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Human IL-3 and GM-CSF Act Synergistically in Stimulating Hematopoiesis in Primates

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Interleukin-3 (IL-3) is a member of a family of growth factors, each of which supports the proliferation and development of hematopoietic precursors in culture. Although the biologic effects of the different hematopoietic growth factors have been well documented in different culture systems, it has only recently become possible to study the activities of these molecules in vivo. In comparison with the later acting hematopoietic growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor, IL-3 elicited a delayed and relatively modest leukocytosis when continuously infused intravenously in primates. The IL-3 infusion, however, greatly potentiated the responsiveness of the animal to subsequent administration of a low dose of GM-CSF. These results suggest that IL-3 expands an early cell population in vivo that subsequently requires the action of a later acting factor such as GM-CSF to complete its development. Optimal stimulation of hematopoiesis may be achieved with combinations of hematopoietic growth factors.

INTERLEUKIN-3 (IL-3) (*1*), ALSO known as multilineage colony-stimulating factor (multi-CSF), is a member of a complex network of interactive cytokines that play an important role in regulating the hematopoietic and immune systems (*2*). Previous studies have shown that IL-3 interacts with early progenitor cells common to most if not all of the myeloid cell lineages (*3–7*). We, as well as others, have reported on the effects of GM-CSF (*8, 9*) and granulocyte colony-stimulating factor (G-CSF) (*10*) in nonhuman primates. We now report the effects of IL-3 alone and in combination with GM-CSF on hematopoiesis.

Continuous intravenous infusion of recombinant human IL-3 expressed either in mammalian cells or bacteria, at a rate of 20 $\mu g\ kg^{-1}\ day^{-1}$, elicited a modest and delayed leukocytosis in normal macaques (Fig. 1). Typically, the white cell counts increased gradually during the 7-day infusion from a mean baseline of 7,750 cells per microliter (range 6,000 to 8,000) to a mean maximum of 17,000 (range 12,000 to 26,000), 1 to 3 days after termination of the treatment in four animals. The increase in leukocyte count was predominantly due to increased levels of neutrophils, eosinophils, and lymphocytes, and an unusual population of leukocytes containing toluidine blue–staining granules (Fig. 2). Although toluidine blue staining is characteristic of normal basophilic granulocytes, the morphology of these cells is atypical in that they are hypogranulated and have a more diffuse chroma-

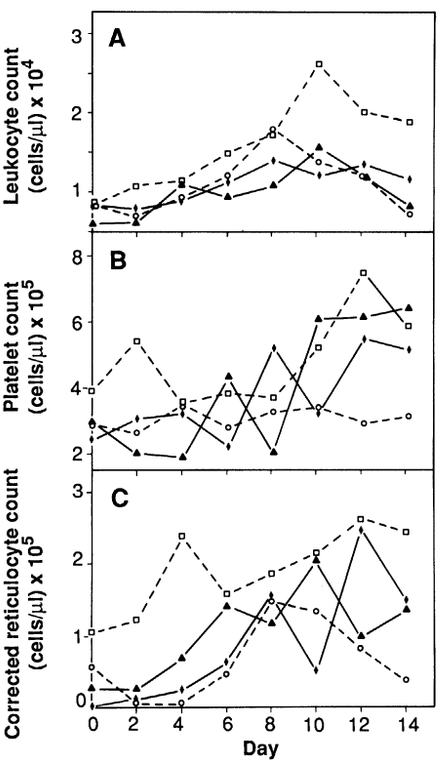

Fig. 1. Effects of continuous infusion of human IL-3 on the levels of circulating blood cells in the cynomolgus macaque (*Macaca fasicularis*). The IL-3 was continuously infused intravenously for 7 days in four different animals (*21*). Daily blood samples were drawn for complete and differential blood cell count analysis. The results from the sample drawn immediately before the infusion was started are indicated as day 0. The data represent the actual values for (**A**) complete white blood cell, (**B**) platelet, and (**C**) corrected reticulocyte counts for four different animals, each infused with IL-3 at the rate of 20 $\mu g\ kg^{-1}\ day^{-1}$.

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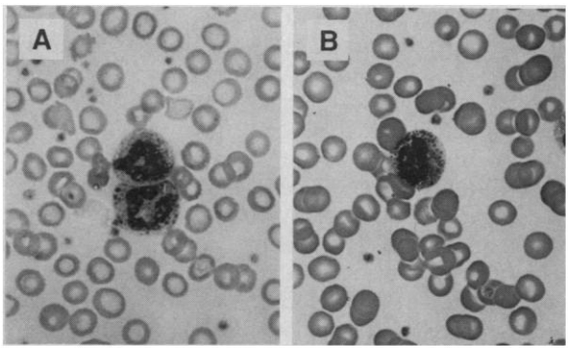

Fig. 2. (**A**) The atypical basophils from blood smears of IL-3–treated animals, were stained with Wright-Giemsa and photographed (×100). These cells also stained positively with toluidine blue. (**B**) The typical basophil was similarly stained and photographed.

tin pattern in comparison with normal basophils. Although human IL-3 has been found in vitro to support the proliferation of mixed colonies containing basophilic granulocytes (*6*), the origin of the atypical basophils in the primates remains to be determined. Within the lineages that were most consistently expanded by IL-3 treatment (in four of the four animals of Fig. 1), the eosinophil levels increased from a mean baseline of 350 per microliter (range 150 to 700) to a mean maximum of 2100 per microliter (range 1400 to 3700); the lymphocyte levels increased from a mean baseline of 3700 (range 2500 to 4200) to a mean maximum of 6000 (range 4900 to 8100); and the atypical basophils increased from less than 100 per microliter to a mean maximum of 1850 (range 500 to 3700).

We have also observed consistent increases in the corrected reticulocyte counts and occasional increases in platelet counts (Fig. 1, B and C). All animals in this study experienced a modest fall in hemoglobin (from a mean of 11.6 g/dl, range 11.3 to 12.2 g/dl before the study to a mean of 10.2 g/dl, range 9.9 to 10.5 g/dl on day 14) and hematocrit levels (from a mean of 40.9%, range 37.3% to 41.5% before the study to a mean of 37.8%, range 36.4% to 39.1% on day 7; the hematocrits had returned to normal by day 14), with the red blood cells becoming slightly macrocytic, perhaps because of repeated bleeding. To minimize the effects of repeated bleeding, the reticulocyte counts were corrected by multiplying the actual counts by the ratio of the hemoglobin values on each day to the value at the start of the infusion. For the group receiving 20 μg kg^{-1} day^{-1}, all four individuals and four of the five in a lower dosage group (10 μg kg^{-1} day^{-1}) experienced increases in corrected reticulocyte counts (mean baseline 60,000, range 25,000 to 100,000, to a mean maximum of 175,000, range 80,000 to 260,000). At the higher dosage, three of four animals also displayed an increase in platelet count (for the group, the mean baseline value of 310,000 per microliter, range 240,000 to 390,000, to a mean maximum of 550,000, range 300,000 to 740,000). When IL-3 was tested at a lower dose (10 μg kg^{-1} day^{-1}), only one of five animals had an increase in platelet count (*11*).

The continuous administration of IL-3 was well tolerated, and no systemic toxicity was observed over the dosage range studied (5 to 50 μg kg^{-1} day^{-1}); circulating liver and kidney enzyme levels remained unaltered. However, the four animals that received IL-3 at the rate of 20 μg kg^{-1} day^{-1} developed modest increases in serum levels of the acute phase reactants α_1-antitrypsin (from a mean of 33% of normal values, range 25 to 40% before the study to a mean of 51% of normal on day 7), orosomucoid (from a mean of 47% of normal, range 22 to 75% before the study to a mean of 80% of normal; range 51 to 107% on day 7), and haptoglobin (from a mean of 207 mg/dl, range 160 to 254 mg/dl before the study, to a mean of 287 mg/dl, range 253 to 320 mg/dl on day 7), without evidence of fever. The levels of all of the acute phase reactants declined to normal values by day 14.

The low magnitude of the IL-3–induced leukocytosis and the delayed response time when compared with the effects of either G-CSF or GM-CSF suggested that IL-3 alone may expand the number of early progenitors but is not sufficient for generating mature cells that are released into the periphery. To test this possibility, we examined the effect of IL-3 administration on the ability of GM-CSF to elicit leukocytosis in primates. In these experiments, IL-3 was administered continuously for 7 days (20 μg kg^{-1} day^{-1}) before the infusion of a low dose of GM-CSF (2 μg kg^{-1} day^{-1}) for 4 days (Fig. 3C). The low-dose GM-CSF treatment elicited a dramatic leukocytosis in the IL-3–treated animal far greater than achieved with this dose of IL-3 alone (Fig. 3A) or with GM-CSF alone (Fig. 3B). The leukocytosis resulted from substantial increases in the levels of neutrophils, banded neutrophils, eosinophils, lymphocytes, monocytes, and basophils. As has been observed with higher doses of GM-CSF (*8, 9*), the leukocytosis resolved within 3 days after termination of the infusion. In one experiment in which the order of administration of the IL-3 and GM-CSF was reversed, the magnitude of the resulting leukocytosis was not different from that achieved with IL-3 alone (*11*). These studies provided further evidence that IL-3 acts predominantly on earlier populations of hematopoietic progenitors than does GM-CSF.

To test the duration of the IL-3–induced enhancement of the responsiveness to GM-CSF, we continuously infused the low dose of GM-CSF to an animal for 14 days after a 7-day treatment with IL-3 (Fig. 4). We expected that as the population of IL-3–stimulated cells became depleted during the GM-CSF administration, the peripheral white cell count would gradually return to the level achievable with GM-CSF alone

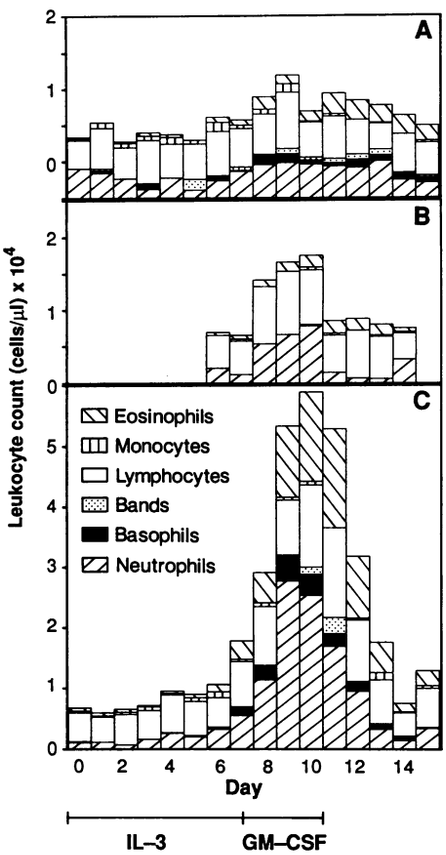

Fig. 3. IL-3 enhancement of the hematopoietic stimulatory effects of GM-CSF. The same individual was (**A**) administered IL-3 alone (20 μg kg^{-1} day^{-1}) for 7 days; (**B**) GM-CSF alone for 4 days (2 μg kg^{-1} day^{-1}); or (**C**) IL-3 (20 μg kg^{-1} day^{-1}) on days 1 through 7 followed by GM-CSF (2 μg kg^{-1} day^{-1}) on days 7 through 10. The animal was rested for at least 14 days between each set of infusions. The preparation of mammalian cell–derived human GM-CSF (*8*) was pyrogen-free and had a specific activity of 1.2×10^7 U/mg measured in the CML proliferation assay (*1*). The differential cell counts were performed on daily blood samples (*21*).

(about 20,000 cells per microliter). However, the leukocytosis was maintained at 45,000 cells per microliter or above for the duration of the GM-CSF administration (Fig. 4A), demonstrating that the treatment with IL-3 primed the hematopoietic system to respond to GM-CSF for at least 14 days. There were also increases in the platelet and corrected reticulocyte counts of this animal (Fig. 4, B and C).

The inability of IL-3 alone to elicit a substantial leukocytosis in primates is consistent with many of the biologic effects of this cytokine in different culture systems. For example, IL-3 has been more effective than GM-CSF in supporting colony formation by multipotential blast cells, supporting the hypothesis that the targets of IL-3 include more primitive progenitors than those of GM-CSF (*6*). In addition, Sonada *et al.* (*12*) found that IL-3 alone in serum-free culture is ineffective in supporting colony formation and that stimulation by a second late-acting growth factor such as G-CSF or erythropoietin is also required to yield colonies. Detailed mapping analysis of the IL-3–containing cultures revealed that IL-3 alone supports several divisions of the progenitor cells but the resulting cluster of cells rapidly regresses and disappears in the absence of the second growth factor. This synergy between growth factors has also been observed in serum-containing cultures: IL-3 acts synergistically with IL-6 in support of early blast cell colony formation (*13, 14*), with G-CSF in support of neutrophilic granulocyte colony formation (7), and with GM-CSF in support of both granulocyte and macrophage colony formation (7). Lopez *et al.* (*3*) reported that mature eosinophils but not neutrophils can be activated by exposure to IL-3, providing further evidence that IL-3 responsiveness is lost during neutrophil differentiation. Although eosinophils retain IL-3 responsiveness, these cells also appear to grow optimally in the presence of both IL-3 and IL-5 (*15*), thereby implicating multiple factors in the development of cells within this lineage. These results are consistent with a requirement for the participation of several factors for supporting the complete developmental program of early hematopoietic progenitors in the primate system.

Although it has been difficult to compare hematopoiesis in primates and rodents directly, the effects of IL-3 in the mouse have been similar to our results in monkeys. Daily intraperitoneal injection of IL-3 in mice had a relatively small effect on the levels of circulating blood cells, with the most consistent increases observed in the numbers of circulating granulocytes (*16–18*). In normal mice, the IL-3 administration had little effect on the cellularity and progenitor numbers in the bone marrow, but increases were noted both in the spleen weight and the frequency of erythroid and myeloid progenitors in the spleen. These effects were greatly amplified in irradiated mice in which splenic progenitor cell numbers were greater in animals treated with IL-3 than in untreated controls (*16*). One of the major effects of IL-3 administration in normal mice was the appearance in the spleen of large numbers of small, incompletely granulated mast cells (*17*). These cells were not found in the circulation of the mice, but it will be of interest to determine whether they are related to basophils that we have observed in the peripheral blood from monkeys treated with IL-3. In short-term experiments, combinations of IL-3, GM-CSF, and M-CSF were more effective than the individual factors in inducing hematopoietic progenitor cells to cycle as well as in increasing the absolute numbers of colony-forming cells in the spleens of lactoferrin-treated mice (*19*).

These results provide further evidence that combinations of CSFs may be most effective in stimulating hematopoiesis in vivo, an expectation supported by our studies with IL-3 and GM-CSF in normal cynomolgus macaques. It will be interesting to test these CSFs in combination with IL-1 or IL-6, cytokines that act synergistically in culture in supporting the growth of primitive, multilineage hematopoietic progenitor cells (*14, 20*). The ability to analyze the hematopoietic effect of the different recombinant cytokines singly and in combination in primates will provide insight into the mechanisms regulating blood cell production and provide information for designing novel therapies for many clinical situations.

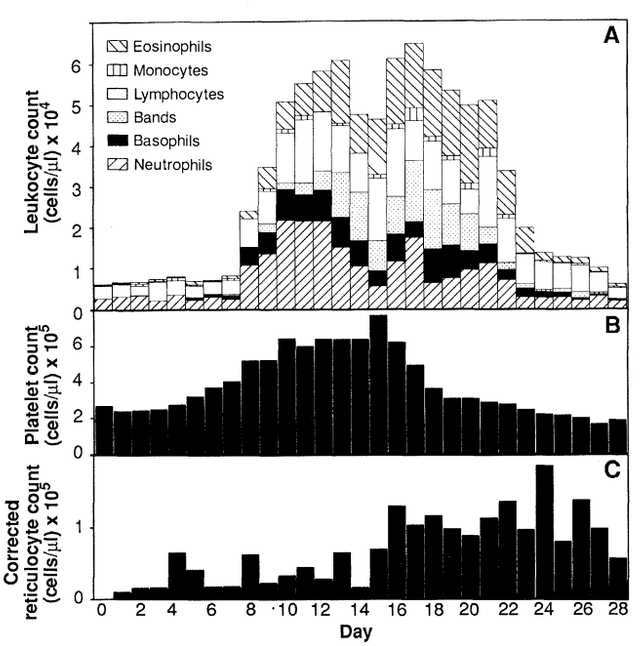

Fig. 4. Serial (**A**) differential cell, (**B**) platelet, and (**C**) corrected reticulocyte counts of a healthy cynomolgus macaque that was infused with IL-3 (10 μg kg^{-1} day^{-1}) for 7 days, followed immediately by GM-CSF (2 μg kg^{-1} day^{-1}) for 14 days (*21*). Blood samples were drawn daily during the infusion and for 7 days after termination of the GM-CSF treatment. Similar results have been obtained with three other animals with IL-3 doses between 5 and 50 μg kg^{-1} day^{-1}.

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21. Indwelling intravenous Broviac catheters (Davol Evermed) were placed in either the iliac or jugular veins of the animals. The protein solutions were infused at a constant rate of 0.25 ml/hour with a battery-operated continuous infusion pump (Abbott Laboratories). Daily blood samples (1 ml) were drawn after anesthetizing the animal with ketamine hydrochloride (Bristol Laboratories) and were submitted in EDTA for serial blood counts. On days 0, 7, and 14, 10 ml of blood were drawn and serum was collected for routine serum biochemistry (Bioran, Cambridge, MA, or Metpath, Teterboro, NJ) and serum protein analysis (Center for Blood Research, Boston, MA). The total volume of blood drawn during the experiment represented approximately 10% of the blood volume of the animal. The IL-3 [3×10^6 to 5×10^6 U/mg measured in the chronic myelogenous leukemia (CML) blast proliferation assay (*1*)] was isolated from an

Escherichia coli strain in which the synthesis of IL-3 was driven by the bacteriophage λ pL promoter. The intercellularly expressed IL-3 was purified to homogeneity by reversed-phase high-performance liquid chromatography in the presence of urea and dithiothreitol. The biologically active protein was produced by dilution of the denaturant, addition of oxidized glutathione, and dialysis against decreasing concentrations of urea. The purified IL-3 was formulated in physiological saline, and the final preparation had less than 1 U of endotoxin per milligram as measured by the *Limulus* amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD).

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Developmental Expression of PDGF, TGF-α, and TGF-β Genes in Preimplantation Mouse Embryos

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Control of growth and differentiation during mammalian embryogenesis may be regulated by growth factors from embryonic or maternal sources. With the use of single-cell messenger RNA phenotyping, the simultaneous expression of growth factor transcripts in single or small numbers of preimplantation mouse embryos was examined. Transcripts for platelet-derived growth factor A chain (PDGF-A), transforming growth factor (TGF)–α, and TGF-β1, but not for four other growth factors, were found in whole blastocysts. TGF-α, TGF-β1, and PDGF antigens were detected in blastocysts by immunocytochemistry. Both PDGF-A and TGF-α were detected as maternal transcripts in the unfertilized ovulated oocyte, and again in blastocysts. TGF-β1 transcripts appeared only after fertilization. The expression of a subset of growth factors in mouse blastocysts suggests a role for these factors in the growth and differentiation of early mammalian embryos.

BECAUSE PREIMPLANTATION MOUSE embryos grow and differentiate in the absence of exogenous factors, endogenous factors must sustain the embryo during the first seven to eight cleavage divisions (*1*). Indirect evidence indicates that preimplantation embryos may make growth factors. Cultured embryos from around the time of implantation produce transforming growth factor–like bioactivity that promotes anchorage-independent growth (*2*). Teratocarcinoma cells, which are thought to be similar to primitive ectoderm, produce PDGF (*3*), insulin-like growth factor–II (IGF-II) (*4*), and three stem cell growth factors (*5*). After implantation in the uterus, mouse embryos produce the fibroblast growth factor homolog *int-2*, TGF-α, TGF-β (*6*), and IGF-II (*4*), but the presence of these growth factors cannot be extrapolated to the preimplantation embryo. Direct evidence for growth factor transcripts of low copy number has been impossible to obtain in preimplantation embryos, because thousands of embryos are required to detect even high copy number transcripts such as histone or actin by RNA blot analysis (*7*).

We recently developed a sensitive and quantitative method for assaying the accumulation of mRNA transcripts in small numbers of cells. Our procedure, single-cell RNA phenotyping (*8*), can detect mRNA in a single cell and in ≤10 RNA transcripts, and resolves threefold differences in input over two orders of magnitude. Thus, this method overcomes the difficulties inherent in the analysis of growth factor transcripts in early embryos. It consists of a microtechnique for isolation of total RNA from 1 to 100 mouse embryos, coupled with two enzymatic steps (*9*): reverse transcription (RT) and amplification of the transcribed cDNA in a polymerase chain reaction (PCR) (*10*). The positions of the target sequences for the transcripts are shown in Table 1. Fragments were selected for inclusion of restriction sites, identification by existing cDNA clones by DNA blot analysis, and/or inclusion of introns that would generate longer fragments if DNA or unprocessed RNA contaminated the reaction. When mouse cDNA sequences were not available, oligonucleotide sequences from other species were chosen to cover areas of conserved sequence with nondegenerate amino acids on the 3′ inside ends.

We detected β-actin RNA transcripts isolated from a single mouse blastocyst by RT-PCR; the signal became stronger when the number of PCR cycles was increased from 30 to 60 (*11*). We then used RT-PCR to detect growth factor transcripts in blastocysts and determined the growth factor mRNA phenotype of the blastocysts. Three growth factor genes, TGF-α, TGF-β1, and PDGF-A, were expressed in mouse blastocysts (Fig. 1A). The blastocysts were uncultured; thus expression was not induced by prolonged handling of embryos. The cDNA

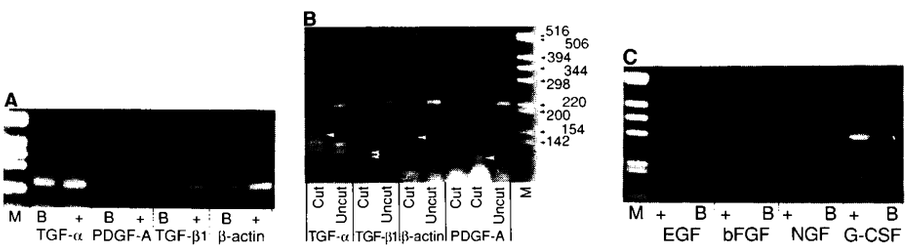

Fig. 1. Detection of mRNA transcripts in mouse blastocysts by RT-PCR. (**A**) Expression of three growth factor transcripts in mouse blastocysts demonstrated by agarose gel electrophoresis of the RT-PCR amplified reaction products obtained after 60 cycles of PCR. The PCR reaction mixtures for the blastocysts (B) contained cDNA from the RNA of 2.2 embryo equivalents in each reaction, and the PCR reaction mixtures for the positive controls (+) contained cDNA from 10 ng of total RNA (*9*). The molecular size markers (M) are Hae III–digested φX174 replicative form DNA. (**B**) Restriction enzyme analysis of cDNA fragments generated by RT-PCR. Messenger RNA transcripts were expressed by blastocysts incubated with the enzymes indicated in Table 1. The diagnostic fragments (arrowheads) are TGF-α, 159 bp; TGF-β1, 119 + 125 bp; β-actin, 151 bp; and PDGF-A, 129 bp. (**C**) Lack of expression of four growth factors in mouse blastocysts demonstrated by agarose gel electrophoresis of the RT-PCR amplified reaction products obtained after 60 cycles of PCR. The PCR reaction mixtures for blastocysts (B) contained cDNA from the mRNA of 2.2 embryo equivalents in each reaction, and the PCR reaction mixtures for the positive controls (+) contained cDNA from 10 to 100 ng of total RNA. The threshold for detection of the growth factors is generally <1 to 100 pg of total RNA; for NGF, as few as ten RNA molecules can be detected by RT-PCR (*8*).

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