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Multiple In Vivo Effects of Interleukin- 3 and Interleukin-6 on Murine Megakaryocytopoiesis

By Patrick A. Carrington, Ronald J. Hill, Paula E. Stenberg, Jack Levin, Laurence Corash, Jolanda Schreurs, Georgiann Baker, and Francine C. Levin

The in vivo effects of interleukin-3 (IL-3), interleukin-6 (IL-6), and a combination of IL-3 plus IL-6 on murine megakaryocytopoiesis and thrombopoiesis were examined. Human recombinant IL-6 was administered subcutaneously as 14 equal injections of 5,000 units each during a 102-hour period. Murine recombinant IL-3 was given as 8 injections of 80,000 units each during the first 54 hours. Megakaryopoiesis and thrombopoiesis were evaluated 120 hours after initial administration of the cytokines. Platelet levels increased by 20% following IL-3 alone, 35% following IL-6 alone and 61% after administration of both IL-3 and IL-6. Platelet production, as measured by ^{75}Se-selenomethionine incorporation, increased by approximately 120% in animals that had received IL-6 or IL-3 plus IL-6. Megakaryocyte ploidy analysis by two-color flow cytometry showed a shift in the modal ploidy class from 16N to 32N and a significant increase in the frequency of 64N cells only in IL-6 treated animals. Both bone marrow and splenic megakaryocyte colony-forming cells were significantly increased following either IL-3 or IL-6. Bone marrow megakaryocyte size increased 18%, 43%, and 38%, respectively, after administration of IL-3, IL-6, or the combination of IL-3 plus IL-6. Leukocyte counts and hematocrits were unaffected by either cytokine. Additional groups of mice received the same injection schedule as above and the serial effects on peripheral blood cell levels were assessed for 30 days. Platelet levels, which had been elevated by IL-3 or IL-6, fell to control values within 4 days following the last injection. Animals given IL-6 or IL-3 plus IL-6 were subsequently thrombocytopenic relative to controls on days 7 through 9 following cessation of treatment. Temporary 'cycling' of platelet levels was observed for 3 weeks following treatment with IL-6 or the combination of IL-3 plus IL-6. We conclude that IL-6 and to a lesser extent IL-3 stimulate platelet production in vivo and that their combined effects on platelet levels are approximately additive. Following discontinuation of IL-3 or IL-6, the effects are rapidly reversed, presumably by negative feedback mechanisms, resulting in a period of 'rebound thrombocytopenia' in mice that had received IL-6.
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THE COMPLEX regulation of megakaryocytopoiesis has been extensively reviewed.[1-4] Although megakaryocyte development is a continuous process, it has been useful to consider early and late stages. The early stage comprises the transition of stem cells to committed megakaryocyte precursors and is measured by megakaryocyte colony number and size in culture. In vitro, this stage is stimulated by one or more megakaryocyte colony-stimulating factors (Meg-CSF) that have been identified in the urine and plasma of patients with aplastic anemia,[5,6] amegakaryocytic thrombocytopenia,[7] and thrombocytopenia following chemotherapy.[8] The late stage comprises the maturation of megakaryocyte precursors into platelet producing cells and may be assessed by megakaryocyte number, size, ploidy or acetylcholinesterase activity (in certain species) or by measuring isotope incorporation into newly forming platelets.[9] Megakaryocyte maturation and platelet production are thought to be mediated by a circulating hormone, thrombopoietin, the levels of which increase in response to thrombocytopenia. Thrombopoietic activity has been detected in the plasma of thrombocytopenic rabbits,[10,11] humans,[12] and in the supernatant of human embryonic kidney cells.[13]

More recently, the study of megakaryocytopoiesis has been enhanced by the availability of recombinant hemopoietic growth factors and interleukins that influence megakaryocyte development. Of cytokines studied to date, IL-3 is apparently the most potent murine Meg-CSF[14-16] while IL-6 has the most potent thrombopoietic activity.[17,18] However, it should be emphasized that neither cytokine is specific for the megakaryocyte lineage and that each may affect both early and late stages of megakaryocytopoiesis.[18,19] More importantly, their activities, both in vitro and in vivo, do not necessarily imply a physiologic role in megakaryocyte regulation. Despite these considerations, IL-3 and IL-6 are potential therapeutic agents, and it is possible that a combination of the two cytokines would be of most benefit, because IL-3 is believed to stimulate early events in megakaryocytopoiesis while IL-6 primarily affects the later stages. Therefore, we have studied the ability of IL-3, IL-6 and a combination of both factors to stimulate megakaryocytopoiesis and thrombopoiesis in vivo. In addition to measuring platelet levels and ^{75}Se-selenomethionine incorporation, several other parameters of megakaryocyte development were assessed, namely megakaryocyte colony-forming cells, ploidy, frequency, size and ultrastructure. Furthermore, the temporal effects of IL-3 and IL-6 on platelet levels were studied serially during a 4-week period.

From the Department of Laboratory Medicine, University of California School of Medicine, and the Veterans Administration Medical Center, San Francisco, CA; the Department of Pathology, Oregon Health Sciences University, Portland, OR; and the DNAX Research Institute, Palo Alto, CA.

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Address reprint requests to Ronald J. Hill, PhD, Veterans Administration Medical Center, 113A, 4150 Clement St, San Francisco, CA 94121.

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MATERIALS AND METHODS

Animals. Female Swiss-Webster (SW) mice (25 to 30g) were obtained from Bantin and Kingman, Fremont, CA.

Cytokines. Human recombinant IL-6, expressed in SF9 insect

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cells using a baculovirus vector, was kindly provided by Cetus Corp, Emeryville, CA. IL-6 bioactivity was 1 to 2 × 10^6 U/mg as measured using the IL-6-dependent B9 cell line,[20] using commercially available IL-6 (Amgen Biologicals, Thousand Oaks, CA) as the assay standard.

Murine recombinant IL-3 was produced in silkworms[21] and purified by affinity and C8 reverse phase chromatography. An immunoaffinity chromatography column was prepared using the anti-IL-3 monoclonal antibody 8F8.1.[22] Ten milligrams of 8F8.1 was conjugated to Affi-Gel Hz Hydrazide resin (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. The resultant anti-IL-3 immunoaffinity gel, equilibrated with PBS, was incubated with 1 mL of IL-3-containing silkworm hemolymph overnight at 4°C. The gel was washed with 40 mL of PBS in an open column until no further protein elution could be detected by the mini Bio-Rad protein assay, followed by a series of 50 mmol/L citrate-phosphate buffer washes of decreasing pH (10 mL each, pH 6.0, 5.0 and 4.0, respectively). IL-3 was then eluted using 1 mL aliquots of citrate-phosphate buffer, pH 3.0. Each aliquot was assayed for protein content as above, and for biologic activity utilizing MC/9 cells in a colorimetric assay using the tetrazolium salt, MTT (Sigma, St Louis, MO) to detect living cells.[23] Aliquots were pooled, concentrated by centrifugation using a Centricon 10 (Amicon, Danvers, MA) and applied to a C8 reverse phase column (Pharmacia, Piscataway, NJ), equilibrated with 0.1% trifluoroacetic acid (TFA). The sample was separated using a gradient of 29% to 37% acetonitrile (in 0.1% TFA). IL-3 was eluted between 32% to 34% acetonitrile and shown by SDS polyacrylamide gel electrophoresis with silver stain to consist of two predominant bands of approximately 21 and 20 Kd; several lower molecular weight species (17 to 19 Kd) were also detected. Specific activity of the final product was 3 × 10^9 U/mg. The purified IL-3 was lyophilized on a Spin Vac (Savant Instruments Inc, Farmingdale, NY) and frozen at −70°C until use.

Administration of cytokines. IL-3 and IL-6 were given as separate subcutaneous injections of 0.5 mL three times daily, until the final day of administration when two injections were given at 8 AM and 2 PM. IL-6 in 0.9% saline for injection (Travenol Laboratories, Deerfield, IL), containing 100 μg/mL bovine serum albumin (Fraction V, Sigma, St Louis, MO), was given at a dose of 5,000 U (3.3 μg) per injection for 14 injections over a 102-hour period (total dose, 70,000 units). IL-3 in saline was given at the same time as the IL-6, at a dose of 80,000 U (0.027 μg) per injection for 8 injections during the first 54 hours (total dose, 640,000 units). These doses were chosen on the basis of previous studies that had demonstrated stimulation of megakaryocytopoiesis and thrombopoiesis by IL-6[18] and stimulation of megakaryocytopoiesis by IL-3 (Hill, unpublished observations) at the doses used. Two other groups of mice received either IL-3 or IL-6 and a second injection of the carrier solution for the other factor (Fig 1). Control animals were given serial injections of saline, using the same injection schedule. All injection solutions were demonstrated to be essentially endotoxin-free (<10 pg/mL) by the Limulus amebocyte lysate test.[24] There was no evidence of inflammation at any injection site.

A separate four groups were given the same injection schedule as the four groups in Fig 1. These mice were bled serially by retro-orbital puncture during a 4-week period to determine peripheral blood cell levels.

Peripheral blood cell levels. Blood was obtained by cardiac puncture from the mice studied at 120 hours. Hematocrit values were determined with a microhematocrit centrifuge (Model MB, International Equipment Co., Needham Hts, MA). Platelet and leukocyte levels were determined using an electronic particle counter (Coulter Electronics, Hialeah, FL).[25] Differential leukocyte counts were performed on 100 cells using slides stained with May-Grunwald-Giemsa. In the serially bled animals, 50 μL samples of blood were diluted with 100 μL of Isoton (Coulter Electronics, Hialeah, FL) and complete blood counts were determined with an H1 whole blood counter (Technicon Instruments, Tarrytown, NY). These studies also provided the opportunity for a comparison of platelet counts obtained with the H1 whole blood counter (Technicon) and an electrical impedance cell counter (Coulter Model ZH, Coulter Electronics, Hialeah, FL). A total of 250 platelet counts were carried out on cardiac blood samples using both machines, in this and other studies.

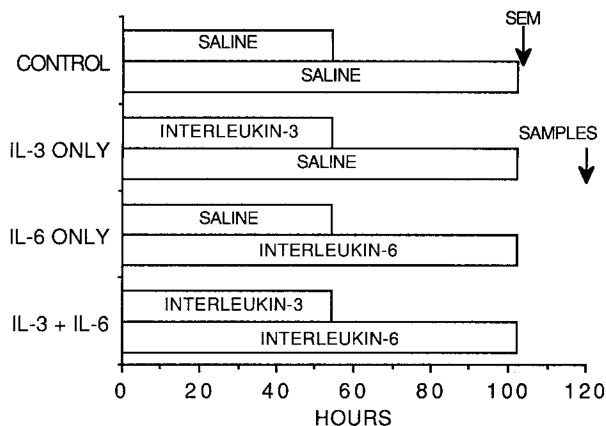

Fig 1. Injection schedule. IL-3 (total dose, 640,000 U), IL-6 (total dose, 70,000 U), or injections of saline were administered to four groups of mice as shown. Details of the timing of injections are provided in Materials and Methods.

Platelet production. ^{75}Se-selenomethionine (^{75}SeM) incorporation into newly forming platelets was used as a measure of platelet production.[9] ^{75}SeM was injected intraperitoneally into control and experimental animals 104 hours following the first injection of cytokine (Fig 1). Sixteen hours later, whole blood was collected by cardiac puncture, and the percentage of injected ^{75}SeM present in platelets was calculated.[26]

Determination of megakaryocyte ploidy. Bone marrow megakaryocyte ploidy distribution was determined by two-color flow cytometry as previously described.[27,28]

Determination of megakaryocyte size. Femurs were split lengthwise and placed in 4% paraformaldehyde, 100 mmol/L phosphate buffer, pH 7.4, at 4°C for 4 to 6 hours. After fixation, tissue samples were plastic embedded as previously described.[29] Cells were visualised with hematoxylin-eosin-azure stain and megakaryocytes were identified morphologically. The cross-sectional areas of recognizable bone marrow megakaryocytes were determined using a Vidas image processing system with Videoplan software (Kontron Bildanalyse, Eching, FRG).

Megakaryocyte ultrastructure. Bone marrow samples were fixed in Karnovsky's fixative, osmicated (2% OsO_4 in veronal-acetate buffer, pH 7.4), stained in block with uranyl acetate, dehydrated in a graded series of ethanol, then infiltrated with propylene oxide, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate, and examined with a JEOL 100-CX transmission electron microscope.

Colony-forming cells. Megakaryocyte and granulocyte-macrophage colony-forming cells (Meg-CFC, GM-CFC) were measured using a soft agar culture system.[30] Bone marrow and spleen cells were collected when animals were sacrificed for platelet isolation (ie, 120 hours after the start of the cytokines and 18 hours after the last IL-6 injection). Cell suspensions were combined from 3 mice in each group in two separate experiments; each sample was cultured using at least two cell concentrations to ensure cultures contained

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numbers of colonies suitable for precise counting. Megakaryocyte and granulocyte-macrophage colonies were identified and counted after 7 days of culture as previously described.[18] Total numbers of colony-forming cells per femur of spleen were calculated by multiplying the frequency (total number of colonies per number of cells plated) by the total number of cells obtained from a single femur or spleen.

Statistical methods. Data on platelet levels, platelet production, megakaryocyte size and ploidy, and sequential platelet counts were assessed by the Mann-Whitney U-test; probabilities were calculated by two-tailed analysis.

RESULTS

Platelet levels. The mean (±1 SE) platelet count in control animals was 1,614 ± 77 × 10^9/L. IL-3, IL-6 and the combination of IL-3 plus IL-6 increased platelet levels by 20% ($P < .005$), 35% ($P < .005$) and 61% ($P < .005$), respectively (Fig 2). The combination of IL-3 plus IL-6 was approximately additive, and was significantly greater than following administration of either IL-3 or IL-6 alone ($P < .005$). The effect of IL-6 alone was similar to the 25% increase previously observed when IL-6 was given for 54 hours and platelet levels measured at 72 hours.[18] Although the H1 whole blood cell counter generated platelet counts approximately 10% lower than did the standard electrical impedence method (correlation coefficient 0.905), the H1 counter was used for determination of serial values because the small sample required (50 μL) allowed the sequential bleeding of mice without the production of anemia or thrombocytosis in controls.

Other blood cells. Hematocrit levels and total and differential leukocyte counts on the day of sacrifice were unaffected by IL-3 or IL-6, either alone or in combination. The mean control leukocyte count was 5.6 ± 1.7 × 10^9/L with

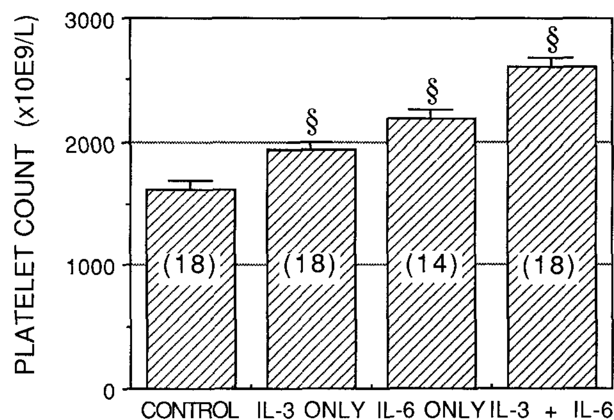

Fig 2. The effects of IL-3 and IL-6 on platelet levels 5 days after the start of injections. Mean platelet counts (±1 SE) are shown for the four groups of mice described in Fig 1. For statistical analysis, treatment groups were compared with controls using the Mann-Whitney U-test. All groups that received cytokines had platelet levels significantly greater than controls (§$P < .005$). Platelet levels of mice that received IL-6 alone were significantly greater than those that had received IL-3 alone ($P < .005$); IL-3 plus IL-6 produced levels significantly greater than did IL-6 alone ($P < .005$). The number of mice in each group is shown in parentheses. The values presented are the results of three separate experiments. Platelet counts were determined using electronic particle counting of blood obtained by cardiac puncture, as described in Materials and Methods.

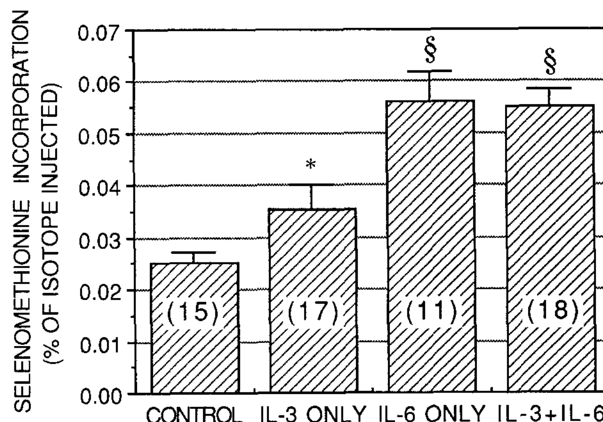

Fig 3. The effects of IL-3 and IL-6 on platelet production. The mean (±1 SE) percentage incorporation of ^{75}SeM into platelets, isolated 16 hours after injection of the isotope, is shown. The combined data from three separate experiments are summarized. Treatment groups were compared to controls by the Mann-Whitney U-test (*$P = .05$, §$P < .005$). Levels of ^{75}SeM in platelets of mice that received IL-6 or IL-3 plus IL-6 were significantly greater than those of mice that received only IL-3 ($P < .01$). The number of mice in each group is shown in parentheses. The values presented are the results of three separate experiments.

20% neutrophils, 75% lymphocytes and 5% monocytes; the mean hematocrit was 39.3 ± 2.6% (SD).

Platelet production. The increase in ^{75}SeM incorporation resulting from administration of IL-3, IL-6 or IL-3 plus IL-6 is shown in Fig 3. In all experiments the results were concordant with the exception of the effect of IL-3. In two of the three experiments, IL-3 caused no rise in ^{75}SeM incorporation, whereas in one experiment IL-3 produced an increase, resulting in an overall rise in the mean that achieved minimal statistical significance ($P = .05$). In addition, no effect on ^{75}SeM incorporation was observed in two previous experiments in which 543,000 units of murine recombinant IL-3 were given during a 54-hour period and levels of ^{75}SeM in platelets measured 18 hours later (Hill, unpublished observations). In contrast, the increases of approximately 120% after IL-6 or IL-3 plus IL-6 were consistent and represent large increases in the rate of thrombopoiesis.

Megakaryocyte frequency and ploidy distribution. The combination of IL-3 plus IL-6 resulted in a shift in the modal ploidy class from 16N to 32N and a significant increase ($P < .005$) in the percentage of 64N cells (Table 1). This is similar to the change following administration of IL-6 alone.[31] Initial assessment of the effect of IL-3 on ploidy (experiment A) demonstrated a reduction in 2N cells and a significant increase ($P < .05$) in 16N cells. Since this experiment was carried out 66 hours after IL-3 was discontinued, at a time when thrombocytosis was present, it was possible that megakaryocytes were under the influence of negative feedback mechanisms acting to reduce platelet production. Therefore, the ploidy analysis was repeated in a separate experiment (experiment B) carried out 72 hours after the start of IL-3 (18 hours following its discontinuation). This also demonstrated a similar change in megakaryocyte ploidy (Table 1). Thus, at two separate time

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Table 1. The In Vivo Effects of IL-3 and IL-6 on Bone Marrow Megakaryocyte Frequency and Ploidy Distribution

Group/No. Studied	Freq.	2N	4N	8N	16N	32N	64N	128N
Control/16	0.12	14.5	11.1	10.1	35.0	24.4	3.1	1.7
SD	0.05	2.1	2.3	4.3	6.1	6.6	0.8	0.9
IL-3 + IL-6/10	0.14	11.8†	8.8*	7.7	26.1‡	36.7‡	6.0‡	2.1
SD	0.02	1.8	1.2	0.9	5.1	3.8	2.0	0.4
IL-3 (Exp A)/5	0.12	12.1*	12.2	9.1	43.3*	19.6	2.5	1.2
SD	0.02	0.5	2.7	1.8	3.4	4.7	0.6	0.8
IL-3 (Exp B)/5	0.10	10.0†	9.8	9.8	44.5*	22.9	2.3	0.7*
SD	0.02	2.1	1.1	1.5	4.2	4.1	1.1	0.1

IL-3, or IL-3 plus IL-6 were administered to Swiss-Webster mice as shown in Fig 1. Bone marrow megakaryocyte frequency and ploidy distribution were measured as described in Materials and Methods. Measurements were performed at either 120 hours (IL-3 plus IL-6; IL-3, Exp A) or 72 hours (IL-3, Exp B) following initiation of cytokines. Mean values and the standard deviation (SD) are shown for each measurement. IL-3 plus IL-6 values represent results from two separate experiments and IL-3 experiments A and B represent one experiment each. Megakaryocyte frequency is expressed as a percentage of the total nucleated cell population. The relative frequency of each ploidy class is expressed as a percentage of the megakaryocyte population. Total nucleated cell counts ($\times 10^6$) obtained from 2 femora and 2 humeri of each animal were: Control 53.1 ± 11.4 (SD); IL-3 plus IL-6 50.9 ± 7.2; IL-3 Exp A 55.8 ± 3.5; IL-3 Exp B 64.4 ± 4.5. Average cell recoveries after labeling procedures were 66 ± 9% (SD) for controls; 65 ± 4% for IL-3 plus IL-6 treated mice; 73 ± 9% for IL-3 Exp A and 62 ± 2% for IL-3 Exp B. The ploidy class of each treated group was compared with that of controls using the Mann-Whitney U-test.

*$P < .05$.

†$P < .01$.

‡$P < .005$.

points, IL-3 caused similar alterations in the ploidy distribution (primarily an increase in the frequency of 16N cells), but no increase in the proportion of 32N or 64N megakaryocytes, as was observed following IL-6[31] or IL-3 plus IL-6.

Megakaryocyte size. The mean cross-sectional area of mature bone marrow megakaryocytes increased by 18% following IL-3 ($P < .005$), 43% with IL-6 ($P < .005$) and 38% with IL-3 plus IL-6 ($P < .005$) (Table 2). In addition, bone marrow megakaryocyte size was greater in mice given IL-6 or IL-3 plus IL-6 than in those given IL-3 alone ($P < .005$). Splenic megakaryocyte area increased by 38% ($P < .005$) following IL-6 alone and 19% after IL-3 plus IL-6 ($P < .005$) (Table 2).

Table 2. The In Vivo Effects of IL-3 and IL-6 on Megakaryocyte Cross-Sectional Area in the Bone Marrow and Spleen

	Megakaryocyte Size (μm^2)	
	Spleen	Bone Marrow
Control	449 ± 19.1	406.3 ± 12.9
IL-3 only	487 ± 20.8	477.7 ± 14.8*
IL-6 only	619 ± 32.0*	582.3 ± 19.6*
IL-3 + IL-6	535 ± 23.7*	560.6 ± 18.8*

IL-3 or IL-6 was administered as shown in Fig 1. Bone marrow and spleen sections were prepared and analyzed as described in Materials and Methods. Data shown are the means ± 1SE for 200 bone marrow megakaryocytes and 100 spleen megakaryocytes obtained from two mice in each group in two separate experiments. Statistical analysis was performed by the Mann-Whitney U-test. Bone marrow megakaryocyte size was significantly increased in all treatment groups ($P < .005$) and was greater in mice given IL-6 or IL-3 plus IL-6 than in those given IL-3 alone ($P < .005$). Spleen megakaryocyte size was significantly increased in mice given IL-6 ($P < .005$) or IL-3 plus IL-6 ($P < .005$).

*$P < .005$.

Megakaryocyte ultrastructure. The electron microscopic appearances of bone marrow megakaryocytes were examined in two mice from each of the four groups identified in Fig 1. Nuclear morphology and distribution of the demarcation membranes were not affected by administration of either IL-3 or IL-6. However, both IL-3 or IL-6 alone and the combination of IL-3 plus IL-6 caused an increase in the percentage of cells with a large peripheral organelle-deficient zone.[31] This was present in only 16% of control megakaryocytes but was identified in 47% to 60% ($P < .005$) of megakaryocytes in mice that had received IL-3, IL-6, or IL-3 plus IL-6 (Table 3).

Colony-forming cells. Total detectable Meg-CFC and GM-CFC in the bone marrow and Meg-CFC in the spleen were significantly increased ($P < .05$) following either IL-6 or IL-3 plus IL-6 (Table 4). Only bone marrow Meg-CFC and GM-CFC were significantly increased following IL-3 alone ($P < .05$). No significant synergism between IL-3 and IL-6 was demonstrated.

Duration of effect of IL-3 and IL-6 on platelet levels. Platelet counts of mice treated with IL-3 plus IL-6 were significantly greater than control values on days 5, 6, 7 and 17 ($P < .05$), and significantly below controls ($P < .05$) on days 12, 13, 14, 23 and 25 (Fig 4). Platelet levels of IL-6 treated animals were significantly above control levels on days 5, 6 and 7 and below controls on days 12, 13, 25 and 27 ($P < .05$). Thus, two periods of relative thrombocytopenia occurred in mice that received either IL-6 alone or IL-3 plus IL-6. Hematocrit levels and white blood cell counts did not change significantly throughout the 4-week observation period.

DISCUSSION

Several conclusions may be drawn from these studies. First, pharmacologic doses of IL-3 caused a modest but significant increase in peripheral platelet levels. To our knowledge, only two other studies of platelet levels following IL-3 administration have been carried out, both in

Table 3. Frequency of Bone Marrow Megakaryocytes With Enlarged Peripheral Organelle-Deficient Zones

	Absent	Present	Total
Controls	31 (84)	6 (16)	37 (100)
IL-3 only	18 (53)*	16 (47)*	34 (100)
IL-6 only	12 (40)*	18 (60)*	30 (100)
IL-3 + IL-6	15 (48)*	16 (52)*	31 (100)

Bone marrow megakaryocyte ultrastructure was assessed as described in Materials and Methods. Megakaryocytes were obtained from two mice in each group in two separate experiments. The numbers and percentages (in parentheses) of bone marrow megakaryocytes in which an enlarged peripheral organelle-deficient zone was absent or present are indicated. Statistical analysis was performed by the chi-square test; all treated groups were significantly different from control.

*$P < .01$.

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Table 4. The In Vivo Effects of IL-3 and IL-6 on Murine Megakaryocyte and Granulocyte-Macrophage Colony-Forming Cells (CFC) in the Bone Marrow and Spleen

	Bone Marrow			Spleen		
	Total Meg-CFC	Total GM-CFC	Total Cells per Femur ($\times 10^{-6}$)	Total Meg-CFC	Total GM-CFC	Total Cells per Spleen ($\times 10^{-6}$)
Control	3,699 ± 457	31,534 ± 1,083	11.4	5,637 ± 599	11,693 ± 3,758	92.4
IL-3 only	8,595 ± 441*	60,351 ± 297*	17.1	6,551 ± 1,274	13,985 ± 5,022	97.2
IL-6 only	10,320 ± 1,756*	52,478 ± 671*	16.8	9,009 ± 1,396*	12,831 ± 3,274	111.4
IL-3 + IL-6	8,833 ± 311*	51,322 ± 5,164*	13.5	11,617 ± 561*	16,495 ± 2,068	82.8

IL-3, IL-6 or IL-3 plus IL-6 were administered as shown in Fig 1. Total CFC per femur or spleen were calculated as described in Materials and Methods. Cell suspensions were combined from 3 mice in each group in two separate experiments. Each sample was plated in triplicate using at least two cell concentrations. The data shown are the means ± 1SE derived from the values obtained for each dilution in the two experiments. Statistical analysis was performed by the Mann-Whitney U-test. Total Meg-CFC and GM-CFC in the bone marrow and Meg-CFC in the spleen were significantly increased ($P < .05$) following either IL-6 alone or the combination of IL-3 plus IL-6. IL-3 alone caused a significant increase only in bone marrow Meg-CFC and GM-CFC ($P < .05$).

*$P < .05$.

primates. Donahue et al[32] observed an increase in the platelet level following continuous intravenous infusion of IL-3, 80,000 U/kg/day for 14 days, whereas Mayer et al[33] did not detect any change in platelet levels following daily subcutaneous injections of up to 460,000 U/kg/day of IL-3 for 14 days. The mechanisms by which IL-3 mediates the in vivo effects observed by Donahue et al and ourselves are unknown. IL-3 has been shown to act as a Meg-CSF in vitro.[14-16] However, the in vivo effects of IL-3 on Meg-CFC we have observed do not necessarily account for the increase in peripheral cell levels because there is no direct relationship between numbers of colony-forming cells and peripheral blood cell levels.[30,34,35] Indeed, in the present studies we observed an increase in GM-CFC but no corresponding increase in peripheral white cell counts on the day of killing. Hence the increase in platelet levels after IL-3 administration may imply an effect of IL-3 on a later stage of megakaryocyte development. This is supported by the changes in bone marrow megakaryocyte size, ultrastructure and ploidy distribution observed following IL-3 administration, and is in agreement with other reports that IL-3 promotes differentiation of megakaryocytes.[19,36] However, our observations do not establish that IL-3 caused these effects directly because indirect effects of IL-3, potentially mediated by IL-6,[37] may operate in vivo.

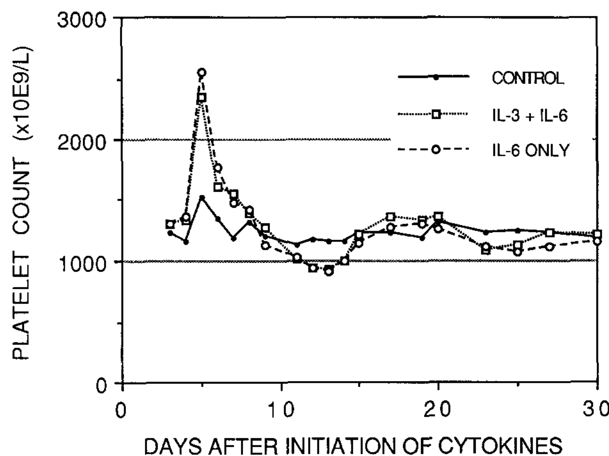

Fig 4. Serial observations of the effects of IL-6 or IL-3 plus IL-6 on peripheral platelet levels. IL-3 plus IL-6, IL-6, or control injections were administered to mice as shown in Fig 1. Animals were then bled retroorbitally and platelet counts determined serially (using the H1 whole blood counter) until day 30. The mean platelet counts of 8 animals in each group are shown. The values presented represent the results of two separate experiments. Platelet counts of mice given IL-3 alone were significantly different from control only on day 5 and have been omitted for clarity. Platelet counts in IL-3 plus IL-6 treated animals were significantly ($P < .05$) above control values on days 5, 6, 7, and 17 and significantly ($P < .05$) below controls on days 12, 13, 14, 23, and 25. IL-6 treated animals had platelet levels significantly above controls on days 5, 6, and 7 and below on days 12, 13, 25, and 27 ($P < .05$).

IL-6 has a much more potent thrombopoietic effect than IL-3, causing a marked ploidy shift and greater increases in platelet production, platelet count and megakaryocyte size (current data and Hill et al[31]). IL-6 also produced an increase in Meg-CFC in vivo, an effect noted in previous experiments.[18] It is possible that this effect is also indirect, because in vitro data suggest that IL-6 has no direct megakaryocyte colony-stimulating activity.[38-41]

The administration of either IL-3 or IL-6 caused significant increases in the number of megakaryocytes with a wide peripheral organelle-deficient zone. Similar changes have also been noted at 48 and 72 hours after induction of acute immune thrombocytopenia in mice.[42] Therefore, this morphologic appearance is associated with two experimental models in which platelet production is increased; hence it is possible that megakaryocytes with this appearance are actively producing platelets.

The injection schedule was designed to achieve maximum expansion of the megakaryocyte progenitor pool capable of maturation under the continued influence of IL-6. In addition, IL-6 was given simultaneously with IL-3 at the beginning of the injection schedule because IL-6 increases Meg-CFC[18] and spleen colony-forming cells[43] in vivo, and because of evidence that IL-6 and IL-3 act synergistically to stimulate hematopoietic stem cells.[44-49] However, we have not demonstrated significant in vivo synergism between IL-3 and IL-6 at the doses used in this

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study. The increase in platelet level following IL-3 plus IL-6 was only slightly greater than the sum of the effects of IL-3 alone and IL-6 alone, and the increases in Meg-CFC and megakaryocyte size were similar following IL-6 alone or the combination of IL-3 plus IL-6. The shift in megakaryocyte ploidy with IL-3 plus IL-6 was similar to that previously reported with IL-6 alone.[31] However, demonstration of synergism is dose-dependent because sub-optimal levels of test materials must be used. Our study has not excluded the possibility that the combined administration of IL-3 and IL-6 might allow the use of lower doses of IL-6 to achieve the same level of stimulation of thrombopoiesis.

The data obtained from the serially studied mice indicate that the effects of large doses of both cytokines on platelet production were rapidly reversed, because platelet levels fell to control values within 4 days (approximately the life span of murine platelets[50,51]) after discontinuation of IL-3 or IL-6. The marked decrease in platelet production was probably caused by negative feedback mechanisms, as manifested by "rebound thrombocytopenia" that was most evident at 7 to 8 days after the peak of the thrombocytosis. The degree and duration of the rebound thrombocytopenia was similar to that observed following hypertransfusion of platelets in rats[52] and rabbits.[9] Moreover, platelet levels "cycled" at approximately 6-day intervals for several weeks in animals given IL-3 plus IL-6 and to a lesser extent following IL-6 alone (Fig 4). Because 6 days is the approximate time required for a murine megakaryocyte progenitor cell to generate platelet producing megakaryocytes, we postulate that this temporary cycling of the platelet count is a manifestation of successive stimulation and suppression of megakaryocyte precursors by endogenous mechanisms in order to re-establish steady state production. Feedback inhibition would act to reduce the overall effect of IL-3 and IL-6. Thus, if IL-3 or IL-6 were administered in situations in which feedback inhibition was not operating (eg, chemotherapy-induced thrombocytopenia) their effects might be greater than those observed in this study of mice with normal hematopoiesis.

Whether the stimulation of megakaryocytopoiesis by IL-3 and/or IL-6 will have any clinical value remains unknown. Granulocyte growth factors are being used increasingly in the management of chemotherapy-induced neutropenia and have proved valuable for the treatment of congenital and cyclic neutropenias.[53] Analogous situations exist for platelets with a similar need for factors capable of increasing platelet production in the management of thrombocytopenia induced by chemotherapy or radiotherapy. The combination of IL-3 and IL-6 provides the most potent stimulus for platelet production so far described. However, the effects of both IL-3 and IL-6 are very short-lived; hence in a therapeutic situation continuous infusion would probably be necessary. Also, neither IL-3 nor IL-6 is specific for megakaryocytopoiesis[54-56] and side effects from other biologic activities may limit their clinical use. There is also evidence that malignant cells are capable of responding to growth factors,[57,58] and IL-6 is a candidate autocrine growth factor for several malignancies.[59-61] Clearly it would be preferable to use factors that are specific for megakaryocytopoiesis. Hence the identification of factors that specifically mediate physiologic regulation of megakaryocytes remains an important goal in hematopoietic research.

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