Hematopoietic effects of recombinant human interleukin-3 and interleukin-6

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Density and CD11b expression of eosinophils from ovarian cancer patients receiving recombinant human interleukin-3 after chemotherapy


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Submitted
Abstract

Several studies have shown that interleukin-3 (IL-3) enhances eosinophil activity in vitro. The in vivo effects of IL-3 on circulating human eosinophils, however, may be modulated by the presence of other cell-types and cytokines. Activation of eosinophils during recombinant human IL-3 (rhIL-3) administration is likely to occur since allergy-like side effects have been reported in previous studies in men. In the present study, the in vivo effects of rhIL-3 administration were evaluated with regard to density and CD11b expression of eosinophils and neutrophils. Patients (n=14) with newly diagnosed ovarian cancer received cyclophosphamide/carboplatin based chemotherapy (day 1, 6 cycles) followed by subcutaneous rhIL-3 (5 μg/kg/day, days 3-12) or no rhIL-3. In the first cycle, blood samples were collected before chemotherapy (day 1), after chemotherapy but before rhIL-3/control administration (day 3), and on day 8. Activation of eosinophils and neutrophil was determined by the number of hypodense cells and CD11b expression in whole blood samples. Priming was measured by CD11b expression induced in vitro after incubation with N-formyl-methionyl-leucyl-phenylalanine (fMLP) or platelet-activating factor (PAF). No allergy-like side effects were observed in cycle 1. RhIL-3 treated patients showed a significant increase in eosinophil count (day 8), concurrent with an increase (p<0.05) in the percentage of hypodense eosinophils and neutrophils. At day 8, the constitutive and the inducible CD11b expression of the rhIL-3 group was increased compared to day 1. Furthermore, the relative CD11b expression on eosinophils (359±62%) and neutrophils (471±109%) in the rhIL-3 group was higher (p<0.05) than in the control group (166±41 and 166±23%, respectively). This study showed that eosinophils can be activated and primed in vivo during rhIL-3 treatment, thereby confirming in vitro studies.
Introduction

Interleukin-3 (IL-3) is a hematopoietic growth factor that acts on numerous target cells. It can stimulate the generation and differentiation of macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytes and erythroid cells by acting on multipotent hematopoietic stem cells and committed progenitor cells [1-4]. Treatment of patients with recombinant human IL-3 (rhIL-3) resulted in multilineage hemopoietic effects [5-8]. These patients frequently reported flu-like symptoms but also experience, although less frequent, allergy-like side effects, such as facial erythema, urticaria, conjunctivitis, dyspnea, edema of the lips and eyelids [9-16]. These side effects were observed more often when more than two chemotherapy and rhIL-3 cycles were administered [13]. Furthermore, rhIL-3 administration consistently resulted in increases in eosinophils [8,10,12-14,16]. The observation of allergy-like phenomena and eosinophilia during and after rhIL-3 administration prompted us to further investigate eosinophil activation and priming during rhIL-3 treatment.

In vitro, IL-3 does affect immature and mature peripheral cells. Eosinophils retain responsiveness for IL-3, while neutrophils lose their responsiveness during differentiation [17]. The modulation of eosinophil function by IL-3 is one of the mechanisms involved in allergic diseases [18,19]. Increased numbers of cells expressing IL-3 mRNA have been found in broncho-alveolar lavage fluid from patients with symptomatic allergic asthma compared to patients free of symptoms [20]. In vitro, IL-3 enhances eosinophil chemotaxis, respiratory burst, degranulation, cytotoxicity and leukotriene C4 production induced by a stimulus [21-24]. IL-3 is also able to induce adhesion of eosinophils [25]. One of the adhesion molecules involved is CD11b, the α subunit of Mac-1 [26]. In vitro, CD11b can be up-regulated on eosinophils by IL-3 [27,28]. This up-regulation may be mediated by fast release from intracellular stores or more slowly by de novo synthesis [29]. In vitro up-regulation of CD11b by N-formyl-methionyl-leucyl-phenylalanine (fMLP) on eosinophils from children with allergy is primed, probably by cytokines such as IL-3 [30].

In vitro IL-3 decreases buoyant density of eosinophils [24]. This phenomenon is also believed to occur in vivo. High numbers of low density, so-called hypodense, eosinophils have been isolated from the blood of patients with allergic asthma and
hypereosinophilic syndromes [31-34]. These hypodense eosinophils express an activated phenotype as shown by increased leukotriene C4 production, cytotoxic capacity, and expression of surface receptors [33-35].

The in vitro effects of IL-3 on eosinophils may be different from the in vivo effects of IL-3. In vivo production of other cytokines induced by rhIL-3 treatment, may modulate activation of eosinophils [10,36]. The aim of the present study was to evaluate whether activation or priming of eosinophils also occurs in vivo during rhIL-3 treatment. Therefore, density and CD11b expression of eosinophils in ovarian cancer patients who were treated with chemotherapy and rhIL-3 were investigated. Neutrophils were also evaluated, although a direct effect on neutrophils is not expected, since rhIL-3 may induce endogenous production of other cytokines [10,37]. In order to investigate whether priming of the inducible expression of CD11b occurred, cells were incubated in vitro with fMLP and platelet-activating factor (PAF).

**Materials and methods**

**Patients.** Patients older than 18 years of age, with newly diagnosed stage IIc-IV ovarian cancer according to the International Federation of Gynaecologists and Obstetricians (FIGO) were eligible to participate in this study. Patients underwent optimal tumor reductive surgery before the start of the chemotherapy, whenever possible. A leukocyte count of ≥3×10⁹/l and a platelet count of ≥100×10⁹/l was required at entry. Patients with severe heart, lung, liver (serum bilirubin ≥40 mmol/l) and renal impairment (creatinine clearance <60 ml/min) were excluded from the study, as were patients with a WHO performance score grade 3-4. Those previously treated with chemotherapy, or on treatment with steroids, morphine, cimetidine or other H₂-histamine blockers were not eligible for the study, as were patients with a history of serious allergies and those known to be hypersensitive to exogenous protein administration.

**Study design.** Combination chemotherapy comprised 6 cycles of cyclophosphamide (750 mg/m²) and carboplatin (dose adjusted to creatinine clearance at 100 ml/min: 507 mg) both administered day 1 on an outpatient basis. The dosing regimen of carboplatin was derived
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from Calvert et al. [38], and calculated for creatinine clearances between 60 and 190 ml/min with steps of 10 ml/min. Cyclophosphamide (ASTA Pharma A.G., Frankfurt, Germany) dissolved in 250 ml saline 0.9%, was administered intravenously (iv) over 15 min, carboplatin (Bristol-Myers Squibb, Regensburg, Germany), dissolved in 250 ml dextrose 5%, was administered iv over 30 min.

Eight patients participating in this study concerning eosinophil analysis were randomized double blind to receive placebo or E. coli derived nonglycosylated rhIL-3 (2-10^6 U/mg), which was provided by Sandoz (Basel, Switzerland) in vials of 750 µg/ml. Placebo or rhIL-3 was reconstituted for subcutaneous (sc) administration with 1 ml of sterile water. After instructions by the oncology nurse, placebo or rhIL-3 was self-administered sc in the upper leg by the patient on an outpatient basis. RhIL-3 was given in a dose of 5 µg/kg body weight/day (µg/kg/d). The injections started 2 days after chemotherapy (day 3) for 10 days. Blood was drawn for differential counts, as well as the study of granulocyte density and CD11b expression during the first cycle only, at day 1 (before chemotherapy, visit I), day 3 (start of rhIL-3 treatment, visit II) and 5 days after the start of rhIL-3 treatment (visit III). In addition, six patients, who had received the same chemotherapy as mentioned above, were entered. Three of these patients received rhIL-3 as an open label, and three received nothing and were used as controls. Therefore a total of seven rhIL-3-treated patients and seven control patients could be evaluated. The study was approved by the Medical Ethical Committee of the University Hospital Groningen. Written informed consent was obtained from all patients.

Density determination. The density profiles of eosinophils and neutrophils were determined using a previous described method [39]. Briefly, 6 ml ethylene diamine tetra acid (EDTA) anticoagulated blood was diluted with an equal volume of 0.9% NaCl. This mixture was subsequently layered on 6 discontinuous density gradients of isotonic Percoll (Pharmacia LKB, Uppsala, Sweden). The gradients were centrifuged at 1000g for 20 min at room temperature. The cells were carefully collected from each interface and excess Percoll was removed by washing in sterile phosphate buffered saline (PBS). Contaminating erythrocytes were lysed by isotonic lysis with ice-cold ammonium chloride (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Subsequently, the cells were washed once and resuspended in PBS. The leukocyte count in each fraction was quantified with a Coulter counter (Model S880, Coulter Electronics Hialeah, Fla). Differential cell counts were determined by examining 200
cells on cytocentrifuge preparations stained with May-Grünwald Giemsa stain. From these data, the percentages of total neutrophils and eosinophils recovered at each density interface were calculated. In our experiments with isotonic Percoll eosinophils and neutrophils with a density of <1.085 g/ml were defined hypodense. This density was based on results from healthy controls in which about 10% hypodense cells were found [39].

Percentages of eosinophils and neutrophils in blood were determined by counting 500 cells on blood films after staining with May-Grünwald Giemsa stain. The eosinophil and neutrophil counts in blood were calculated from these percentages and the total leucocyte counts quantified with the Coulter counter.

**Stimulation and labeling.** Stimulation and labeling whole blood cells was performed as described previously [30]. Samples of 1.5 ml EDTA-anticoagulated blood were washed twice with excess PBS, at room temperature. Thereafter, the cells were resuspended in 1.5 ml PBS. Portions of this suspension (100 μl) were added to 400 μl of Hanks' balanced salt solution (HBSS, GIBCO, Paisley, UK). These samples were incubated 15 min at 37°C, in the presence of HBSS for measurement of constitutive expression or different concentrations of fMLP (0.002 or 0.02 μM) or PAF (0.01 or 0.1 μM) for measurement of induced expression. Reactions were stopped by adding ice-cold PBS, containing a 2.5% (vol/vol) solution of bovine serum albumin solution (BSA, Organon Teknika, Boxtel, The Netherlands). After fixation with 0.01% formaldehyde, the cells were incubated 15 min at room temperature with 10 μl anti-CD11b (Becton Dickinson, Mountain View, CA) or control antibody of the same mouse isotype (IgG2a, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). This was performed in the presence of 0.1 μl of diluted human AB-positive serum (20%) to prevent non-specific binding of antibody. After lysis of red blood cells with lysis buffer (155 mmol/l NH₄Cl, 10 mmol/l KHCO₃, 0.1 mmol/l EDTA), each sample was labeled 15 min with 50 μl fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (GAM-FITC, Becton Dickinson, 20% vol/vol in PBS with BSA), in the presence of 5% AB-serum. Flow cytometric analysis was performed within 4 h after staining.

**Flow cytometry.** Flow cytometric measurements were performed with a Becton-Dickinson FACStar. Fluorescence gain was standardized with Quick Cell 3 beads (Flow Cytometry Standards Co., San Juan, PR). Eosinophils and neutrophils were discriminated by means of
depolarized orthogonal light scattering as described previously [30,39,40]. In each sample, 10,000 events were analyzed. The mean specific fluorescence on neutrophils and eosinophils was evaluated with a personal computer (Hewlett Packard) and the Lysis program (Becton Dickinson). During evaluation neutrophils and eosinophils were gated based on forward scatter/sideward scatter (FSC/SSC) and further discriminated by SSC and depolarized light scattering.

Expression of specific receptors is presented as mean fluorescence intensity (MFI ± SEM), after subtraction of the fluorescence with control antibody. Since CD11b expression shows a great inter-individual variability [41], MFIs on visits II and III were calculated as a percentage of MFI on visit I.

Statistical analysis. Differences between the visits were evaluated using the Student’s *t*-test for paired samples. The Student’s *t*-test for independent samples was used to investigate differences between rhIL-3 treated patients and controls. Eosinophil counts were log-transformed before analysis. A *p*-value below 0.05 was considered to be significant.

Results

Clinical manifestations. All patients receiving rhIL-3 experienced fever, accompanied by headache, with chills and myalgia. These symptoms subsided after discontinuation of the rhIL-3 injections. These flu-like symptoms were absent in the control patients. No allergy-like side-effects were observed in this group of patients.

Eosinophil and neutrophil number. At visit I, the eosinophil counts (mean±SEM) in blood were similar for patients treated with rhIL-3 (0.217±0.063 ×10⁹/l) and control (0.117±0.052 ×10⁹/l). A significant difference between visit III and visit I is indicated by *.

Figure 1. Mean eosinophil counts before chemotherapy (visit I), after chemotherapy (visit II), and after treatment (visit III) with control (○) or 5.0 μg/kg/d rhIL-3 (■). A significant difference between visit III and visit I is indicated by *.

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and controls (0.204±0.048 \times 10^{9}/l). After chemotherapy, at visit II, a decrease in eosinophil count was observed. At this time, the eosinophil count in the rhIL-3 group was 0.189±0.38 \times 10^{9}/l and in controls 0.148±0.013 \times 10^{9}/l. At visit III the eosinophil count in the rhIL-3 group increased to 0.419±0.139 \times 10^{9}/l, compared to visit II (p=0.03). In contrast, the eosinophil counts in the control group at visit III further decreased to 0.128±0.023 \times 10^{9}/l, which was lower than in the rhIL-3 group (Figure 1).

No differences were observed in the neutrophil counts before treatment between the two groups (not shown). Two days after chemotherapy, at visit II, the neutrophil counts were not significantly changed in both groups. At visit III a decrease in the neutrophil count was found (p<0.05) compared to visit I. No significant difference was observed between patients treated with rhIL-3 and control patients.

**Eosinophil and neutrophil density.** At visit I, 31±9% (n=5) and 35±8% (n=7) of the eosinophils (mean±SEM) were found to be hypodense in the rhIL-3 and control group respectively. Two days after chemotherapy (visit II), these percentages were not significantly changed. At visit III, still no significant effect of chemotherapy on eosinophil density was found, since the percentage of hypodense eosinophils was not changed in the control group. In contrast, the patients treated with rhIL-3 showed more than a doubling in the number of hypodense eosinophils; from 31±9% at visit I to 69±15% at visit III (p<0.05). However, no significant differences were found between the rhIL-3 and the control group (Figure 2).

Similar to eosinophils, the percentage of hypodense neutrophils increased from 29±9% at the start of the study to 68±9% (mean±SEM) after treatment with rhIL-3 (p<0.05). The percentage of hypodense neutrophils decreased at visit II in the control group but this change was not significant. No significant differences were found between the two groups (Figure 3).
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As shown in Figure 4, the CD11b expression of eosinophils (mean±SEM) increased after visit I in the rhIL-3 group, resulting in a significant difference at visit III for these patients (359±62%) compared to the control group (166±41%, p<0.05). The CD11b expression on neutrophils showed a pattern similar to that observed for eosinophils (Figure 5). In the rhIL-3 group and the control group, a significant increase was found on visits II and III, compared to visit I. This increase was more pronounced in the rhIL-3 group than in the control group, resulting in a higher relative CD11b expression at visit III (471±109%) compared to the control group (166±23%, p<0.05).

**Figure 2.** Percentage of hypodense eosinophils (density <1.093 g/ml or 1.085 g/ml, see Materials and Methods) before chemotherapy (visit I), after chemotherapy (visit II), and after treatment (visit III) with control (○) or 5 µg/kg/d rhIL-3 (■). A significant difference between visit III and visit I is indicated by *.

**Figure 3.** Percentage of hypodense neutrophils (density <1.090 g/ml or 1.085 g/ml, see Materials and Methods) before chemotherapy (visit I), after chemotherapy (visit II), and after treatment (visit III) with control (○) or 5.0 µg/kg/d rhIL-3 (■). A significant difference between visit III and visit I is indicated by *.
As shown in Figure 6, eosinophils from both groups showed an increased expression of CD11b to PAF (0.1 and 0.01 μM) at visit II when compared to visit I (NS). In the rhIL-3 group the relative expression of CD11b continued to increase to 211±99% (p<0.05) at visit III. Whereas for the control group a decrease was noted at this visit. This difference between both groups at visit III did not reach statistical significance. Similar results were found for 0.01 μM of PAF, in the rhIL-3 treated group, the response of eosinophils at visit III was 426±180% of the response found at visit I (not shown). After exposure of 0.2 μM or 0.02 μM fMLP no differences in CD11b expression of eosinophils were observed for both groups.
The response of neutrophils to 0.1 μM and 0.01 μM PAF tended to increase after visit I and remained stable thereafter. This was the same for both groups. Similar results were found for fMLP. The response of neutrophils to 0.02 μM fMLP in the rhIL-3 group at visit II was 129±21% of the response at visit I. This response at visit III was 115±11% (n=6) of that at visit I. This small increase at visit II and III was also found in the control group, 115±27% and 121±22% respectively. The increases compared to visit I, however, were not significant. Minor responses were found with 0.002 μM fMLP and only data of four rhIL-3 treated patients could be obtained. Therefore, these results were not further evaluated.

**Discussion**

In this study the effects of rhIL-3 treatment (5 μg/kg/d) after chemotherapy on peripheral eosinophils and neutrophils compared to no rhIL-3 were evaluated. Activation of these cells by rhIL-3 was assessed with quantitative methods. Despite chemotherapy an increase in the eosinophil count was observed within 5 days after the start of the rhIL-3 treatment, which was not observed in the control group. This is in line with earlier studies. Aglietta, for instance, who analyzed rhIL-3 effects without chemotherapy, reported an increased eosinophil count within 3 to 6 h after the first IL-3 dose (0.25 to 10 μg/kg/d), followed by a dose dependent eosinophilia the next 7 days [42]. Biesma et al. showed that after the same chemotherapy as in the present study, an increase in eosinophils was observed 3 days after the start of rhIL-3 administration (1 to 15 μg/kg/d) [10]. After 7 days of rhIL-3 (5 μg/kg/d)
administration and chemotherapy a 408% increase in eosinophils was observed [13]. After 5 cycles chemotherapy plus rhIL-3 this increase in eosinophil count was diminished but still present [13].

In the current study, before chemotherapy, 30 to 35% of eosinophils were found to be hypodense, compared to 10% in healthy controls [39]. Increased numbers of hypodense eosinophils have also been observed in patients with malignant diseases [33,34]. All patients underwent surgery for their ovarian cancer, which may also have affected the number of hypodense eosinophils at the start of the treatment. A further increase of the percentage of hypodense eosinophils was only observed in patients who received rhIL-3. This showed that rhIL-3 enhances the effector function of eosinophils. However, the density of eosinophils alone is not conclusive for their activational state, since immature, non-activated, eosinophils can also have a decreased density [43]. A decreased eosinophil density in vivo has also been reported after treatment with rhIL-2 [44,45]. This is probably mediated by endogenous IL-5 production [46-48], indicating a similarity between the in vivo effects of IL-3 and IL-5, which are both involved in allergic inflammation [18].

The percentage of hypodense neutrophils also increased during rhIL-3 treatment compared to before chemotherapy. Increased numbers of hypodense neutrophils have been reported in allergic rhinitis and rheumatoid arthritis [49,50] and these neutrophils appeared to be activated as shown by increased myeloperoxidase activity, chemotaxis, and complement-dependent cytotoxicity [51-53].

Constitutive CD11b expression of eosinophils and neutrophils was increased after 5 days of rhIL-3 administration and in the control group. However, significantly more pronounced in the rhIL-3 group (p<0.05). In vitro experiments have shown that IL-3 is able to enhance the constitutive CD11b expression on eosinophils [27,28]. Therefore, this increased CD11b expression may indicate in vivo activation of eosinophils and neutrophils by rhIL-3. The up-regulation of CD11b can be mediated by rapid release from intracellular stores and de novo synthesis as was shown by in vitro work reporting the effects of GM-CSF on neutrophils [29]. The mechanisms by which IL-3 increases CD11b on eosinophils are probably similar because rapid up-regulation within 1 h [28] as well as slow up-regulation during 1 day [27] have been observed. Up-regulation of CD11b on eosinophils has also been described during
Density and CD11b expression of eosinophils from ovarian cancer patients receiving rhIL-3 after chemotherapy [27]. In contrast to eosinophils, in vitro experiments have shown no direct effects of IL-3 on neutrophil CD11b expression [17]. Therefore, the increase of CD11b on neutrophils during rhIL-3 treatment may be due to other endogenous produced cytokines. TNF-alpha plasma levels are slightly increased during rhIL-3 administration and TNF-alpha secretion by monocytes is enhanced during rhIL-3 treatment [10, 36]. In vitro, TNF does increase CD11b expression on neutrophils in vitro [28]. This could explain an indirect effect of rhIL-3 on neutrophil CD11b expression.

Apart from the constitutive CD11b expression, the inducible expression of CD11b on eosinophils was also increased by rhIL-3 treatment. It enhanced or maintained priming of eosinophils and not that of neutrophils. So, rhIL-3 selectively primes eosinophils in vivo for PAF-induced CD11b up-regulation.

The observed increased activity and priming of eosinophils may be involved in the development of rhIL-3 related side-effects such as urticaria and facial erythema [10,19,54,55]. This is supported by the finding that urinary leukotrienes excretion was increased during treatment with rhIL-3 [56]. To obtain consistent data, results were obtained in the first chemotherapy cycle. However, in this particular period, no allergy-like side effects were observed. In previous studies, most allergy-like side effects were observed after 2 or more cycles of chemotherapy and rhIL-3 administration [10,13,16]. An explanation for this may be the fact that priming has to occur first, before rhIL-3 is able to stimulate eosinophils in susceptible individuals. This may consequently result in rhIL-3 related allergy-like symptoms. Other factors could also be involved. A separate role for basophils, another potentially important effector cell of hypersensitivity reactions, in causing these allergy-like side-effects cannot be excluded. Histamine release of basophils has been shown to be inducible by IL-3 [57]. This was also reported in allergic asthmatic patients [58]. Chemotherapy alone and especially platinum based compounds may also result in hypersensitivity reactions [59,60]. These reactions occur in general during or shortly after the chemotherapy, while the rhIL-3 related side-effects occur many days later during rhIL-3 treatment.

In conclusion, this study showed that rhIL-3 administered after chemotherapy increased eosinophil numbers and induced an activated and primed phenotype of
eosinophils. And it furthermore demonstrated that the effects of rhIL-3 on eosinophils found in vitro are similar to the effects found in vivo. This supports the role of IL-3 in the modulation of eosinophil function in allergic diseases.

References


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**Chapter 2**
Density and CD11b expression of eosinophils from ovarian cancer patients receiving rhIL-3 after chemotherapy

