In Vivo Synergism of Recombinant Human Interleukin-3 and Recombinant Human Interleukin-6 on Thrombopoiesis in Primates

By Klaus Geissler, Peter Valent, Peter Bettelheim, Christian Sillaber, Brunhilde Wagner, Paul Kyrle, Wolfgang Hinterberger, Klaus Lechner, Ekke Liehl, and Peter Mayer

Using a primate model, we examined the effect of recombinant human interleukin-3 (rhIL-3) and rhIL-6 on thrombopoiesis in vivo. Administration of 33 ng/kg/d of rhIL-3 for 11 to 14 days increased levels of circulating colony-forming units megakaryocyte (CFU-Mk) by approximately 15-fold in five rhesus monkeys without raising their platelet counts. In contrast, administration of 30 ng/kg/d of rhIL-6 for 10 days in four animals did not increase CFU-Mk levels but significantly raised platelet counts from a mean pretreatment value of 460 x 10^3/µL (range 360 to 610) to a mean maximum of 746 x 10^3/µL (665 to 790) on day 8. If monkeys were pretreated with rhIL-3 (33 or 100 ng/kg/d for 11 days) to expand their CFU-Mk compartment, the thrombopoietic effect of rhIL-6 was synergistically enhanced leading to platelet counts above 1,000 x 10^3/µL (mean maximum value 1,247) in all three primates studied. The sequential administration of rhIL-3 and rhIL-6 might represent a powerful strategy to stimulate thrombopoiesis in vivo.

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A substantial amount of information suggests that the process of platelet production is regulated by distinct factors acting at different levels of cellular development.1 Several cytokines have been shown to stimulate the in vitro growth of bone marrow (BM) megakaryocyte progenitor cells (CFU-Mk) and thereby possess megakaryocyte colony stimulating-activity (MK-CSA).1

Recombinant human interleukin-3 (rhIL-3) has proven to be an excellent stimulator of CFU-Mk growth in vitro.3 Recently, we could demonstrate a similar stimulatory effect of rhIL-3 in vivo leading to profound increases of CFU-Mk levels in rhesus monkeys.3

IL-6 is a cytokine originally defined by its ability to induce the final maturation of B cells into antibody-forming cells.5 Its wide range of biologic activities also includes promotion of the maturation of megakaryocytes in vitro.6 Furthermore, it was demonstrated that IL-6 is an in vivo thrombopoietic factor inducing increases of blood platelet counts in mice.7,8 and monkeys.9

In this study we pretreated rhesus monkeys with rhIL-3 to expand their CFU-Mk compartment and subsequently administered rhIL-6 to promote the terminal phase of thrombopoiesis.

MATERIALS AND METHODS

Animals. Eighteen adult rhesus monkeys, Macaca mulatta, approximately 4 to 10 years old and weighing between 5 and 16 kg, were housed in individual stainless cages. Monkeys were provided with 10 changes per hour of fresh air conditioned to 23 ± 2°C with a relative humidity of 60% ± 10%. They were maintained in a 12-hour light/dark cycle and provided with tap water ad libidum and commercial primate chow and fruit. Research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council.

Recombinant cytokines. The rhIL-3 used in this study was provided by Genetics Institute (Cambridge, MA). The non-glycosylated protein was extracted from Escherichia coli cells expressing the IL-3 cDNA from a plasmid vector. The rhIL-3 that accumulated intracellularly was purified to homogeneity by a series of chromatographic steps, including high-performance liquid chromatography (HPLC). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed the presence of a single Coomassie blue staining band with a purity ≥ 95%. The in vitro biologic activity was found to be 4.6 x 10^7 U/mg protein assayed by thymidine incorporation by chronic myelogenous leukemia (CML) myeloblasts as described.11 The endotoxin content was 30 ± 5 pg/ng as determined by the limulus assay (limulus amebocyte lysate; Whittaker, MA, Bioproducts, Walkersville, MD). rhIL-6 was also provided by Genetics Institute. The nonglycosylated protein extracted from E coli was purified as described above. Coomassie blue staining after SDS-PAGE showed a purity of greater than 98%. The in vitro biologic activity was found to be 1 x 10^6 U/mg protein, assayed by thymidine incorporation by the B9 cell line as described,15 the endotoxin content was <2.9 ng/mg protein.

Cell preparation. Five milliliters of peripheral blood (PB) was collected into sterile tubes containing 1 mL EDTA. BM samples were obtained by aspiration into sterile tubes containing heparin with no preservative (Seromed, Berlin, Germany). PB mononuclear cells (MNC) and BM MNC were harvested after a Ficoll-Hypaque gradient centrifugation (400g, for 40 minutes, 1,077 g/mL).

Progenitor cell assay. CFU-Mk progenitor cells were assayed using a modification of the clonal assay described by Fauser and Messner.14 Each plate contained 0.9% methylcellulose, 30% autologous serum which was harvested before the administration of cytokines, 10% bovine serum albumine (BSA; Behring, Marburg, Germany), α-thioglycerol (10^-4 mol/L), 5% phytohemagglutinin-leukocyte-conditioned medium (PHA-LCM), and Iscove's modified Dulbecco's medium (IMDM; Gibco, Paisley, Scotland). PB MNC and BM MNC were plated in triplicate at 0.7 to 2.0 x 10^5/mL. After a culture period of 14 days (37°C, 5% CO2, full humidity), cultures were examined under an inverted microscope. Aggregates containing at least four megakaryocytes (large, translucent, and polymorphic cells with distinct borders) were scored as CFU-Mk (Fig 1). CFU-GM were cultured as described above except using fetal calf serum (FCS) instead of autologous serum.

From the First Department of Medicine, University of Vienna; and Sandoz Research Center, Vienna, Austria. Submitted February 11, 1991; accepted October 22, 1991.

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Address reprint requests to Klaus Geissler, MD, First Department of Medicine, Division of Hematology, University of Vienna, A-1090 Vienna, Austria.

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Aggregates containing more than 40 cells were counted as CFU-GM.

The number of progenitors per 1 mL of PB was calculated on the basis of the number of colonies per number of PB MNC plated, the white blood cell (WBC) count, the percentage of MNC in the blood, and the percentage of MNC in the population of cells obtained by Ficoll-Hypaque centrifugation. BM progenitors were expressed as colonies per $1 \times 10^3$ BM MNC plated.

**Immunophenotyping of colonies.** To establish the validity of quantitating CFU-Mk in unstained cultures we classified 78 consecutive colonies by their morphologic appearance before plucking them, staining them with a monoclonal antibody recognizing the glycoprotein Iib (CD41) by an immunoalkaline phosphatase technique, and reclassifying them by two independent persons according to their immunophenotype (Fig 2). As shown in Table 1 there was a high correlation between both methods, indicating that the visual technique was very reliable in predicting the megakaryocytic lineage of colonies just by their morphologic appearance in situ.

**Hematologic examinations.** For hematologic examinations, blood was collected in EDTA-coated tubes. The parameters measured...
translucent and polymorphic cells were morphologically classified as megakaryocytic (Fig 1A). After classification by the visual technique colonies were plucked and stained with a monoclonal antibody recognizing the glycoprotein Ilb using an immunoperoxidase method (Fig 2) as described. The colonies were then reclassified according to their immunophenotype by two independent observers.

**Determination of acute-phase reactants.** Serum C-reactive protein, α-antitrypsin, haptoglobin, and prealbumin were assayed by a nephelometric method using antisera raised in rabbits against the human proteins (Behring Nephelometer; Behring, Marburg, Germany). Serum ceruloplasmin levels were determined by a radial immunodiffusion assay (LC Partigen, Behring).

**Administration of rh cytokines to animals.** rhIL-3 and rhIL-6 were prepared for subcutaneous (sc) administration by thawing the protein, a-antitrypsin, haptoglobin, and prealbumin were assayed by a nephelometric method using antisera raised in rabbits against the human proteins (Behring Nephelometer; Behring, Marburg, Germany). Serum ceruloplasmin levels were determined by a radial immunodiffusion assay (LC Partigen, Behring).

**RESULTS**

**Effect of recombinant cytokines on the CFU-Mk compartment.** In five rhesus monkeys treated with 33 μg/kg/d of rhIL-3 for 11 to 14 days and in four rhesus monkeys treated with 30 μg/kg/d of rhIL-6 for 10 days, the levels of circulating CFU-Mk were determined before and immediately after treatment. As shown in Fig 3A the PB levels of CFU-Mk markedly increased in all five animals receiving rhIL-3. There was an approximately 15-fold increase from a mean pretreatment value of 41/mL to a mean value of 598/mL at the end of rhIL-3 administration (P < .01 by the Student’s test for paired samples). In contrast, no consistent changes in the number of CFU-Mk were observed during administration of rhIL-6 with a mean pretreatment value of 27/mL and a posttreatment value of 37/mL, respectively (Fig 3B). Also, in four control monkeys receiving saline supplemented with an adequate amount of endotoxin, no significant changes were seen (mean pretreatment value, 17/mL, and posttreatment value, 32/mL, respectively).

To confirm this results by a more objective quantification of CFU-Mk, all of the colonies grown in culture from a monkey receiving rhIL-3 and another animal treated with rhIL-6 were phenotyped as described. Whereas only a minor change in the number of CD41-positive colonies was seen under rhIL-6 (from 2 to 6/10^5 PB MNC), a profound increment (from 3 to 21/10^5 PB MNC) in such colonies was observed in the animal treated with 33 μg/kg/d of rhIL-3.

In two animals receiving a daily dose of 33 μg/kg rhIL-3 and in one monkey treated with 30 μg/kg rhIL-6, BM CFU-MK were also determined. As shown in Table 2, a moderate increase in the concentration of BM CFU-Mk was found in both animals receiving rhIL-3, reaching significance (P < .05 by the Student’s test) in one of them (no. 1). No increment in the concentration of BM CFU-Mk was seen in the animal treated with rhIL-6.

**Effect of recombinant cytokines on PB platelet counts.** As shown in Fig 4A, no consistent changes in platelet counts were observed in the five rhesus monkeys receiving 33 μg/kg/d of rhIL-3 (mean pretreatment value, 350 × 10^9/...
rhIL-3 and rhIL-6 sequentially, no consistent synergistic effect on WBC or RBC counts was observed as it has been seen for platelet counts. Table 3 also shows that the stimulatory effect of rhIL-3 on the progenitor cell compartment was not restricted to CFU-Mk but also induced marked increases in numbers of circulating CFU-GM. In contrast, rhIL-6 had no significant effect on PB CFU-GM counts. In animals receiving rhIL-3 and rhIL-6 sequentially, blood CFU-GM levels increased after rhIL-3 but fell back under the administration of rhIL-6.

Effect of recombinant cytokines on nonhematologic parameters. Most monkeys receiving rhIL-3 developed transient urticaria-like skin lesions but no other toxicity was seen by clinical or laboratory examination. Rhesus monkeys receiving rhIL-6 appeared clinically normal, although showing significant increases in serum levels of positive acute-phase reactants (C-reactive protein from undetectable levels to detectable levels; α-antitrypsin by 3-fold; haptoglobin by 4-fold; ceruloplasmin by 3.3-fold; fibrinogen by 1.8-fold) and decreases of negative acute-phase reactants (prealbumin by 2-fold). Serum levels of IgG in these animals remained unchanged.

Serum antibodies against rhIL-3 and rhIL-6. All mon-
keys receiving rhIL-3 and/or rhIL-6 developed IgG antibodies against the respective human protein. Serum antibodies became detectable by day 10 of rhIL-3 treatment and by day 8 of rhIL-6 treatment, respectively. The sera containing antibodies had at least partial (30% to 90%) neutralizing activity as determined in bioassays by reduction of thymidine incorporation by the appropriate target cell population (CML myeloblast assay for anti-IL-3 antibodies, B9 cell line for anti-IL-6 antibodies). Despite the development of anti-IL-6 antibodies in rhIL-6-treated monkeys, thrombocytosis was observed in all of them.

**DISCUSSION**

We show that rhIL-3 potentiates the thrombopoietic effect of rhIL-6 in rhesus monkeys. The synergistic effect of rhIL-3 pretreatment on rhIL-6–induced thrombocytosis might be due to the stimulation of the early phase of thrombopoiesis by rhIL-3. Here we demonstrate that rhIL-3 markedly expands the pool of megakaryocytic progenitors in primates, predominantly in the peripheral blood, and an increment in the number of BM megakaryocytes in some patients receiving rhIL-3 has been reported recently. The in vivo stimulatory effect of rhIL-3 on the CFU-Mk compartment is not unexpected because rhIL-3 is also an excellent stimulator of megakaryocyte colony formation from BM cells in semisolid cultures. Despite its effect on the CFU-Mk compartment, rhIL-3 did not consistently increase platelet counts in rhesus monkeys. Thus, rhIL-3 apparently lacks significant stimulatory activity on the terminal phase of platelet production. In contrast, such “thrombopoietin-like” activity was observed with rhIL-6, which caused a dose-dependent increase of blood platelets but failed to expand the pool of megakaryocytic progenitors. In mice, rhIL-6 increased CFU-Mk numbers in some studies but did not do so in other reports. An action on a rather late than early phase of thrombopoiesis and maturation promoting effect by rhIL-6 in rhesus monkeys is also suggested by the observation that animals receiving rhIL-6 showed a marked increment in the size but not in the number of megakaryocytes in their BM biopsies.

From our study we cannot exclude that the in vivo effects of rhIL-3 and/or rhIL-6 on thrombopoiesis are mediated by the release of other cytokines, but at least some direct effects are likely. Recently, the presence of IL-6 receptors on human megakaryocytes was demonstrated by in situ hybridization. The observation that IL-3 can induce megakaryocyte colony formation from enriched bone marrow progenitors also suggests a direct effect on the CFU-Mk.

Both cytokines were well tolerated at dosage and duration used in this study. Although marked basophilia may occur during rhIL-3 administration, no serious allergic side effects were yet observed in monkeys or humans. Major systemic effects resembling Castleman’s disease have been reported in congenitally anemic W/W mice reconstituted with hematopoietic cells transfected with the coding sequences of murine IL-6. The relatively brief administration of rhIL-6 in this study clearly induced changes in the profile of acute-phase reactants and caused mild anemia but did not increase IgG levels in primates. Side effects due to increased platelet activation were not observed in our animals. In vitro we were unable to find any stimulatory effect of rhIL-6 on adenosine diphosphate or collagen-induced platelet-aggregation or P-thromboglobulin release from human platelets (P.K., B.W., unpublished data, 1991). Thus, rhIL-6 seems to promote platelet production but apparently does not affect mature platelets.

Interestingly, platelet counts began to decrease before the end of rhIL-6 administration, which could be due to neutralization of the biologic effects of rhIL-6 by anti-rhIL-6 antibodies. Antibody production against human proteins has been observed with most recombinant cytokines used in monkey models. In fact, all rhIL-6–treated animals in this study developed substantial titers of anti-IL-6 antibodies in the second week of treatment, showing some neutralizing activity in the respective bioassay. The fact that these neutralizing antibodies did not cause thrombocytopenia in animals may suggest that IL-6 is not required for steady-state thrombopoiesis, but may play a role in situations of hematopoietic stress.

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**Table 3. Effects of rhIL-3 and rhIL-6 Alone or in Combination on Other Hematologic Parameters**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WBC 10^3/μL</th>
<th>RBC 10^6/μL</th>
<th>PB CFU-GM/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>8.5 ± 2.3</td>
<td>6.1 ± 0.3</td>
<td>193 ± 153</td>
</tr>
<tr>
<td>rhIL-3 33 μg (n = 5)</td>
<td>10.8 ± 2.2</td>
<td>6.5 ± 0.5</td>
<td>384 ± 228</td>
</tr>
<tr>
<td>rhIL-6 30 μg (n = 4)</td>
<td>8.1 ± 1.5</td>
<td>5.8 ± 0.4</td>
<td>340 ± 151</td>
</tr>
<tr>
<td>Animal 14</td>
<td>10.9</td>
<td>5.3</td>
<td>283</td>
</tr>
<tr>
<td>Animal 15</td>
<td>16.2</td>
<td>4.2</td>
<td>919</td>
</tr>
<tr>
<td>rhIL-3 33 μg</td>
<td>10.8</td>
<td>6.5</td>
<td>155</td>
</tr>
<tr>
<td>rhIL-6 30 μg</td>
<td>14.7</td>
<td>5.1</td>
<td>245</td>
</tr>
</tbody>
</table>

Five rhesus monkeys received 33 μg/kg/d of rhIL-3 for 11 to 14 days, four animals received 30 μg/kg/d of rhIL-6 for 10 days, and two monkeys (nos. 14 and 15) were treated with 33 μg/kg/d of rhIL-3 for 11 days and subsequently received 30 μg/kg/d of rhIL-6 for another 10 days. Values are given before (Pre) and at the end (Post) of cytokine treatment. The Student’s test for paired samples was used for statistical analysis.
Our results provide further evidence that combinations of rh colony-stimulating factors (rhCSFs) may be most effective in stimulating hematopoiesis in vivo. Due to its profound stimulatory effect on early hematopoiesis, rhIL-3 seems to prime the hematopoietic system to be more responsive to later-acting and more lineage-restricted cytokines, leading eventually to maximal generation of mature blood cells of various lineages. Considering this concept it is not surprising that the sequential administration of rhCSFs has been shown to be superior to the simultaneous administration. In fact, no major synergistic effect on thrombopoiesis has been observed if rhIL-3 and rhIL-6 were administered simultaneously to monkeys (K. Welte, personal communication, 1991). Optimal in vivo stimulation of myelopoiesis and erythropoiesis has been already achieved with the sequential application of rhIL-3 and rhGM-CSF and rhIL-3 and rh erythropoietin, respectively. The sequential administration of rhIL-3 and rhIL-6 might represent a novel and powerful strategy to stimulate thrombopoiesis in vivo, which may have therapeutic potential in conditions with decreased platelet production.

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REFERENCES

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