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In Vivo Effect of Interleukin-6 on Cycling Status of Hematopoietic Progenitors from Adults and Neonates[1]

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ABSTRACT. ***In vitro*, IL-6 can induce hematopoietic progenitors to progress from G_0 into cycle, but a role for IL-6 in regulating cycling status of progenitors *in vivo* has not been established. In our studies, groups of five to six adult and newborn rats received i.v. injections of either IL-6 (1 ng/g body wt) or the vehicle (control), after which cycling of hematopoietic progenitors was evaluated by tritiated thymidine suicide. Progenitors from adult rats injected with the control had thymidine suicide rates of 7 ± 1% (mean ± SEM), compared with 23 ± 7% in the IL-6 recipients ($p < 0.02$). Progenitors from newborn rats injected with the control had thymidine suicide rates of 19 ± 2%, compared with 29 ± 1% in the IL-6 recipients ($p < 0.003$). In addition, IL-6 administration resulted in release of cells from the neutrophil storage pool into the circulation, as evidenced by fewer polymorphonuclear cells flushed from the long bones (neonates, $p < 0.001$; adults, $p < 0.003$), a rise in blood neutrophil concentration (neonates, $p < 0.001$; adults, $p < 0.05$), and a leukocyte "left shift" (neonates, $p < 0.001$; adults, $p < 0.01$). Thus, the effects of IL-6 *in vivo* in newborn and adult rats include cycle induction of hematopoietic progenitors and release of neutrophils from the storage pool into the circulation. (*Pediatr Res* 28: 323–326, 1990)**

Abbreviations

^{3}Htdr, tritiated thymidine
PMN, polymorphonuclear cell
MEM, minimum essential medium

Multipotent hematopoietic progenitors, obtained from the marrow of healthy adults, generally reside in either a slowly cycling or nonproliferating (G_0) state (1–8). Evidence supporting this interpretation includes the relative insensitivity of these progenitors to cycle-active cytotoxic agents (1–3), their relative lack of thymidine incorporation (1, 4, 5), and their prolonged survival in the absence of hematopoietic regulatory factors (8). In contrast, active cycling is observed in a substantial fraction of the multipotent progenitors obtained from the blood, liver, marrow, or spleen of fetal subjects (5, 9–11). The mechanisms that result in the induction of cycling in otherwise nonproliferating adult progenitors, and in maintenance of active cycling of fetal progenitors, are not completely known. Studies by Ikebuchi *et al.* (12, 13) and Leary *et al.* (14) demonstrated that, *in vitro*, IL-6 is capable of inducing cycling of hematopoietic progenitors from adult mice and adult humans. Similarly, studies by our group (15) demonstrated that IL-6 can accelerate cycling of fetal hematopoietic progenitors *in vitro*. However, the action of IL-6 upon cycling status *in vivo* has not been evaluated. Thus, we injected groups of adult and newborn rats with recombinant IL-6 or a vehicle control and assessed the cycling status of their hematopoietic progenitors.

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MATERIALS AND METHODS

Animals. Sprague-Dawley rats, weighing approximately 100 g, were obtained from Charles River Laboratories (Stone Ridge, NY). Groups of six animals received single i.v. injections (tail vein) of either IL-6 (1 ng/g body wt, in a volume of 4 μL/g body wt), or the same volume of the vehicle used to suspend the IL-6 (PBS containing 0.1% BSA). Before the injections, the tails were prepared by washing with a 10% povidine-iodine solution, followed by a wash with 70% isopropyl alcohol. Four h after the injections, the animals were killed by CO_2 inhalation, after which blood was drawn from the inferior vena cava and both tibias were removed.

Timed-pregnant Sprague-Dawley rats were allowed to deliver at the University of Utah Vivarium. Twenty-four to 48 h after delivery, groups of five to six pups, each weighing 5–8 g, received i.v. injections (tail vein) of either IL-6 (1 ng/g body wt, in 4 μL/g body wt), or the same volume of the vehicle used to suspend the IL-6 (16). Four h after the injections, pups were killed by CO_2 inhalation, after which blood was drawn from the internal jugular vein and the spleen and both femurs were removed.

Quantification of circulating and myeloid pools. Concentrations of nucleated cells in blood and bone marrow were determined electronically (Coulter Electronics, Hialeah, FL), after which differential counts (300–500 cells) were performed on Wright stained smears. Marrow cells were flushed from the two tibias in adult animals, and from the two femurs in neonatal animals, into α-MEM (HyClone, Logan, UT), using the method of Chervenick *et al.* (17). Cells that had a fine, filamentous strand separating the lobes of the nucleus were defined as PMN. Band neutrophils were defined as cells in which beginning segmentation was evident but a fine filament was not observed. Metamyelocytes were defined as cells in which nuclear segmentation was not evident and the diameter of the nuclear opening was ≥½ the diameter of the nucleus. Myelocytes were defined as cells with neutrophilic granulation in which the nuclear hole had a diameter <½ the diameter of the nucleus. Promyelocytes were defined as cells with azurophilic granulation in which a small nuclear opening might or might not be evident. Myeloblasts were defined as cells with fine nuclear chromatin, without a nuclear opening or cytoplasmic granules (17). The circulating leukocyte "left shift" was quantified by the ratio of immature neutrophils (band

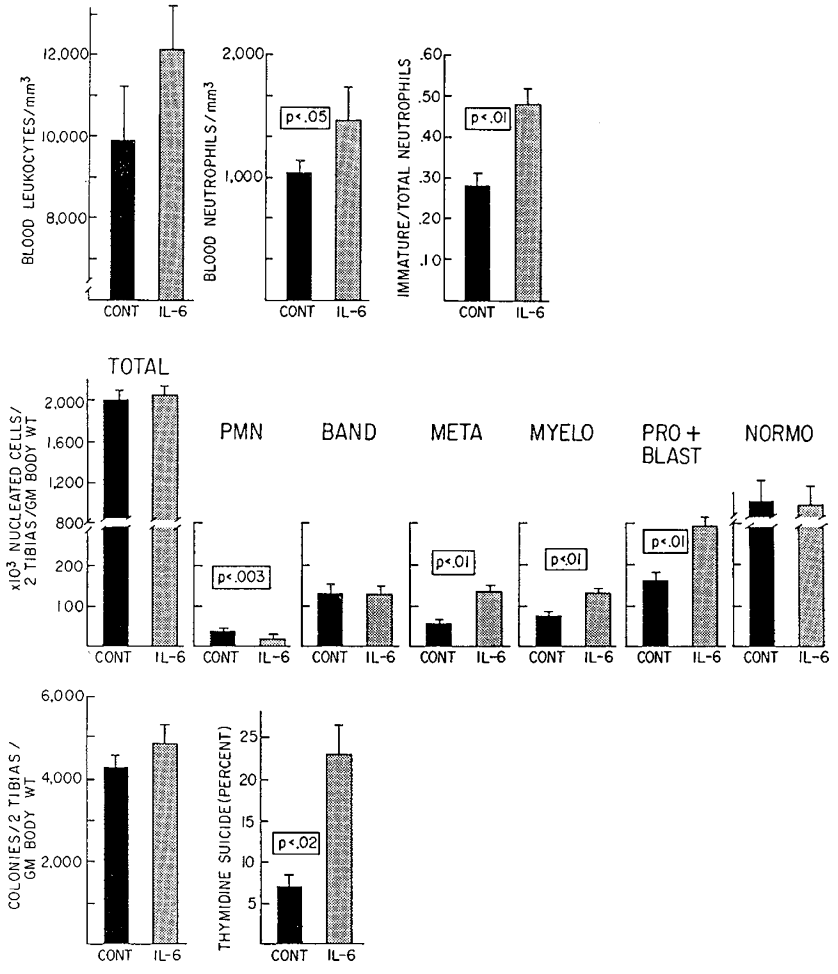

Fig. 1. Adult rats, injected with IL-6 or the vehicle (control), were killed 4 h after the injections. Displayed on the *upper panel* are the blood leukocyte concentrations, blood neutrophil concentrations, and degree of leukocyte left shift (quantified by the ratio of immature to total circulating neutrophils). Marrow cells are displayed on the *middle panel*, and expressed as cells flushed from 2 tibias/g body wt. Total nucleated marrow cells, PMN, band neutrophils, metamyelocytes, myelocytes, promyelocytes plus myeloblasts, and normoblasts are displayed. Displayed on the *lower panel* are the quantity of hematopoietic progenitor cell-derived colonies (expressed as colonies/2 tibias/g body wt), and the ^{3}Htdr suicide rate. The *bracket* indicates the SEM for groups of six rats.

neutrophils plus metamyelocytes) to total neutrophils (PMN plus band neutrophils plus metamyelocytes) on blood films (18).

^{3}Htdr studies. Cell-cycle status was evaluated on marrow cells from adult rats, but on spleen cells from newborn rats due to the small number of marrow cells obtained from the newborn animals. Suspensions of spleen cells in α-MEM were prepared by passing spleens through serially smaller needles (from 18- to 25-gauge). The marrow or spleen cells from rats injected with IL-6, anti-IL-6 antibody, or the control, were incubated with either 0.1 mCi of ^{3}Htdr (sp act, 80 Ci/mmol, New England Nuclear, North Billercia, MA) or the same amount of nonradioactive thymidine. After a 20-min incubation, thymidine (3–4 mg) in cold α-MEM was added (10, 19, 20). The cells were then washed twice, resuspended, counted, and plated in clonogenic assays, as described below. The ^{3}Htdr suicide rate was calculated by subtracting the number of colonies/10^5 plated cells subjected to ^{3}Htdr from the number of colonies/10^5 plated cells subjected to "cold" thymidine, divided by the number of colonies/10^5 plated cells subjected to cold thymidine.

Hematopoietic growth factors. Recombinant human IL-6 (R & D Systems, Minneapolis, MN), produced in *E. coli*, was purified to homogeneity (≥95% by SDS-PAGE) by sequential chromatography and then sterile filtered. The endotoxin level was ≤0.1 ng/μg IL-6.

Purified recombinant human erythropoietin (kindly provided by Dr. Steven Clark, Genetics, Institute, Cambridge, MA) had a spec act of 3.5×10^5 U/mg, with a purity by SDS-PAGE of >99.3%. Pokeweed-mitogen-stimulated spleen cell-conditioned media was prepared by incubating 2×10^7 spleen cells, obtained from a normal adult Sprague-Dawley rat, in α-MEM containing 10% FCS (HyClone), 10^{-5} M β-mercaptoethanol (Eastman Chemical Co., Rochester, NY), and 0.3 mL pokeweed mitogen (Gibco Labs, Grand Island, NY). After 7 d, the supernatant was removed, passed through 0.45 μm filters (Millipore Products, Bedford, MA), and stored at −70°C until used.

Clonogenic cultures. Cells were cultured at a density of 10^4/mL in quadruplicate 1-mL culture dishes containing α-MEM, 5×10^{-4} M β-mercaptoethanol, 30% FCS (HyClone), 1% BSA (Sigma Chemicals, St. Louis, MO), 10% pokeweed-mitogen-stimulated spleen cell-conditioned media, 3 U erythropoietin, and 1.1% methylcellulose (Sigma). After 10 d of culture, colonies were counted with the aid of an inverted microscope. All groups containing >50 cells were scored as a colony.

Statistical analysis. Differences between circulating concentrations of leukocytes, marrow cell populations, hematopoietic progenitor cells, and ^{3}Htdr suicide rates were assessed using *t* test.

RESULTS

Effect of IL-6 on circulating leukocyte concentration. Four h after injection, total blood leukocyte concentrations in the adult IL-6 recipients did not differ from controls (Fig. 1, *upper panel*).

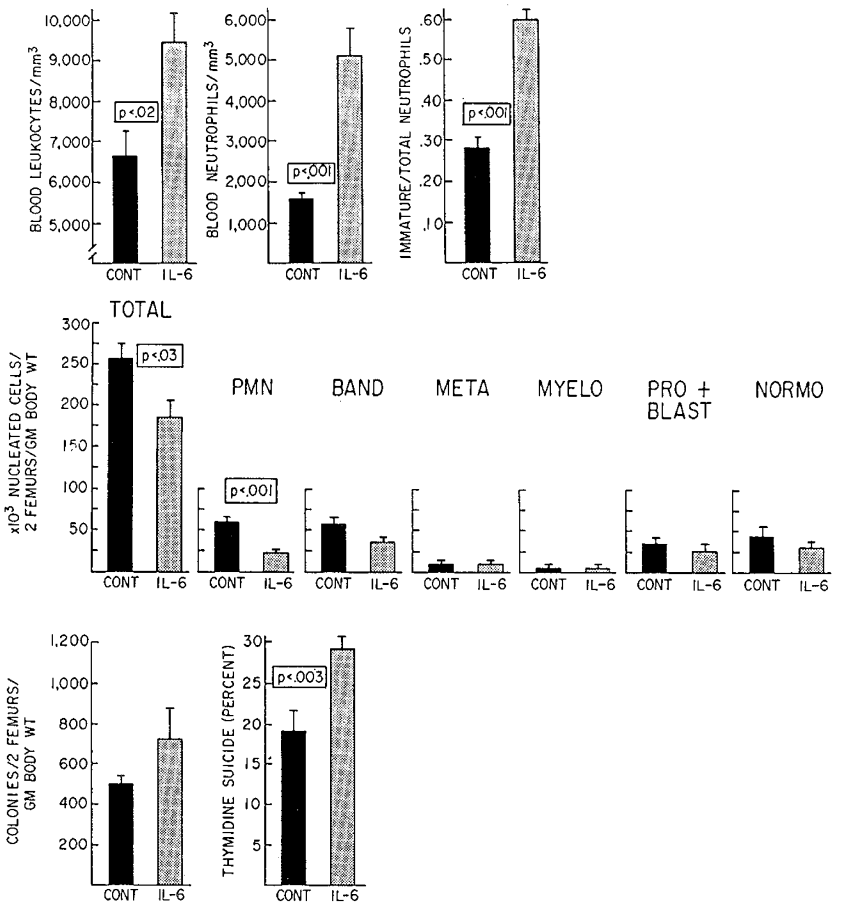

Fig. 2. Newborn rats, injected with IL-6 or the vehicle (control), were killed 4 h after the injections. On the *upper panel* are displayed the blood leukocyte concentrations, blood neutrophil concentrations, and degree of leukocyte left shift (quantified by the ratio of immature to total circulating neutrophils). Marrow cells are displayed on the *middle panel*, and expressed as cells flushed from 2 femurs/g body wt. Total nucleated marrow cells, PMN, band neutrophils, metamyelocytes, myelocytes, promyelocytes plus myeloblasts, and normoblasts are displayed. Displayed on the *lower panel* are the quantity of hematopoietic progenitor cell-derived colonies (expressed as colonies/2 femurs/g body wt) and the ^{3}Htdr suicide rate. The *bracket* indicates the SEM for groups of five to six rats.

Blood neutrophil concentrations, however, were greater in the IL-6 recipients (1460 ± 317/mm^3, mean ± SEM) than controls (1050 ± 180/mm^3, $p < 0.05$). A greater leukocyte left shift was observed in IL-6 recipients, as evaluated by the increase in proportion of immature/total neutrophils on blood films (0.48 ± 0.05 *versus* 0.28 ± 0.3 in controls, $p < 0.01$). No differences were observed between IL-6 recipients and controls in circulating concentrations of lymphocytes, monocytes, or eosinophils.

Changes in blood leukocytes after IL-6 injections were more pronounced in newborn animals than in adults (Fig. 2, *upper panel*). Newborns had a marked increase in total blood leukocyte concentration relative to control animals, a 3-fold increase in blood neutrophil concentration (1560 ± 460/mm^3 in controls *versus* 5130 ± 1390/mm^3 in IL-6 recipients, $p < 0.001$), and a marked left shift (0.28 ± 0.16 in controls *versus* 0.60 ± 0.04 in IL-6 recipients, $p < 0.001$). As in the adult rats, no differences were observed in concentrations of lymphocytes, monocytes, or eosinophils.

Effect of IL-6 on neutrophil storage and proliferative pools. No difference in number of nucleated cells flushed from the tibias was observed between the IL-6 recipients (2140 ± 90 × 10^3 cells/g body wt) and the control recipients (2090 ± 300 × 10^3 cells/g body wt) 4 h after injecting the adult animals (Fig. 1, *middle panel*). The number of PMN flushed from the tibias, however, was less in IL-6 recipients (19 ± 4 × 10^3/g body wt) than in controls (39 ± 4 × 10^3/g body wt, $p < 0.003$). Correspondingly, the numbers of metamyelocytes, myelocytes, promyelocytes, and myeloblasts flushed from the tibias were greater in IL-6 recipients than in controls. The number of normoblasts was not different.

In contrast to adult animals, fewer total nucleated cells were flushed from the marrow of neonatal IL-6 recipients (185 ± 42 × 10^3 cells/g body wt) than from the controls [261 ± 46 × 10^3 cells/g body wt, $p < 0.03$ (Fig. 2, *middle panel*)]. Similar to the adults, the number of PMN flushed from the femurs of IL-6 recipients (23 ± 7 × 10^3/g) was less than controls (58 ± 14 × 10^3/g, $p < 0.001$). No differences in quantities of metamyelocytes, myelocytes, promyelocytes, and myeloblasts were observed.

Effect of IL-6 on concentration and cell cycle characteristics of hematopoietic progenitors. The quantity of hematopoietic colony-forming cells flushed from the tibias of adult rats is shown in Figure 1, *lower panel*. No difference in the number of colonies generated/2 tibias/g body wt was observed between adult animals injected with IL-6 and controls. However, hematopoietic progenitors from animals injected with IL-6 had a higher ^{3}Htdr suicide rate (23 ± 5%) than controls (7 ± 1%, $p < 0.02$).

Similar to the adults, IL-6 injections did not alter the quantity of hematopoietic progenitors flushed from the femurs of newborn rats (Fig. 2, *lower panel*). The ^{3}Htdr suicide rate of hematopoietic progenitors obtained from control neonatal rats (19 ± 2%) was higher than that of control adult rats (7 ± 1%, $p < 0.001$). Similar to the adults, IL-6 injections increased ^{3}Htdr suicide of progenitors from neonatal rats (29 ± 1%, $p < 0.003$ *versus* control).

DISCUSSION

IL-6 is a multifunctional cytokine that appears to be centrally involved in regulation of the immune system (21–23). Its recognized actions include induction of antigen-specific IgG production from B lymphocytes (24), induction of T lymphocyte growth and differentiation (25), and induction of acute phase proteins by liver cells (26). In addition, studies *in vitro* by Ikebuchi *et al.* (12, 13), Leary *et al.* (14), and our group (15) suggest that IL-6 is involved in the process of inducing hematopoietic progenitors from G_0 into active cycling. Whether IL-6 is a physiologic regulator of cycling status of hematopoietic progenitors *in vivo* has not been established. Indeed, whether administration of IL-6 results in enhancement of progenitor cell cycling *in vivo* has not been reported.

To assess its effect on circulating and marrow neutrophil populations, Ulich *et al.* (27) injected adult rats with IL-6. They observed neutrophilia between 1½ and 12 h after a single dose of 1 ng IL-6/g body wt. They also observed that, 12 h after injection, a greater quantity of myeloblasts, promyelocytes, and myelocytes were flushed from humeri of IL-6 recipients than from rats receiving control injections. In our study, we sought to extend those observations by examining the cycle status of hematopoietic progenitors after injection of IL-6. In addition, we sought to determine whether administration of IL-6 to neonatal rats would further increase the proportion of cycling progenitors. This was of interest because we previously reported that, compared with adult rats, neonatal rats have a large proportion of hematopoietic progenitors that are actively cycling (11, 19).

Indeed, we observed that 4 h after IL-6 injection (1 ng/g body wt), the proportion of progenitors killed by exposure to ^{3}Htdr of high spec act was significantly greater than that from animals receiving control injections. Progenitors from newborn rats had a much higher baseline rate of cycling than did those from normal adult rats; nevertheless, IL-6 injections into newborns further increased this cycling.

In addition to its action on progenitors, IL-6 administration resulted in release of neutrophils from the storage pool into the blood. Evidence for this includes the observations that fewer PMN/g body wt were flushed from the marrow and that the circulating neutrophil concentrations and degree of leukocyte left shift were increased in the IL-6 recipients. Whether this was a direct effect of IL-6 as a neutrophil releasing factor (28) is not clear.

Like Ulich *et al.* (27), we observed that IL-6 injections into adult rats resulted in increased marrow quantities of morphologically recognizable neutrophil precursors. The observation that this increase occurred after only 4 h suggests that, in addition to its action on hematopoietic progenitors, IL-6 enhanced proliferation of the neutrophil precursor population. It is not clear why IL-6 administration to newborn rats did not result in increased quantities of morphologically recognizable neutrophil precursors. Perhaps the cycling rates of these populations were already near-maximal in the control newborn rats (11).

In summary, injection of IL-6 into adult and newborn rats resulted in accelerated cycling of hematopoietic progenitors and release of neutrophils from the storage pool into the circulation. Because these kinetic events are also observed during experimental bacterial infection (11, 18, 29), we speculate that IL-6 might be involved in mediation of these events during infection *in vivo*. The data presented here demonstrate that alterations in IL-6 concentration could affect such changes.

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