Expression in Asthma

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Asthma is characterized by the accumulation of activated T cells and eosinophils within the airway. Eosinophils derive from CD34⁺ bone marrow progenitor cells under the influence of hematopoietic growth factors, subsequently migrating to the airways under the cooperative influence of interleukin (IL)-5 and chemokines, including eotaxin. We compared the relative effects of systemic versus local IL-5 on progenitor-cell mobilization and mature eosinophil phenotype by using flow cytometry, following the administration of intravenous (2 μg) or inhaled (15 μg) IL-5 to nine patients with mild asthma. Intravenous IL-5 induced a rapid reduction in circulating eosinophil counts followed by prolonged blood eosinophilia. Both intravenous (p < 0.002) and inhaled (p < 0.05) IL-5 significantly increased CD34⁺/CD45⁺ lymphoblastoid eosinophil progenitors. Intravenous IL-5 increased mature eosinophil CCR3 expression from a baseline mean fluorescence intensity (MFI) of 658 ± 51.7 to 995 ± 93.2 at 24 h (p < 0.05), but had no effect on interleukin-5 receptor subunit α or CD11b expression. Lymphocyte CCR3 MFI was increased by intravenous IL-5 from 38.5 ± 13.6 at baseline to 73.6 ± 14.3 at 24 h (p < 0.05). Systemic IL-5 increased circulating eosinophil progenitors, suggesting a key role for systemic IL-5 in eosinophil mobilization. Further, IL-5 causes terminal maturation of the eosinophil by increasing CCR3 expression, potentially affecting CCR3-dependent chemotaxis by eosinophils and lymphocytes.

Keywords: interleukin-5; eosinophil; eosinophil progenitors; chemokine receptors; asthma

The inflammation of chronic asthma is associated with activation of peripheral blood T lymphocytes and eosinophils and subsequent infiltration of these cells into the airway (1). T-helper type 2 (Th2) cells, the predominant source of the cytokine interleukin (IL)-5, are also increased in the asthmatic airway (2), and this cytokine plays an important role in the mobilization, terminal differentiation, and maintenance of mature eosinophils (3).

IL-5 levels have been measured in the circulation of asthmatic individuals (4) and rise during asthma exacerbations (5). Additionally, IL-5 messenger RNA (mRNA) expression is increased in bronchial mucosa and in bronchoalveolar lavage CD4⁺ T cells in asthma (2, 6), and IL-5 mRNA levels in bronchiolar biopsy specimens have been positively correlated with asthma severity (7). IL-5 protein levels are increased in bronchiolar biopsy specimens (8) and sputum (9) during exacerbations of asthma, and are further increased after allergen challenge (10, 11).

Mature eosinophils develop from pluripotent hematopoietic progenitor cells that bear the cell-surface glycoprotein CD34⁺ (12). Increased numbers of CD34⁺ cells have been demonstrated in both blood and bone marrow from atopic individuals as compared with normal subjects (13) and in the bronchial mucosa of atopic asthmatic subjects (14). The number of CD34⁺ cells coexpressing the IL-5 receptor α-subunit (IL-5Rα) is increased after allergen challenge of these subjects (15). Eosinophils mature from bone marrow–derived CD34⁺ precursors under the early influence of IL-3 and granulocyte–macrophage colony-stimulating factor (GM-CSF) (16), with IL-5 inducing a late specific expansion of eosinophil lines (17). The subsequent mobilization and activation of circulating eosinophils after stimulation by IL-5 appears to be a crucial precondition to the accumulation of activated eosinophils in the allergic airway.

We hypothesized that IL-5 has a central role in the mobilization of eosinophil precursors (CD34⁺/CD45⁺ progenitors) into the peripheral blood, and that systemic rather than airway IL-5 activity may be more potent in this role. In addition, as a feature of the terminal differentiation of mature eosinophils, systemic IL-5 may increase the expression of the chemokine receptor CCR3 and influence the IL-5Rα subunit and the integrin CD11b, thereby facilitating the trafficking of mature eosinophils from the peripheral circulation to the airway. We therefore compared the effect of intravenous versus inhaled IL-5 administration in patients with mild asthma.

METHODS

Study Design

In a double-blind and placebo-controlled investigation, we studied nine subjects during three separate periods at intervals of 2 wk. The subjects received intravenous IL-5 and nebulized placebo, intravenous placebo and nebulized IL-5, or double placebo, in randomized order. IL-5 (and placebo) administration cycles were separated by at least 2 wk. Whole blood was collected for full blood count, differential leukocyte count, and IL-5 assay at baseline and at 0.5, 1, 2, 3, 4, 5, 24, and 72 h after IL-5 or placebo administration. The study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospital Trust.

Subjects

Nine volunteers with mild atopic asthma were recruited for the study (Table 1). Inclusion criteria were a diagnosis of asthma as previously defined (18), with intermittent asthma symptoms, FEV₁ > 70% predicted, and bronchial hyperreactivity (a provocative concentration of methacholine that decreased FEV₁ by 20% [PC₂₀] < 8 mg/ml), and positive skin prick reactivity to common aeroallergens. Subjects were stable for 4 wk before entering the study, with no other respiratory disease, no recent history of respiratory tract infection (6 wk), and a need only for inhaled short-acting β₂-agonists (<1,000 μg albuterol/wk) for symptom control.

Materials

Recombinant human IL-5 was purchased from Genzyme (Cambridge, MA). Unconjugated rat anti-human CCR3 (clone 61545) antibody was purchased from R&D Systems (Abingdon, Oxon, UK). Fluorescein isothiocyanate (FITC)-conjugated anti-CD9 (clone MM2/57), RPE-conjugated anti-CD9 (clone MM2/57), RPE–Cy5-conjugated...
anti-CD16 (clone 3G8), FITC-conjugated rabbit antirat IgG (Fab2), FITC-conjugated anti-CD11b (clone ICRF44), and FITC-conjugated anti-CD34 Class III (clone 581) antibodies were purchased from Sero-tec (Kidlington, Oxon, UK). RPE-conjugated mouse antihuman IL-5Ra (CD125, clone A14) antibody was purchased from Pharmingen (San Diego, CA). RPE IgG1 isotype control was purchased from Becton Dickenson (St. Louis, MO). FITC-conjugated IgG and IgG2a (clone UPC-10) isotype controls were purchased from Sigma (Poole, Dorset, UK). An IL-5 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Pharmingen (Cambridge, UK). Red cell lysing buffer (Erythrolyse) was purchased from Serotec. Fluorescence events were recorded with a FACS Scan (Becton Dickenson, St Louis, MO) flow cytometer, and fluorescence activated cell sorting (FACS) data were analyzed with Cell Quest software (Becton Dickenson).

IL-5 Administration

IL-5 doses were chosen after a preliminary dose escalation study in which 0.1, 0.5, 1, or 2 μg IL-5 were given intravenously and 5, 10, or 15 μg were given by nebulization to seven separate, nonasthmatic normal individuals. The highest doses were chosen as those having the greatest effect on peripheral blood or induced sputum eosinophil levels. Intravenous IL-5 (15 μg) for inhalation was reconstituted in 2 ml of 0.9% sodium chloride. IL-5 or placebo (2 ml of 0.9% sodium chloride) was then nebulized via a nebulizer (MEDIC-AID, Pagham, Sussex, UK) from a mouthpiece, using a one-way exhaust valve (LC for a further 30 min. To avoid cross-reactivity between rat and mouse antibodies, we performed incubations with anti-CCR3 and FITC-conjugated rabbit antirat antibodies before incubations with mouse antihuman CD9 and CD16 antibodies. Cells were washed after secondary incubations in PBS staining buffer, and were centrifuged for 5 min at 400 × g. The cell pellet was resuspended in 2 ml of 1× erythrocyte lysis buffer (Erythrolyse) and incubated in the dark at RT for 10 min. Tubes were centrifuged for 10 min at 400 × g and the pellet was resuspended in PBS staining buffer, washed once in PBS staining buffer, and centrifuged. The cell pellet was finally resuspended in 0.5 ml of FACScan (Becton Dickenson) containing 1% paraformaldehyde and was stored at 4°C in the dark until analysis. All samples for each individual and for each treatment were then analyzed together, within 26 h of sample collection. Isotype-matched control samples with FITC-conjugated primary antibodies were included for each analysis.

Flow Cytometry and Gating Strategy

We acquired 50,000 events for CD34+ cell measurements and 10,000 events for eosinophil measurements. CD34+ cells were gated according to a previously described protocol (13, 15) entailing logical sequential gating (19). Gating was based on CD34 and CD45 positivity combined with forward scatter (FSc) and SSC characteristics of CD34+ progenitor cells. Briefly, CD45+ cells (total leukocytes) were collected in a rectangular gate (R1) and projected into a second dot-plot of CD45+ positivity versus SSC (Figure 1). CD45-bright cells were then gated (R2) and projected into a third dot-plot of CD45+ positivity versus SSC. CD45+ cells with mononuclear morphology were then gated (R3) and projected into a fourth plot of FSC versus SSC. Cells with mononuclear cell morphology were then gated (R4), and the gate statistics for R1 to R4 were determined. A negative control was created by using the same gating strategy backgated, in which the IgG2a isotype control was substituted for anti-CD34 antibody. CD34+ events were then calculated as the means of duplicate-test CD34+ /CD45+ events minus events from isotype control tubes.

Eosinophils were identified by granulocyte morphologic characteristics, CD16-negativity (20) and CD9-positivity (21), and the respective fluorescence intensities for CCR3, IL-5Ra, and CD11b were obtained.

**TABLE 1. SUBJECT CHARACTERISTICS**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>FEV1 ( % pred)</th>
<th>PC20 (mg/ml)</th>
<th>Serum IL-5 (pg/ml)</th>
<th>Blood Eosinophils (&lt;10^6 cells/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>26</td>
<td>94</td>
<td>0.37</td>
<td>0.0</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>25</td>
<td>85.6</td>
<td>2.79</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>36</td>
<td>79</td>
<td>1.44</td>
<td>90.9</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>29</td>
<td>95</td>
<td>3.73</td>
<td>7.4</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>26</td>
<td>97</td>
<td>0.19</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>41</td>
<td>117</td>
<td>8.0</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>35</td>
<td>106</td>
<td>0.15</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>28</td>
<td>97</td>
<td>6.68</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>27</td>
<td>85</td>
<td>0.42</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>SF-4M</td>
<td>30.3 (5.6)</td>
<td>95.1 (3.8)</td>
<td>1.15* (2.21)</td>
<td>0.0* (0-0.9)</td>
<td>0.18 (0.03)</td>
</tr>
</tbody>
</table>

*Median (Range).

Definition of abbreviations: IL-5 = interleukin-5; PC20 = provocative concentration causing a 20% decrease in FEV1.
Peripheral Blood Eosinophils

Circulating peripheral blood eosinophils showed a biphasic change after intravenous administration of IL-5 (Figure 2). In our subjects with mild asthma, there was a significant reduction in eosinophil numbers at 0.5 h as compared with baseline numbers (−72.2 ± 12.4% [mean ± SEM], p < 0.01), which was not observed in subjects given placebo (2.7 ± 6.5%) or inhaled IL-5 (−1.8 ± 8.0%). Eosinophils were increased significantly at 3 h after IL-5 administration and remained significantly elevated 72 h afterwards (+42.6 ± 15.5%; p < 0.05). Subjects given placebo and inhaled IL-5 showed no early or late change (−6.4% ± 12.8% and (−3.7% ± 3.7%, respectively) in peripheral blood eosinophil numbers.

Serum IL-5 Levels following IL-5 Administration

Serum IL-5 was detectable in only one subject with mild asthma at baseline, but rose in the group, peaking 0.5 h after intravenous administration of IL-5 (median: 266 pg/ml; range: 0 to 1,306 pg/ml; p = 0.002; Figure 2). Serum IL-5 levels were not significantly increased after inhaled IL-5 or placebo.

CD34+/CD45+ Progenitor Cells

At baseline, there were 3,405 ± 1,431 CD34+ events per million CD45+ leukocytes detected in whole blood in our asthmatic subjects, as compared with undetectable levels of CD34+ events at baseline in nonasthmatic subjects. This compares with previous findings of 1,438 ± 347/10⁶ nonadherent mononuclear cells isolated from the peripheral blood of atopic subjects and 236 ± 77/10⁶ of such cells isolated from nonatopic volunteers (13). Twenty-four hours after intravenous administration of IL-5, CD34+ events increased by fivefold, from 3,405 ± 1,431 to 12,470 ± 6,918 (p < 0.002; Figure 3). Inhaled IL-5 doubled baseline CD34+ events at 24 h (from 3,826 ± 1,224 to 5,989 ± 966, respectively; p < 0.05), whereas placebo had no effect on eosinophil progenitor numbers.
Expression of CCR3, IL-5Rα, and CD11b in Peripheral Blood

CCR3 was expressed on 98 to 100% of CD9
/CD16
 eosinophils from our atopic asthmatic subjects. One hour after intravenous administration of IL-5, CCR3 expression fell from a baseline mean fluorescence intensity (MFI) of 658 ± 51.7 to 444 ± 76.4, and then rose significantly, peaking 24 h after IL-5 administration at 995 ± 93.2 pg/ml (p < 0.05) (Figure 4). The MFI for CCR3 was increased after intravenous IL-5 as compared with that for the placebo and inhaled IL-5 groups at 24 h but did not change significantly at any time points after inhaled IL-5 or placebo administration. The MFI for eosinophil CCR3 at 24 h after intravenous IL-5 was weakly but not significantly correlated with peak serum IL-5 levels (r = 0.53, p > 0.05).

The IL-5Rα subunit was variably expressed on 19.5 to 90.7% of peripheral blood eosinophils. Intravenous IL-5 caused a small but insignificant increase in the MFI for IL-5Rα expression, from 58.3 ± 19.9 at baseline to 106.6 ± 49.5 at 4 h and to 97.0 ± 30.7 at 24 h (Figure 4). The MFI for IL-5Rα was not changed by inhaled IL-5 or by placebo.

The activation marker CD11b was expressed on 8.5 to 50.1% of peripheral blood eosinophils at baseline. Placebo and inhaled IL-5 caused no change in the MFI for CD11b. Following intravenous administration of IL-5, the MFI for CD11b rose from its baseline value (67.3 ± 13.7) to 89.7 ± 18.6, 87.5 ± 11.6, and 101.2 ± 16.8 at 1, 4, and 24 h, respectively, but this was not significant (Figure 4).

IL-5Rα was expressed at negligible levels on unseparated lymphocytes (MFI = 12.6 ± 3.2, MFI for isotype control = 6.8). CCR3 was expressed on 19.2 to 47.6% of lymphocytes. The MFI for CCR3 was increased from 38.5 ± 13.6 at baseline to 73.6 ± 14.3 at 24 h after intravenous administration of IL-5, but was not changed from baseline after inhaled IL-5 or placebo (Figure 5). The MFI for lymphocyte CCR3 at 24 h after intravenous administration of IL-5 was significantly correlated with peak serum IL-5 levels at 0.5 h after IL-5 administration (r = 0.80, p < 0.05).

DISCUSSION

In this first comparative investigation of the effects of both inhaled and intravenous IL-5 in patients with mild asthma, we observed that intravenous and, to a lesser extent, inhaled IL-5 caused significant and profound increases in the number of circulating CD34
 cells with lymphoblastoid morphology. This is the first indication that progenitor cells with the potential for eosinophilic differentiation are mobilized from the bone marrow by IL-5 in humans. A similar expansion of circulating bone marrow progenitor cells has previously been observed after allergen challenge of asthmatic individuals, with increases in the ratio of CD34
 cells expressing IL-5Rα seen at 24 h after challenge (15).

We also observed a profound early decrease in mature peripheral blood eosinophils after intravenous but not inhaled IL-5, followed by a prolonged (72 h) peripheral eosinophilia. These observations differ from those made previously in animal models, of no early decrease in eosinophil numbers and of a peripheral eosinophilia of relatively short duration (< 2 h) (23). The early decrease that we observed in mature eosinophils may result from a transient clearance of eosinophils from the circulation by margination, with possible trafficking of eosinophils to the lung. This may occur through the action of endogenous chemokines such as eotaxin mediated by the CCR3 receptor, given our finding that eosinophil CCR3
 expression was significantly reduced at 1 h after intravenous IL-5 administration. Indeed, the density of CCR3 expression on circulating eosinophils was reduced after intravenous IL-5, coincident with the reduction in circulating eosinophils. Although this could be explained by a reduction in surface expression of CCR3, a more likely explanation is the clearance from the circulation, under the influence of endogenous chemokines, of eosinophils having a higher level of CCR3 expression.
IL-5 also induced late phenotypic changes in eosinophils, consistent with the terminal differentiation of the maturing eosinophil. We observed increased CCR3 expression at 24 h, with nonsignificant increases in the expression of IL-5Rα and CD11b. By increasing the expression of CCR3, IL-5 may prime circulating eosinophils for tissue migration by increasing the potential for chemotaxis under the influence of endogenous chemoattractants such as eotaxin. This concept is supported by a study in which local instillation of IL-5 into the lungs of asthmatic individuals induced airway mucosal eosinophilia (24).

The observation of CD34+ progenitor mobilization after inhaled and systemic IL-5 implies a role in this process for both lung-derived and systemically derived IL-5. In a prior study, peripheral eosinophilia was observed after inhalation of IL-5 (25). In our study, systemic IL-5 had a greater effect on CD34+ cell mobilization than did inhaled IL-5, suggesting a potent role for systemic or bone marrow–derived IL-5. Significantly, IL-5 production by human bone marrow microvascular endothelial cells has been demonstrated in vitro (26), and by bone marrow Th2 cells after allergen challenge of sensitized mice in vivo (27).

We presume that CD34+ progenitor cells may either mature into eosinophils in the circulation upon exposure to IL-5, or alternatively may accumulate at inflammatory sites, such as in the airways (14, 17). Notably, CD34 is a functional ligand for L-selectin, and may thereby actively contribute, as an adhesion molecule, to such leukocyte accumulation. Marked attenuation of airway eosinophilia has been observed in CD34-deficient mice undergoing airway allergen challenge, despite normal hematopoiesis and hematopoietic progenitor-cell recovery (28), suggesting a functional role for CD34 in tissue eosinophil accumulation in vivo.

Our observations accord with the suggestion that a sequential action of IL-5 and eotaxin is required for the accumulation of eosinophils at inflammatory sites. In this schema, an early increase in circulating IL-5 induces peripheral eosinophilia (29), while eotaxin operates primarily at local tissue sites to induce their chemoattractive effect (23). Eotaxin induces tissue eosinophilia when given to mice that overexpress IL-5, but not to wild-type mice (30). A dependence on the CCR3 receptor is also demonstrated by studies showing inhibition by CCR3-specific antibodies of chemotaxis and calcium flux responses to eotaxin, regulated on activation, normal T cell expressed and secreted (RANTES), macrophage chemotactic protein (MCP)-2, MCP-3, and MCP-4 (31). Previous in vitro studies have shown induction of CCR3 expression on developing eosinophils after stimulation with IL-5, with a consequent increase in binding of eotaxin to eosinophils (32). These findings provide further support for a role of IL-5 in priming CCR3-mediated eosinophil chemotaxis and activation.

Interestingly, IL-5 increased the MFI of CCR3 on lymphocytes. CCR3 has previously been identified on the Th2 cell surface, and these cells migrate under the influence of CCR3-dependent chemokines (33, 34). CCR3 expression on these lymphocytes may therefore contribute to the tissue-selective migration of T cells. However, although IL-5 receptor expression has been documented on B cells (35, 36), it has not to our knowledge been reported on T cells. In preliminary experiments, we observed IL-5Rα expression on 3.4% of CD4+ lymphocytes, and in view of this low level of expression of IL-5Rα, we cannot exclude the possibility that induction of CCR3 expression may occur by a mechanism independent of IL-5Rα.

We found that IL-5 had a lesser effect on blood eosinophil counts in nonatopic normal volunteers than in patients with mild asthma (−24 versus −72% at 0.5 h), suggesting that the systemic effects of IL-5 may depend on the presence of primed eosinophil precursors or of mature eosinophils. Indeed, higher numbers of bone marrow cells expressing CD34+ have been reported in patients with atopice asthma, with these CD34+ precursor cells demonstrating an increased ability to prolifer-
ate into eosinophil colonies \textit{in vitro} (13, 37, 38). In addition, a positive correlation between serum IL-5 and peripheral blood CD34$^+$ cells has been noted in normal and asthmatic subjects (38), and a greater proportion of these CD34$^+$ precursor cells have been reported to express IL-5R$\alpha$ in asthmatic subjects than in normal subjects (39).

We observed no significant changes in IL-5R$\alpha$ expression on circulating eosinophils after IL-5 administration. The expression of receptors for hematopoietic growth factors such as GM-CSF, IL-3, and IL-5 on progenitor cells evolves with their maturation. Culture of bone marrow–derived CD34$^+$ cells with hematopoietic growth factors (IL-3 and granulocyte colony-stimulating factor) induces the acquisition of responsiveness to IL-5 and loss of CD34$^+$ immunoreactivity (40). In the case of IL-3 and GM-CSF, very low levels of expression of their respective receptors has been demonstrated on the most immature CD34$^+$ progenitor cells, but this increases as these progenitor cells develop into committed precursors (41). However, a negative feedback effect of GM-CSF, IL-3, and IL-5 on IL-5R$\alpha$ mRNA expression, together with an inhibition of IL-5 binding, has been reported in \textit{in vitro} studies of human eosinophils (42).

In summary, we have shown that systemically administered IL-5 increases the number of CD34$^+$ progenitor cells and mature eosinophils expressing CCR3 in asthmatic subjects. This shows that IL-5 reaching the systemic circulation has an important role in eosinophil progenitor mobilization and terminal differentiation and in the priming of mature eosinophils for chemokine-induced trafficking and activation.

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