Effects of Interleukin-3 and Erythropoietin on In Vivo Erythropoiesis and F-Cell Formation in Primates

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To test the in vivo cooperativity between interleukin-3 (IL-3) and erythropoietin (Epo) in stimulating erythropoiesis and hemoglobin F (HbF) production in primates, we administered recombinant human IL-3 and recombinant human Epo to baboons and macaques. The effect of these treatments was assessed by serial bone marrow cultures and by measuring HbF production in the progeny of bone marrow progenitors and in peripheral-blood reticulocytes. Administration of IL-3 alone to hematologically normal or anemic baboons produced an early increase in erythroid colony-forming units (CFUe) and erythroid clusters (e-clusters) and precursors with an increase in reticulocyte counts and a late increment in the relative frequency of erythroid burst-forming units (BFUe).

In parallel to the increase in peripheral-blood reticulocytes, IL-3 increased the frequency of F reticulocytes in the normal and anemic animals. When administration of IL-3 was followed by administration of Epo, expansion in all classes of erythroid progenitors and increase in reticulocytes occurred, beyond the levels observed when the animals were treated with Epo alone. The combination of IL-3 and Epo, however, did not increase consistently the rate of F reticulocytes beyond the level induced by Epo alone. These results suggest that IL-3 enhances the effect of Epo on erythropoiesis, but the combination of the two growth factors does not lead to a preferential and significant enhancement of HbF production.

MATERIALS AND METHODS

Two baboons (Papio cynocephalus) and two macaques (Macaca fascicularis), housed in the Regional Primate Research Center of the University of Washington, were used in this study. Purified recombinant human Epo (specific activity, 2.2 × 10^5 IU/mg; Genetics Institute, Cambridge, MA) was diluted in 5% glucose solution with 0.05% human serum albumin and administered with intravenous injection of 3,000 IU/kg at 12-hour intervals. Purified recombinant human IL-3 (specific activity, 1.2 × 10^5 IU/mg; Genentech Institute, Cambridge, MA) was diluted with isotonic saline solution containing 0.05% human serum albumin and administered subcutaneously at eight-hour intervals for a total daily dose of 20 μg per kilogram of body weight.

Bone marrow mononuclear cells were obtained after centrifugation on a cushion of Hypaque-Ficoll (Nygaard, Oslo, Norway). Assays of CFUe and e-clusters were done in plasma clots containing 1 mL of Iscove’s medium (GIBCO Labs, Grand Island, NY), 30% fetal calf serum (Armour Pharmaceutical Co., Tarrytown, NY), 1% bovine serum albumin (Armour Pharmaceutical), 10% human AB serum, 10^{-4} mol/L 2-mercaptoethanol, 10% beef embryo extract (GIBCO), 10% Mo-cell conditioned medium (a kind gift from Dr. David Golde, UCLA), 0.2 IU/mL recombinant human Epo (Genetech).
ics Institute), and 10% bovine citrated plasma (Irvine Scientific, Santa Ana, CA). Cultures were evaluated after 3 days of incubation at 37°C with 5% CO₂ at high humidity. Colonies with eight or more cells were counted, after benzidine staining, as CFUe-derived colonies, and aggregates of three to seven cells were counted as e-cluster-derived colonies. Evaluations of BFUe and granulocyte-macrophage colony-forming unit (CFU-GM) were carried out in 0.9% methylcellulose (Fisher Scientific, Springfield, NJ) cultures¹⁰,¹¹ with Iscove's medium, 30% fetal calf serum, 1% bovine serum albumin, 10% human AB plasma, 2 × 10⁻⁴ M 2-mercaptoethanol, 10% Mo-cell conditioned medium, and 2 IU/mL recombinant human Epo. Cultures were incubated at 37°C with 5% CO₂ at high humidity for 10 to 12 days. Hemoglobinized red colonies were counted as BFUe-derived colonies, and non-hemoglobinized colonies with 40 or more cells as CFU-GM-derived colonies, under a dissecting microscope.

**RESULTS**

**Effects of IL-3 on in vivo erythropoiesis.** Effects of IL-3 on in vivo erythropoiesis were assessed by treating baboons with 20 μg/kg per day (administered subcutaneously in three equal doses) for five days and quantitating the effects on peripheral blood and on progenitor-cell pools. The latter were assessed by measuring the relative numbers of progenitor cells in cultures of bone marrow samples obtained on treatment days 0, 5, 8, 11, and 14.

As shown in Fig 1, in response to IL-3, CFUe increased by 4.2-fold and e-clusters by 1.6-fold at post-treatment day 5; both types of progenitors returned to pretreatment levels by day 8. The increase in late progenitors was followed by an increase in reticulocytes, which peaked on day 9. BFUe and CFU-GM increased slowly and reached maximum levels by day 11. Six other experiments of administration of IL-3 in baboons or macaques provided similar results (Fig 2). Since administration of IL-3 induced myeloid hyperplasia (granulocyte/erythroid [G:E] ratios were changed from 2:1 before treatment to 7:1 on treatment days 3 to 5) and since our measurements of progenitor-cell numbers are expressed per 10⁸ inoculated bone marrow cells, the observed increase of e-clusters and CFUe should be considered an underestimate of the real expansion of late progenitors under the influence of IL-3.

**Cooperation of IL-3 and erythropoietin in vivo.** To test whether IL-3 and Epo act cooperatively on erythroid progenitors, animals were pretreated for five days with IL-3 and subsequently given Epo. Effects on progenitor-cell pools were compared with those obtained when the same animals were treated only with Epo.

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**Fig 1.** Effects of IL-3 on in vivo erythropoiesis. A normal animal received IL-3 (20 mg/kg per day in three subcutaneous injections) on the days indicated by the arrows. In response to this schedule of treatment, leukocytes, eosinophils, and platelets increased as described previously in monkeys.¹⁵ As shown in the upper two panels, in response to administration of IL-3, reticulocytes as well as F reticulocytes increased and reached maximal levels by day 8 or 9 (i.e., four to five days from the end of administration of IL-3). Notice that IL-3 increased CFUe (A) and e-clusters (B) and produced a late increment in BFUe (C) and CFU-GM (D).

**Fig 2.** Comparisons of the numbers of e-clusters and CFUe colonies in baboons and macaques before (a) and after (b) administration of IL-3. All animals received a five-day course of IL-3, consisting of three subcutaneous injections (eight hours apart) of IL-3 for a total dose of 20 μg/kg per day. Notice the increase in numbers of late erythroid progenitors after administration of IL-3.
A single-day administration of Epo (3,000 IU/kg x 2) expanded c-clusters by 7.5-fold and CFUe by 4.1-fold (Fig 3A). When five days of IL-3 preceded the administration of Epo, c-clusters expanded by 19.4-fold and CFUe by 5.4-fold (Fig 3B). The induction of erythropoiesis by the two hematopoietins was also reflected in the increase in reticulocyte output (Fig 3).

Administration of Epo (3,000 IU/kg x 2 per day) for three days increased CFUe by 7.7-fold and c-clusters by 9.9-fold (Fig 4A). When five days of IL-3 treatment preceded the three days of Epo, c-clusters increased by 20.7-fold and CFUc by 11.7-fold (Fig 4B). This combination treatment also resulted in an increase of total reticulocyte output (Fig 4). The combination of IL-3 and Epo also resulted in the expansion of BFUc and CFU-GM beyond the levels attained by Epo alone (Figs 3 and 4).

To examine the effect of IL-3 on a chronically expanded erythropoiesis and in the presence of chronically increased levels of Epo, we administered IL-3 to a baboon kept chronically anemic (hematocrit, 26% to 28%) with frequent phlebotomies. Administration of IL-3 increased CFUe by 2.0-fold, e-clusters by 2.3-fold, and reticulocytes by 1.5-fold. The numbers of BFUe and CFU-GM of this anemic baboon increased by about 1.8-fold (Fig 5).

**Effect on HbF expression.** In parallel to the induction of reticulocytes, IL-3 increased F reticulocytes by 1.8-fold in the anemic animals and 2.8-fold in the non-anemic animals (Figs 1 and 2). To examine whether IL-3 influences fetal globin production in colonies formed by late erythroid progenitors, CFUe and e-cluster colonies were analyzed for HbF expression after labeling with anti-γ chain monoclonal antibodies. There was no consistent change in the frequency of F-positive e-clusters following IL-3 treatment, but there was an increase in the frequency of F-positive CFUe in five of six experiments (data not shown).

The combination of IL-3 and Epo did not increase the frequency of F-positive CFUe or e-clusters beyond the very high levels already attained following treatment with Epo.
alone (data not shown). Since, however, treatment with IL-3 plus Epo increased the absolute numbers of progenitors above the levels reached by Epo alone (see Figs 3 and 4), the absolute number of F-positive erythroid progenitors following IL-3 plus Epo was higher than that obtained following treatment with Epo alone. Animals receiving the combination of IL-3 and Epo had an absolute increase in F reticulocytes above that achieved with Epo alone (Fig 6). The effect of the combination IL-3 plus Epo on the percentage of F reticulocytes, however, was inconsistent. While IL-3 plus one day of Epo increased the percentage of F reticulocytes above the levels induced by Epo alone (Fig 3), the combination of IL-3 plus three days of Epo did not (Fig 4).

**DISCUSSION**

Our results show that IL-3 augments the erythropoiesis of the normal and the anemic baboon and acts synergistically with Epo in achieving further erythropoietic expansion. This is consistent with in vitro observations that recombinant human IL-3 is capable of acting on erythroid progenitors (appearing to be superior to GM-CSF in this capacity) and the earlier in vivo evidence in primates that IL-3 and GM-CSF can induce reticulocytosis. Our results, similarly, are in agreement with previous studies in mice showing expansion of early as well as late erythroid progenitors in the spleens of IL-3-treated animals.

Although it is well established that IL-3 stimulates proliferation of BFUe, the mechanism whereby this hemopoietin leads to an early expansion of late, Epo-dependent erythroid progenitors is unclear. Since CFUe and e-clusters lack the capacity for self-renewal, the expansion of late erythroid progenitors in response to IL-3 should represent influx from the pool of BFUe. Further maturation of the expanded CFUe and e-cluster pools should reflect action of ambient levels of Epo present in the treated animal. Whether IL-3 has a direct effect on late stages of erythroid maturation remains to be determined.

The capacity of IL-3 to expand erythropoiesis is best illustrated in the combination treatments using IL-3 and Epo. In these experiments, the five days of administration of IL-3 were followed by one or three days of administration of Epo, with the expectation that IL-3 would expand the population of target cells on which Epo acts. The sequential administration of IL-3 and Epo increased both early and late erythroid progenitors to levels substantially higher than those obtained when the animals were treated with Epo alone. The net outcome of the combined treatment was a significant increase in total reticulocyte output. Such results were expected in view of previous findings in clonal human cultures and in liquid cultures of BFUe-enriched populations, which showed that maximal expansion of erythroid clusters and CFUe is achieved when both IL-3 and Epo are present in culture. These results add to the growing evidence that IL-3 acts synergistically with lineage-restricted hemopoietins in producing expansions of committed progenitors of various hemopoietic lineages in vitro or in vivo.

Treatment of baboons with pulses of high doses (1,500 to 3,000 IU per injection) of Epo results in reproducible waves of F reticulocytes which peak four to six days from the onset of treatment. F-reticulocyte increase is preceded by a significant expansion of F-programmed late erythroid progenitors, peaking three days before maximum F-reticulocyte output. Assuming that Epo-induced expansion of late erythroid progenitors is due to mobilization from BFUe, BFUe influx and fast downstream differentiation might be the reason for the induction of F-reticulocyte production by erythropoietin.

In view of these hypotheses, we wanted to test whether administration of IL-3, a hemopoietin known to influence BFUe proliferation, would enhance the effect of erythropoie-
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Fig 6. Comparisons of the absolute numbers of reticulocytes and $\alpha$-reticulocytes after administration of IL-3 alone, Epo alone, or combinations of the two hemopoietins. Note the significant increase in absolute reticulocytes following the combination of IL-3 plus Epo.

References


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