Interleukin-1 Accelerates Murine Granulocyte Recovery Following Treatment With Cyclophosphamide

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This study investigated the effects of recombinant human interleukin-1 (rhIL-1α) on granulocyte recovery following treatment of mice with cyclophosphamide (CPM). CF-1 mice were injected with 0.5 µg rhIL-1α or heat-inactivated rhIL-1α according to five different regimens, before and/or following 200 mg/kg CPM. Significant neutrophilia initially developed in treatment mice of all five regimens and accelerated granulocyte recovery occurred in treatment mice of four IL-1 regimens. Significant elevations in serum colony stimulating activity (CSA) occurred in treatment mice at a number of time points studied. In addition, marked increases in the percentage of maturing granulocyte precursors and in the proportion of cells cycling in S and G2/M were observed in treatment marrow throughout the IL-1 regimen. Before granulocyte recovery, premature nuclear segmentation was noted in metamyelocytes of treatment marrow. Concomitant with granulocyte recovery, treatment marrow was significantly more cellular and contained more total CFU-GM, more CFU-GM in S phase, more cells in S and G2/M, and more mitotic figures than control marrow. Splenic myelopoiesis was also enhanced in treatment mice. These data suggest that IL-1 significantly hastens granulocyte recovery following treatment with CPM by enhancing both proliferation and maturation of myeloid precursors.

MATERIALS AND METHODS

Animals. Female CF-1 mice (Harlan-Sprague-Dawley, Inc., Indianapolis), 10 to 18 weeks old, were used in all experiments. They were housed in the AAALAC-accredited Animal Care Facility of the University of Colorado Health Sciences Center and fed standard lab chow and water ad libitum. Control and treatment mice were exposed to identical environmental conditions.

Endotoxin determination. The limulus amebocyte lysate (LAL) coagulation test (E-toxate; Sigma, St Louis) was used to determine the presence of endotoxin (>0.05 to 0.2 ng/mL) in batches of sterile plasticware, needles, phosphate-buffered saline (PBS), and IL-1. All supplies and PBS used in the experiments were endotoxin-free.

IL-1. Recombinant human IL-1 (rhIL-1α) was kindly provided by Hoffmann-LaRoche, Nutley, NJ. It had been purified from Escherichia coli and consisted of the carboxy-terminal 154 amino acids of the 271 amino acid IL-1 precursor. rhIL-1α (Lot JSM63) had a specific activity as measured by Hoffmann-LaRoche of 1 x 10^6 U/mg (D0 assay), and had endotoxin activity of 15 EU/mL (LAL), and was diluted in 30 mmol/L Tris-Cl, 0.4 mol/L NaCl (pH = 7.8). Heat-inactivated (HI) rhIL-1α served as control. Heat-inactivation was achieved by incubation at 90°C for one hour.

Manipulations in vivo. All mice received 200 mg/kg CPM (Bristol-Myers Oncology Division, Syracuse, NY) by intraperitoneal (IP) injection at hour 0. Treatment mice received 0.5 µg rhIL-1α and control mice received 0.5 µg HI rhIL-1α IP during several days before and/or after CPM according to five different regimens (Table 1). The IL-1 and HI IL-1 were diluted in endotoxin-free PBS (pH = 7.2) (GIBCO, Grand Island, NY) immediately before injection and delivered to treatment and control mice as successive alternating injections in 0.3 mL volumes with 28-gauge needles attached to 0.5 mL insulin syringes (Becton-Dickinson, Franklin Lakes, NJ).

Peripheral blood was obtained from the tail snipped at its tip with a scalpel blade. After discarding the first drop of blood, a smear was prepared by allowing the blood to air dry on a glass slide.

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Submitted May 18, 1988; accepted November 8, 1988.

Supported by an American Society of Clinical Oncology Young Investigator Award and a gift from the Veterans of Foreign Wars, Colorado Chapter.


Blood, Vol 73, No 4 (March), 1989: pp 938-944
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Scientific, San Francisco) and sera harvested after clot retraction. To obtain spleen cell suspensions, each spleen was placed on a screen in 1 mL IDMEM. Cells were flushed from the tibial cavity into sterile tubes (Falcon, 5 x 10^7 cells/tube) with tuberculin syringes containing 1 mL IDMEM. To obtain nucleated BM cells, pooled BM from each mouse was placed on a 60 x 15 mm culture dish (Falcon) containing media and gently mashed with the tip of a tuberculin syringe. Differential counts of 500 nucleated cells were made on Wright-stained smears of peripheral blood. In selected mice, spleens were removed by simple excision and placed in 100 cells were made on Wright-Giemsa cytospin preparations of approximately 1 x 10^3 nucleated cells. In this report, the term “ringed” cells refers to metamyelocytes, juvenile and segmented neutrophils that comprise the nonproliferating, maturing marrow granulocyte compartment.24,25

Table 1. IL-1 Regimens

| Regimen No. | -96 | -72 | -48 | -30 | -24 | -3 | 0 | 6 | 24 | 48 | 72 | 96 |
|-------------|-----|-----|-----|-----|-----|----|---|---|---|----|----|----|-----|
| 1           | X   | X   |     |     |     | X  | X |   |   | X  | X  | X  |     |
| 2           |     |     | X   | X   |     | X  | X |   |   | X  | X  | X  | X   |
| 3           | X   | X   |     |     |     | X  | X |   |   | X  | X  | X  |     |
| 4           |     |     | X   | X   |     | X  | X |   |   | X  | X  | X  | X   |
| 5           | X   | X   |     |     |     | X  | X |   |   | X  | X  | X  |     |

Mice received 0.5 μg IL-1 or HI IL-1 IP at the times indicated by X and CPM 200 mg/kg IP at hour 0.

Granulocyte-monocyte colony assays. CFU-GM cultures were prepared as described for the CSA assay above except that 0.1 mL dialyzed, concentrated human urine replaced serum as the source of CSA.28 BM from each mouse was added to quadruplicate cultures at 1 x 10^5 nucleated cells per plate. Spleen cells from each mouse were added to triplicate cultures at 5 x 10^5 cells per plate. Data expressed as % treatment “ringed” cells refers to colonies served as an index of CSA.26

Statistical analyses. The Student’s t test was used to establish statistical significance. Unless otherwise specified, P values reflect two-tailed t distribution. One-tailed t distribution allowed significance at P < .05 for a specific time period examined only if data obtained from the preceding time period began a trend in a similar direction.27

RESULTS

Effect of IL-1 on granulocyte recovery after CPM. Groups of treatment and control mice received IL-1 or HI IL-1 along with CPM according to one of five different IL-1 regimens (Table 1). As shown in Fig 1, significant neutrophilia initially developed in all treatment groups. Significant leukocytosis developed as well (data not shown). Marked neutropenia developed in all treatment and control groups several days following administration of CPM. Interestingly, the ANC nadirs occurred at least 24 hours earlier in treatment than control mice of all regimens except no 3. Granulocyte recovery occurred 24 to 48 hours earlier in treatment mice compared with control mice in four of the five IL-1 regimens (Fig 1). In these four regimens (no. 2 through 5), IL-1 injections continued through at least hour 30 after CPM, whereas in regimen no. 1, the IL-1 injections were completed within six hours of CPM.
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Mechanisms involved in accelerated granulocyte recovery. In view of the above data, various aspects of granulopoiesis were studied in mice treated according to regimen no. 5. In two separate experiments (n = 4 for each treatment and control group), mice began treatment with IL-1 or HI IL-1 and were killed at either -90, -48, 0, 48, 96, or 168 hours.

Serum CSA was measured at the times indicated in Fig 2. A significant increase in CSA of pooled sera was measured by standard bioassay in agar culture (see Materials and Methods). Data represent mean number of colonies ± SEM (n = 4 for each time point studied). Significant differences between treatment and control groups are denoted by * P < .005.

BM cell counts, differentials, and morphology were examined at the times indicated in Fig 3. At -48 hours, treatment BM contained significantly fewer nucleated cells than did control BM, P < .05, (n = 8) (Fig 3A). In contrast, significantly more nucleated cells/tibia were present in treatment than control mice at 96 hours, concomitant with initial recovery of treatment ANC, P < .005 (n = 8) (Fig 3A). As shown in Fig 3B, treatment BM contained a significantly greater proportion of “ringed” cells than did control BM at all time points studied except 168 hours, P < .05 to .005 (n = 8). In addition, a significantly greater number of “ringed” cells/tibia were observed in treatment mice at 0, 96, 168 hours, P < .05 to .01 (n = 8) (Fig 3C). Nuclear morphology of BM “ringed” cells was strikingly different between treatment and control groups at 48 hours. As evident in Fig 4, numerous thick-ringed metamyelocytes in treatment BM (n = 4) contained segmented nuclei, suggesting premature nuclear segmentation. This aberrant morphology was not observed in control mice at 48 hours or any other time points studied. At 96 hours, significantly more mitotic figures were identified in cytospin preparations of treatment than control BM (mean number mitotic figures/500 cells ± SEM, IL-1: 11 ± 2; HI IL-1: 4 ± 1, P < .05 [n = 4]).

BM CFU-GM were evaluated at the times indicated in Fig 5. A significant increase in CFU-GM/tibia was observed in treatment compared with control mice at 96 hours only, P < .005 (n = 8), concomitant with initial recovery of treatment ANC. A significant decrease in CFU-GM/tibia occurred in treatment mice at -48 hours (following the first two doses of IL-1), P < .005 (n = 8), corresponding to the time when a depletion of nucleated BM cells was also observed in treatment mice (Fig 3A).

Cell cycle status of tibial BM cells was evaluated at the times indicated in Fig 6. Treatment BM contained a greater proportion of nonringed cells cycling in S + G2/M than did control BM at all time points studied, P < .005 (n = 4).

Fig 1. Effects of five IL-1 regimens on peripheral neutrophil counts. Treatment (□—□) and control (○—○) mice received IL-1 or HI IL-1 according to regimens no. 1 through 5, as indicated in Table 1. (N = 6 for regimens no. 1, 2, and 4; n = 5 for regimen no. 3; n = 12 for regimen no. 5). Data represent mean ANC ± SEM. Significant differences between treatment and control groups for each time point studied are denoted by * P < .005, † P < .01, ‡ P < .05.
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Fig 3. Effect of IL-1 on BM cellularity and maturing myeloid compartment. Treatment (B—●) and control (O—O) mice received IL-1 or HI IL-1 according to regimen no. 5. Mice were killed at the time points indicated and bone marrows evaluated for (A) nucleated cells per tibia, (B) % 'ringed' cells per tibia, (C) total 'ringed' cells per tibia. 'Ringed' cells refer to metamyelocytes and juvenile and segmented granulocytes that comprise the maturing myeloid BM compartment. X indicates injection of IL-1 or HI IL-1. Data from two separate experiments are combined and represent mean ± SEM (n = 8 for each time point studied). Significant differences between treatment and control groups are denoted by * P < .005, † P < .01, ‡ P < .05.

However, the total number of marrow cells in S + G2/M was significantly greater in treatment than control mice at 48 and 96 hours only (Fig 6B).

The proportion of CFU-GM in S phase was examined at -48, 0, and 96 hours. Too few BM cells were collected from tibiae to determine cell cycling status at 48 hours. A significantly greater percent of CFU-GM in S phase was observed at 96 hours only: mean % in S phase ± SEM: 0 hours, IL-1: 29 ± 4%, HI IL-1: 16 ± 9%, P = .1 (n = 4); 96 hours, IL-1: 54 ± 8%, HI IL-1: 33 ± 5%, P < .05 (1-tailed t) (n = 4).

Spleen cell counts, differentials, and CFU-GM were evaluated at 48, 96, and 168 hours since myelopoiesis readily occurs in murine spleens during states of stressed hematopoiesis. Treatment spleens contained significantly more "ringed" cells than did control mice at 96 and 168 hours: mean number ringed cells/spleen (×10⁶) ± SEM: 96 hours, IL-1: 5 ± 2, HI IL-1: 0.1 ± 0.1 P < .01 (n = 3); 168 hours, IL-1: 42 ± 9, HI IL-1: 9 ± 2 P < .01 (n = 3). At 96 hours, the majority of myeloid forms in treatment spleens were myelocytes and metamyelocytes, while at 168 hours, metamyelocytes and juvenile and segmented neutrophils comprised the splenic myeloid population. Although more CFU-GM were present in treatment than control spleens at 96 and 168 hours, the differences were statistically significant for 168 hours only: mean CFU-GM/spleen (×10⁶) ± SEM: 96 hours, IL-1: 69 ± 8, HI IL-1: 2 ± 1 P < .06 (n = 3); 168 hours, IL-1: 32 ± 8, HI IL-1: 17 ± 2 P < .05 (1-tailed t) (n = 3).

DISCUSSION

Four of the five IL-1 regimens evaluated in the present study hastened granulocyte recovery in mice treated with sublethal doses of CPM. In the one regimen where accelerated recovery did not occur, the last dose of IL-1 was given six hours after CPM. In the other regimens, accelerated recovery was observed whether IL-1 treatments were completed 30, 72, or 96 hours after CPM, and whether IL-1 injections were administered both before and after, or only after the dose of CPM.

Neutrophilia occurred during the initial phase of all five...
regimens, consistent with previous data that IL-1 induces leukocytosis by stimulating neutrophil egress from bone marrow. IL-1 treatments shortened but did not ablate the ANC nadir following CPM injection. Interestingly, in four of the five regimens, the ANC nadir of treatment mice preceded that of control mice by at least 24 hours. Similarly, Moore and Warren observed ANC nadirs develop earlier in 5-FU–treated mice injected with rhIL-1 than in 5-FU–treated controls. These early nadirs probably reflect premature depletion of BM storage pool neutrophils by IL-1. Indeed, in the one regimen where treatment and control ANC nadirs occurred concurrently, the period of neutrophilia in treatment mice was short-lived compared with that of the other regimens.

In the present study, various aspects of granulopoiesis were examined in mice treated with IL-1 and CPM according to regimen no 5. Without such evaluation one could argue that, but for regimen no 2, IL-1 caused earlier recovery of circulating neutrophils merely by enhancing neutrophil egress from BM. One could also argue that, but for regimen no 3, earlier ANC nadirs per se accelerated granulocyte recovery by signalling an earlier need for enhanced production of CSFs. However, significant increases in serum CSA occurred in treatment mice within 24 hours of the IL-1 injections, suggesting that IL-1 stimulated release of CSFs in vivo. Although the effects of IL-1 on bone marrow cellularity, morphology, CFU-GM, and cell-cycling status varied depending on the time point studied, two effects consistently noted during treatment suggest that IL-1 enhanced both proliferation and maturation of granulocyte precursors. Marked increases in the percentage of metamyelocytes and juvenile and segmented granulocytes were observed in treatment marrow throughout the IL-1 regimen. In addition, the proportion of potentially proliferating cells in S and G2/M phases of cell-cycle was significantly greater in treatment than control marrow at all time points studied. Neta et al recently reported a similar increase in the percentage of large murine BM cells cycling in S, G2, and M 20 hours after injection of rhIL-1α.

Before the onset of neutropenia following CPM, the maturing myeloid compartment of treatment marrow was significantly larger than that of controls and premature nuclear segmentation was apparent in treatment metamyelocytes. In addition, treatment BM contained significantly more cells cycling in S and G2/M. Data in A were determined by multiplying % nucleated BM cells in S + G2/M by an adjustment factor (see Materials and Methods) to allow comparison of equivalent numbers of potentially proliferating, nonringed BM cells in treatment and control mice. The results in B were determined by: % cells in S + G2/M (unadjusted) × total nucleated cells/tibia. Data represent mean ± SEM (n = 4 for each time point studied). Significant differences between treatment and control groups are denoted by * P < .005, † P < .01, ‡ P < .05.

Fig 5. Effect of IL-1 on BM CFU-GM. Treatment (●—●) and control (O—O) mice received IL-1 or Hi IL-1 according to regimen no 5. BM of each mouse was plated in standard agar culture in quadruplicate at 1 × 10⁶ nucleated cells/plate. CFU-GM/tibia = CFU-GM/10⁶ cells × mean number nucleated cells/tibia. Data from two separate experiments are combined and represent mean ± SEM (n = 8) of treatment mice as percent mean of control mice, the latter arbitrarily set at 100% for each time point studied. X indicates injection of IL-1 or Hi IL-1. Significant differences between treatment and control groups are denoted by * P < .005.
Several results noted following the first two IL-1 injections deserve discussion. At ~48 hours a significant decrease in BM cells was noted in treatment marrow despite a marked increase in the percentage of ringed cells per tibia. These observations suggest that IL-1 simultaneously caused egress of granulocyte precursors from BM and accelerated progression of cells from the proliferating into the maturing myeloid compartment. In addition, a depletion of CFU-GM per tibia was evident in treatment mice at that time. These findings are reminiscent of those recently reported by Metcalf et al in mice treated for six days with recombinant murine GM-CSF. These authors observed a significant reduction in femur cell counts and in marrow colony-forming cells despite enhanced splenic myelopoiesis and neutrophil infiltration of several organs. The depletion of marrow CFU-GM we observed at ~48 hours may reflect a relative delay in recruitment into the CFU-GM compartment following previously enhanced colony formation. Alternatively, loss of marrow CFU-GM may indicate transfer of progenitors to spleen, in view of the subsequent increase in splenic myelopoiesis of treatment mice.

During the past several years, a series of studies involving mice and nonhuman primates have examined the effects of recombinant G-CSF and GM-CSF on myelopoiesis in vivo. In general, leukocytosis sustained throughout the course of treatment has occurred along with hastened granulocyte recovery following myelosuppression. Numerous human trials of these hematopoietic growth factors have recently been reported or are currently under way. Preliminary data in patients with the acquired immune deficiency syndrome (AIDS), with myelodysplastic syndromes, and with cancer receiving myelosuppressive chemotherapy, or autologous BM transplant suggest potential therapeutic benefit of these proteins. Similarly, the ability of IL-1 to enhance myelopoiesis in various clinical settings deserves further study in both animal and human trials. In addition to its effects on hematopoiesis, IL-1 has recently been shown to cause tumor necrosis and enhance CPM cytotoxicity in tumor bearing mice and to confer radioprotection. Thus, IL-1 has several important therapeutic implications for use as adjuvant therapy in neoplastic disease.

ACKNOWLEDGMENT

We are indebted to Dr Peter Lomedico for donating the rhlL-1, to Shirley Uebelhoer for her secretarial assistance, to Kim Dohren for her artistic skills, and to Phil Jewett for his technical assistance.

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