Interleukin-3 Treatment of Rhesus Monkeys Leads to Increased Production of Histamine-Releasing Cells That Express Interleukin-3 Receptors at High Levels


To understand the hematopoietic and nonhematopoietic responses to interleukin-3 (IL-3), expression of cell-surface IL-3 receptors (IL-3R) was examined on bone marrow (BM) cells and peripheral blood (PB) cells of rhesus monkeys during the course of in vivo IL-3 treatment. Whereas IL-3R expression is low in untreated monkeys, IL-3 administration led to a gradual increase in both low- and high-affinity binding sites for IL-3. This increase reflected the total number of cells expressing IL-3Rs, as detected by flow cytometry using biotinylated IL-3. Most of these IL-3R+ cells in both BM and PB could be characterized as basophilic granulocytes that contained high levels of histamine. In contrast to the effect on these differentiated cells, IL-3 administration did not significantly alter the low level IL-3R expression on immature, CD34+ cells. Further flow cytometric analysis using biotinylated growth factors showed that the IL-3R+ basophils also expressed receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), but not for IL-6 or Kit ligand. These findings indicated that the IL-3R+ cells included neither monocytes, which express GM-CSFRs and IL-6Rs abundantly, nor mast cells, which express c-Kit. By combining flow cytometric and Scatchard data, it was calculated that the basophils contain as many as 1 to 2 x 10^3 high-affinity IL-3Rs and 15 to 30 x 10^2 low-affinity sites. The finding that in vivo IL-3 treatment leads to the production of large numbers of cells that express high levels of IL-3R and are capable of producing histamine provides an explanation for the often severe allergic reactions that occur during prolonged IL-3 administration. It also indicates that IL-3, in addition to its direct effects on hematopoietic cells, may also stimulate hematopoiesis through the release of secondary mediators such as histamine by IL-3-responsive mature cells.

INTERLEUKIN-3 (IL-3) stimulates the survival, proliferation, and differentiation of precursors of granulocytes, monocytes, erythrocytes, and platelets.1-5 In addition, IL-3 is thought to be essential for the survival of immature, multipotent hematopoietic cells and to modulate the functional activities of specific mature blood cell types.6-10 In vivo administration of IL-3 to human patients or experimental animals results in increased proliferation of hematopoietic progenitor cells and production of mature cells of all myeloid lineages in peripheral blood (PB).10-16 A prominent feature of in vivo IL-3 treatment of rhesus monkeys is the appearance of basophilic granulocytes, which are atypical because they do not stain with basophilic dyes such as toluidine and alcin blue.12,16 In addition to its hematopoietic effects, in vivo IL-3 treatment is associated with a wide range of side effects, including urticaria, edema, enlargement of lymphoid tissue, and acute arthritis.11,15-18

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The multiple effects of IL-3 are mediated by high-affinity receptor complexes, which have been identified on normal monocytes, basophils of chronic myeloid leukemia (CML) patients, eosinophils of hypereosinophilic patients, blast cells of acute myeloid leukemia (AML) patients, and various cell lines of leukemic origin.19-23 Results of binding studies with radiolabeled IL-3 have indicated that the average number of high-affinity IL-3Rs in normal human and rhesus monkey bone marrow (BM) is low (<100 sites per cell).24,25 Results of flow cytometric studies using biotinylated IL-3 indicated that IL-3R expression is mainly associated with immature, CD34+ cells.25a In the present study, we have combined the results of equilibrium binding experiments and flow cytometric detection of IL-3R+ BM and PB cells to obtain a quantitative estimate of changes in IL-3 binding affinity, receptor levels, and distribution patterns as a result of hematopoietic stimulation by IL-3 in vivo.

MATERIALS AND METHODS

IL-3. Rhesus monkey IL-3,26,27 expressed in Bacillus licheniformis, was purified to homogeneity as described.24 Human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and c-Kit ligand (KL) were gifts of Dr S.C. Clark (Genetics Institute, Cambridge, MA) and Dr S. Gillis (Immunex, Seattle, WA), respectively. Human recombinant IL-6 was provided by Aresa-Serono (Geneva, Switzerland).

Animals. Young adult rhesus monkeys (Macaca mulatta) bred at the Primate Center TNO (Rijswijk, The Netherlands) were used throughout this study. Body weights were between 2.5 and 4 kg. Animals were free of intestinal parasites, herpes B, simian T-lymphotropic virus (STLV), and simian immunodeficiency virus (SIV). IL-3 administration. Animals received IL-3 at a dose of 15 μg/kg/day subcutaneously (SC) once daily for 14 to 30 consecutive days. Under these conditions, PB cell numbers reached values up to 30,000 cells/μL, similar to previous observations.13

Procurement of BM and PB cells. PB was obtained by venous puncture. BM was collected by piercing the head of the humeral shaft to obtain small samples or by piercing the knee joint into

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the femoral shaft in case larger amounts of BM were needed. Mononuclear cells were isolated from BM or PB by density centrifugation using Ficoll (Organon Teknika, Durham, NC). Before use in binding experiments, interphase cells were incubated for 1 hour at 37°C in 5% fetal calf serum (FCS), HEPES-buffered Hank's Balanced Salt Solution (HF) to allow dissociation of in vivo bound IL-3 from its receptor complex.

Radioiodination of IL-3. IL-3 was radiolabeled with Bolton and Hunter reagentz9 (Amersham Laboratories, Amersham, UK) as described. Specific binding of 125I-IL-3 as determined by self-displacement analysis was 1.4 x 10^4 and 4 x 10^4 cpm/ng for the two batches used in this study. More than 95% of radioactivity was precipitable in 10% (wt/vol) trichloroacetic acid. The radiolabeled IL-3 had retained its biologic activity as shown in a 3H-thymidine uptake assay using the M07E cell line.31

Binding of 125I-IL-3 to cells. To evaluate receptor binding, cells were incubated with 500 pmol/L or titrated concentrations of 125I-IL-3 (25 to 5,000 pmol/L) in 200 μL HF at 4°C for 20 hours. Nonspecific binding was determined in parallel incubations in the presence of 100-fold molar excess of nonlabeled IL-3. To separate cell-bound from free radiolabeled IL-3, cells were centrifuged through a cushion of precooled oil (a mixture of dibutyl-phthalate and dioctyl-phthalate at a ratio of 3:2; Sigma). After freezing in liquid nitrogen, the tips of the microcentrifuge tubes were cut off and radioactivity was measured using a γ-counter. Receptor numbers and apparent kd values were estimated using a γ-counter. Receptor numbers and apparent kd values were estimated after Scatchard plot analysis of the binding data.32 The data were analyzed using the Sigmaplot curve fitting program (Jandel Co) and were found to fit a two-component curve as determined using the least square method and simple equilibrium equations. The best fit for the two-component curves was drawn.

Biotin-labeling of IL-3, IL-6, GM-CSF, and KL. Growth factors were biotin-labeled essentially as described. Bioactivity of biotinylated IL-3, GM-CSF, and KL were measured in a 3H-thymidine uptake assay using M07E cells. Bioactivity of biotinylated IL-6 was measured using the IL-6-dependent B9 hybridoma cell line. Bioactive growth factors were greater than 99% biotin-labeled as tested by absorption to streptavidin-agarose beads (Sigma).

Binding of biotin-labeled growth factors to cells. One million cells were incubated with biotin-labeled IL-3 (5 nmol/L), biotinylated GM-CSF (2 nmol/L), biotinylated IL-6 (1 nmol/L), or biotinylated KL (1 nmol/L), respectively, in HF, 0.05% sodium azide for 20 hours on ice. Nonspecific binding was evaluated in parallel incubations in the presence of a 100-fold molar excess of nonlabeled growth factors. Cells were washed twice and incubated with streptavidin-phycocerythrin (SA-RPE; Molecular Probes, Eugene, OR) for 1 hour on ice. The fluorescence intensity was amplified by one cycle of sequential incubations with a biotin-labeled antibody against RPE and with SA-RPE, as described. To compare IL-3R expression with CD34 expression, BM cells were incubated with a fluoresceinated monoclonal antibody (MoAb) against CD34 (MoAb 566; kindly provided by Dr T Egeland, University of Oslo, Oslo, Norway) during the second incubation with SA-RPE. After washing, receptor-expressing cells were detected by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometry data were analyzed using LYSYS II software (Becton Dickinson).

Cell sorting. Atypical basophilic cells of animals treated with IL-3 were sorted for histamine content determination on the basis of light scatter properties using a FACScan flow cytometer (Becton Dickinson).

Histamine determination. Histamine content of cells was measured using an automated fluorometric method according to Siriganian.38

RESULTS
To examine the ability of IL-3 to stimulate and expand IL-3-responsive cells in vivo, we performed equilibrium

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Fig 1. Increased IL-3R expression during IL-3 treatment. (A) Saturation binding curves of 125I-IL-3 binding to BM cells (specific activity, 14,000 cpm/ng; 5 x 10^6 cells per point) of a rhesus monkey at days 2, 6, and 8 of IL-3 treatment (15 μg/kg/d SC). Results represent the mean of duplicate determinations. Nonspecific binding, as determined with a 100-fold molar excess of nonlabeled IL-3, was subtracted. (B) Scatchard transformation of the data in (A) depicting data points and corresponding fitted curves showing an increase in high- as well as low-affinity binding sites per cell. Estimated kd values were 63, 45, and 54 pmol/L (56, 108, and 181 high-affinity binding sites/cell) and 1,798, 1,784 and 1,782 pmol/L (422, 2,678, and 3,999 low-affinity binding sites/cell) for days 2, 6, and 8, respectively. Specific binding of 125I-IL-3 to day 0 cells was too low for evaluation by Scatchard analysis.
binding studies with $^{125}$I-IL-3 on BM and PB cells of rhesus monkeys during the course of IL-3 treatment. Previous results have indicated that the number of IL-3Rs on normal BM cells is very low. During treatment there was a gradual increase of specific binding of $^{125}$I-IL-3 to BM cells (Fig 1A). Scatchard analysis (Fig 1B) showed that this was due to an increase of both high-affinity sites from ~50 to 180 sites per cell and of low-affinity binding sites from ~400 to 4,000 between days 2 and 8. The respective kd values remained virtually unchanged at ~50 pmol/L and 1,800 pmol/L. Similar results were obtained for IL-3R$^+$ cells in PB (data not shown).

We next examined alterations in IL-3R expression during in vivo IL-3 treatment using biotinylated IL-3 and flow cytometry. The results confirmed those obtained with radiolabeled IL-3, because large numbers of IL-3R$^+$ cells could be identified in BM and PB of IL-3–treated monkeys (Figs 2 and 3). The majority of IL-3R$^+$ cells displayed intermediate-to-high forward and intermediate right-angle light scatter (Figs 2D and 3A). These IL-3R$^+$ cells were not detectable in untreated monkeys, increased gradually in frequency during treatment, but disappeared rapidly after discontinuation of IL-3 administration. In monkeys treated with IL-3 for periods up to 30 days, the frequency of IL-3R$^+$ BM cells continued to increase, but in PB plateau values were usually reached after 8 to 9 days. Maximal numbers of IL-3R$^+$ cells reached values of 18% in BM after 25 days of IL-3 treatment.

As shown in Fig 2A, immature CD34$^+$ hematopoietic cells also expressed IL-3Rs in BM of IL-3–treated monkeys, but at much lower levels than the CD34$^-$ cells. No differences were observed between IL-3R expression levels of CD34$^+$ cells of IL-3–treated monkeys and those of untreated monkeys.

Analysis of other growth factor receptors on IL-3R$^+$ cells indicated that most of these cells expressed low levels of the GM-CSF receptor (GM-CSFR), whereas a minor fraction was GM-CSFR$h^+$ (Fig 3D). IL-6Rs were only present on a small subset of cells in the same light scatter window, whereas c-kit could not be detected (data not shown). The frequency of GM-CSFR$h^+$ and IL-6R$^+$ cells was similar to that of the small subset of IL-3R$^-$ cells in the same window (Fig 3B). Most likely, this minor cell fraction consisted of monocytes, which, in normal rhesus monkeys, have no detectable IL-3R expression, but express GM-CSFRs and IL-6Rs abundantly.

The frequency of IL-3R$^+$ cells in the IL-3–treated monkeys was similar to that of the basophilic granulocytes identified previously in similarly treated monkeys by morphologic analysis. PB cells of an IL-3–treated monkey were sorted on the basis of the specific light scatter properties. These cells expressed histamine at levels that were comparable to those found in human basophils and mast cells, ie, 700 and 1,000 fg/cell in two separate experiments, whereas cells outside the sort window of basophils contained only 140 and 70 fg/cell.

By combining the average number of IL-3Rs per cell as estimated by Scatchard analysis with the frequency of IL-3R$^+$ BM cells determined by flow cytometry, the absolute
numbers of high- and low-affinity receptors on the histamine-containing atypical basophils could be estimated (Table 1). In BM the number of high-affinity receptors ranged between $10^4$ and $2 \times 10^4$ and did not show an increasing trend during IL-3 treatment. However, the number of low-affinity sites increased from $\sim 1 \times 10^4$ at day 2 to $3 \times 10^4$ on day 8. Values for PB cells were $6.5 \times 10^3$ high-affinity sites and $7 \times 10^4$ low-affinity sites after 25 days. This gradual increase in the total number of receptors showed that the increased IL-3R expression during IL-3 treatment, although largely attributable to the expansion of basophilic granulocyte numbers, is at least partly due to increased expression of IL-3Rs on individual cells.

Table 1. Estimate of IL-3R Numbers on Basophilic Granulocytes in BM Produced During In Vivo IL-3 Treatment

<table>
<thead>
<tr>
<th>Day</th>
<th>IL-3R* Cells (%)</th>
<th>High-Affinity Receptors*</th>
<th>Low-Affinity Receptors*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$kd$</td>
<td>Sites/Cell</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>63</td>
<td>1,867</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>45</td>
<td>990</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>54</td>
<td>1,392</td>
</tr>
</tbody>
</table>

* Number of high- and low-affinity sites was calculated by correcting the Scatchard data of Fig 1 for the frequency of IL-3R expressing basophils as determined by flow cytometry.

DISCUSSION

In this study, IL-3R expression on BM and PB cells in rhesus monkeys during the course of IL-3 treatment was shown to be predominantly attributable to the production of large numbers of basophils that express high- and low-affinity IL-3Rs at high levels. High-affinity IL-3Rs have previously been reported for leukemic basophils isolated from CML patients, but low-affinity sites were not detected on these cells. The presence of low-affinity binding sites at high levels in our study could be the consequence of IL-3 treatment, although it could also reflect a qualitative difference between rhesus monkeys and humans or between normal and leukemic basophils. It is difficult to examine IL-3R expression on basophils in healthy human individuals or monkeys because of the paucity of these cells in normal PB. In a series of 140 untreated monkeys, we could detect basophils in PB of only 15 of these monkeys at frequencies of less than 2% (van Gils et al. and unpublished observations).

In this study we found that in vivo IL-3 treatment does not alter IL-3R expression on immature CD34$^{bright}$ BM cells. The presence of IL-3Rs on these cells in BM of normal and of IL-3-treated rhesus monkeys indicates that IL-3 may act directly on immature hematopoietic cells. That this is indeed the case is shown by the ability of IL-3, in conjunction with other cytokines, to stimulate proliferation and differentiation of CD34$^{bright}$/RhLA-DR$^{null}$ cells at the single cell level.
However, our data indicate that other mechanisms of IL-3 action, eg, indirect stimulation through secondary cytokine release by accessory cells, may also be relevant, as has been shown for a number of other growth factors such as IL-1 and IL-6. One candidate mediator is histamine, produced by the basophils, but also by other cell types, on IL-3 stimulation (this study).

Histamine has been shown to stimulate proliferation of immature hematopoietic cells and to play a role in IL-3-mediated activities on these cells in vitro. Other mediators produced on IL-3 stimulation of murine and human cells include IL-1, IL-4, and IL-6, which have potent direct hematopoietic effects on their own and can also induce cytokine production by various cell types. Therefore, it is conceivable that continuous production and activation of basophils and other cells during IL-3 treatment and the resulting systemic reactions are at least partly involved in the hematopoietic effects of IL-3.

The IL-3-induced production of IL-3R+ histamine-producing basophils could play a major role in the wide range of nonhematopoietic systemic effects that are associated with IL-3 in vivo. IL-3 administration to rhesus monkeys that are pancytopenic as a result of a sublethal dose of total body irradiation generally leads to slower increases in cell numbers and considerably less severe nonhematopoietic effects than in nonirradiated monkeys. These nonhematopoietic effects in irradiated monkeys are similar to the relatively mild course observed in human cancer patients treated with IL-3. A possible reason for these differences is the absence of sufficient IL-3-responsive target cells during periods of pancytopenia. In irradiated rhesus monkeys, urticaria and other nonhematopoietic reactions do eventually develop, in parallel with the recovery of white blood cell numbers. That these reactions continue to increase in severity concomitant with the increase in basophils during prolonged IL-3 treatment may also be considered as evidence for a cell-mediated mechanism. For this reason, one may expect that IL-3-stimulated regeneration of IL-3R+ basophils may lead to adverse systemic reactions in human patients as well and thereby limit the therapeutic efficacy of IL-3, particularly at high doses and/or prolonged treatment periods. The in vitro requirement of multiple growth factors to stimulate immature cells indicates that the in vivo efficacy of low dose IL-3 may be enhanced by a combination with IL-6, KL, GM-CSF, or other cytokines that are active on early hematopoietic cells.

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FLOW CYTOMETRIC DETECTION OF IL-3 RECEPTORS

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