In a series of studies designed to extend our understanding of interleukin-2 (IL-2) and to study the effect of biologic response modifiers on bone marrow, we observed that administering recombinant human (rH) IL-2 to normal mice resulted in an increase in the frequency of colony-forming units-culture (CFU-C) in bone marrow. In addition, rH IL-2 was able to accelerate host recovery from cyclophosphamide (CTX)- or radiation-induced bone marrow depression and peripheral blood leukopenia. Not only can rH IL-2 accelerate, in a dose-dependent manner, the return of bone marrow, peripheral blood cellularity, and CFU-C frequency to normal levels following cytoreduction by CTX or irradiation, but it also significantly increases CFU-C frequency to greater than normal levels. Furthermore, rH IL-2 can significantly prolong survival of animals receiving a lethal dose of irradiation or CTX. Thus, multiple mechanisms are responsible for the synergistic therapeutic activity associated with rH IL-2 and CTX. rH IL-2 does not act only as an immunomodulatory agent in the presence or absence of suppressor T cells, but also accelerates host recovery from cytoreductive agents, resulting in decreased leukopenia and perhaps resistance to secondary infection. Thus, rH IL-2 plus chemotherapy may increase therapeutic activity against neoplastic disease, not only by adding immune stimulation to the direct antitumor effect of the drug but also by allowing delivery of higher, more effective doses of chemotherapy. This is a US government work. There are no restrictions on its use.

INTERLEUKIN-2 (IL-2) is a lymphokine produced by helper T cells and large granular lymphocytes and has numerous immunomodulatory properties both in vitro and in vivo. Several laboratories have demonstrated that recombinant human (rH) IL-2, as a single agent, has therapeutic activity against established pulmonary, hepatic, subcutaneous, and peritoneal tumors. In addition, therapeutic activity has been reported when rH IL-2 is administered in conjunction with cultured specific immune cells or lymphokine-activated killer cells, which can mediate the regression of transplantable tumors. Other studies have demonstrated additive or synergistic therapeutic activity with rH IL-2 and cyclophosphamide (CTX). The increase in therapeutic activity has been suggested to be associated with the selective toxicity of CTX for suppressor T-cell activity and the resultant outgrowth or proliferation of helper T-cell activity. There are, in addition, several other potential mechanisms of therapeutic activity for rH IL-2 and CTX, including tumor debulking by the drug, immunorestoration, and myelorestoration by rH IL-2.

Recently, it became apparent that T cells play an important role in hematopoiesis, eg, bone marrow allografts depleted of T cells to prevent graft-versus-host disease (GVHD) frequently fail to engraft. These data suggest that T cells may produce factors that promote hematopoietic stem cell activity and, indeed, GM colony-stimulating factor (GM-CSF) production by T cells has been shown. On the other hand, some patients with aplastic anemia have recovered bone marrow function in response to treatment aimed at destroying or inhibiting T-cell (particularly suppressor T cell) function. We wondered whether rH IL-2 is a T-cell product that might modulate bone marrow activity either directly or indirectly through release of hematopoietic factors.

Evidence from clinical trials with rH IL-2 at the maximum or supramaximum tolerated dose suggested that leukopenia can be induced by rH IL-2 or factors it induces. However, preclinical data suggest that this peripheral blood leukopenia may be due to marginalization of cells to parenchymal organs, most notably lung and liver. The present study demonstrates the ability of rH IL-2 to increase bone marrow cellularity and proliferation of stem cell activity in normal animals as well as in animals receiving lethal or sublethal doses of CTX or lethal doses of γ-irradiation. Northern blotting analysis revealed that rH IL-2 upregulates cytoplasmic RNA levels of GM-CSF and IL-3, suggesting that its effect on colony-forming unit-culture (CFU-C) levels may be secondary to the stimulation of conventional CF Ski activities. We conclude, therefore, that myeloid recovery is an additional mechanism associated with the therapeutic activity of rH IL-2.

MATERIALS AND METHODS

Animals. Specific pathogen-free female C57BL/6 mice (H-2b) and BALB/c athymic nude mice (H-2b) were obtained at age 4 weeks from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility. Recombinant murine (rM) interferon-γ (IFN-γ specific activity –1.3 x 10^7 U/mg) was provided by Biogen (Cambridge, MA). Recombinant murine (rM) interferon-γ (IFN-γ specific activity –1.3 x 10^7 U/mg) was obtained from...
Genentech (South San Francisco). Immunex (Seattle) supplied rM GM-CSF (specific activity 4 × 10^6 U/mg) and rH IL-1β (specific activity 1.2 × 10^6 U/mg). The specific activities of these materials varied slightly from lot to lot and were standardized using an aliquoted source as a reference. CTX was obtained from Dr Ven Narayan, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

Technical approach. The experiments were repeated at least three times, and any study in which either the positive or negative control did not function was repeated.

Assay of CFU-C frequency. Cell suspensions from bone marrow were counted in a hemocytometer, and viability was assessed by trypan blue exclusion. Three mice were used per group, resulting in three pools of two femurs each. Cell pools were diluted to the appropriate concentration in CMRL-1066 medium supplemented with sodium pyruvate, equine serum (10%), fetal bovine serum (FBS 20%), L-serine, and tryptic soy broth (0.6%). For colony growth, medium was supplemented with 0.3% bactoaggar (Difco Laboratories, Detroit) and 20 U/mL rM GM-CSF. Ninety-six-well flat-bottom plates (0.2 mL) were used for culturing, and 24 wells were seeded for each dilution of the cell suspension tested (10,000, 5,000, 1,000, and 100 cells per well). Plates were incubated for eight or nine days in 100% humidity, 5% CO2-air atmosphere. The wells were scored positive or negative for colonies containing >50 cells with a dissecting microscope at 100× magnification. CFU-C precursor frequency was calculated from the limiting dilution data by a probability distribution (described in statistical analysis).

Effect of CTX or γ-irradiation. At time 0, we administered a dose of CTX determined to be 95% lethal (580 mg/kg) for C57BL/6 mice or a lethal dose of γ-irradiation administered from a cesium source at 950 rad per C57BL/6 mouse. With both of these cytotoxic agents, the cytokines were administered by various routes and at different doses and schedules to determine their effect on cellularity, stem cell activity, and host survival. Peripheral blood lymphocyte (PBL) counts were also determined by retroorbital bleeding into a heparin-coated 100-μL capillary pipette, and the number of cells was counted in a Coulter counter.

Statistical analysis. The average number of leukocytes, CFU-C frequency, and total number of CFU-C were analyzed with the paired Student’s t test to determine treatment groups differed significantly from the appropriate control groups and reported as mean ± SE. We used ten animals in each group for the therapy studies and either three or five animals per group for the studies of CFU-C number and frequency. Pearson’s correlation coefficient was used to determine relationships between any two parameters. CFU-C precursor frequency was calculated from the limiting dilution data by a linear-regression analysis, the calculations being performed using the BMDP program and the IR module and shown with 95% confidence limits. The precursor frequency can be shown to equal the slope of the line of the logarithm of the fraction of nonresponding cultures v the mean number of cells per culture.28 We express the data in terms of precursor frequency per 10^6 cells. Confidence intervals were calculated using a form of the Student’s t test. Conformance of the data to linearity was tested with a chi-square analysis.

RESULTS

Dosage-dependent effect of rH IL-2 on bone marrow. The effect of rH IL-2 on bone marrow CFU-C activity in normal mice, as measured by marrow cellularity and CFU-C frequency, was examined 72 hours after intraperitoneal (IP) injection of rH IL-2. As shown in Fig 1, rH IL-2 increased the number of CFU-C per femur. The optimal response was observed after injection of 2.5 × 10^6 U/kg rH IL-2 per animal, and a significant response was also observed after animals received 5 × 10^6 U/kg rH IL-2. A single injection of lower doses of rH IL-2 had minimal effects of CFU-C activity at 24 hours, based on the total number of CFU-C per femur. The increase in total number of CFU-C per femur was associated with CFU-C frequency rather than an increase in total number of cells per femur (Fig 1). Thus, 24 hours after a single high dose of rH IL-2, increase appears to occur in the number of CFU-C but no increase occurs in total number of cells per femur.

Kinetics of CFU-C activity induced by rH IL-2. The kinetics of rH IL-2 augmentation of CFU-C activity was examined by injecting rH IL-2 IP at 5 × 10^5 or 10^6 U/kg at various times and determining the total number of CFU-C per femur, as shown in Fig 2. The maximal increase in CFU-C per femur at the higher dose of rH IL-2 was observed on day 6 and had not declined to control levels by day 11.
Animals receiving $5 \times 10^5$ U/kg rH IL-2 had a smaller increase that peaked later (day 8). Since the $T_1$ serum half-life ($t_{1/2}$) of rH IL-2 is short (~55 minutes), the delayed effects on stem cell activity suggest that IL-2 may be initiating or inducing a complex physiologic response in the target tissue, perhaps mediated more directly by another cytokine.

**Effect of multiple injections of rH IL-2.** The effects of multiple injections of rH IL-2 were examined by injection $5 \times 10^4$ or $10^5$ U/kg rH IL-2 per animal IP for one or three
Assay D-0:BRMs QD 4X

Fig 4. C57BL/6 or BALB/c athymic nude mice received an IP injection of either rH IL-2 (2.5 x 10^6 U/kg), rH IL-1 (2.5 x 10^6 U/kg), or rM IFN-γ (2.5 x 10^6 U/kg) daily for four days. The control group received four daily injections of saline. Twenty-four hours after the last injection, femurs were removed, a number of cell pools were formed, and the number of cells per femur was determined. In addition, the CFU-C frequency was ascertained, and the total number of CFU-C per femur was calculated by multiplying the average number of cells per femur by the average CFU-C frequency.

days and measuring the bone marrow CFU-C frequency and bone marrow and PBL cellularity on either day 4 or 8. Multiple injections of rH IL-2 significantly increased CFU-C number and frequency on day 8 but not day 4 at doses that had minimal activity after a single injection \( [P = .027 \text{ (Fig 3)}] \). The increase in CFU-C number and frequency on day 8 was greater than that observed on day 4 for 5 x 10^6 U/kg rH IL-2, but multiple injections of 5 x 10^4 U/kg rH IL-2 produced an earlier peak in CFU-C frequency and total number (Fig 3). Bone marrow cellularity also increased after single or multiple injections of high or low doses of rH IL-2, although the number of PBLs did not change at either time point examined. Thus, three injections of 5 x 10^4 U/kg IL-2 significantly increased CFU-C frequency \( [P = .013] \) and total number \( [P = .007] \) on day 4 and resulted in an increase in the total number of cells per femur on day 8. In contrast, there was no change in the number of PBL on day 4 or 8.

Role of T cells in CFU-C response to IL-2. The ability of nude mice (compared with immunologically intact C57BL/6 mice) to respond to rH IL-2, rM IFN-γ, or rH IL-1β was examined 24 hours after the last of four daily IP injections. Figure 4 shows the results of these studies, including an analysis of total number of CFU-C per femur. Normal immunologically intact C57BL/6 mice had a dramatic five- to 25-fold increase in total number of CFU-C per femur, as compared with a maximum 0.5% increase (nonsignificant) in nude animals receiving rM IFN-γ, rH IL-2, or rH IL-1. A similar lack of response was noted in nude mice injected with rM GM-CSF (results not shown). We conclude from these studies that cytokines require T cells to increase the total number of CFU-C per femur. As discussed previously, only minimal effects on total peripheral blood, spleen, and bone marrow cellularity were observed after administration of these lymphokines to either normal or nude animals.

Effect of rH IL-2 on CTX-induced leukopenia. Dose-limiting toxicity with many chemotherapeutic agents is associated with myelosuppression resulting in neutropenia. CTX is one of the most important antineoplastic agents in the oncologist’s armamentarium, with neutropenia the dose-limiting toxicity for this agent. Thus, CTX was used as the “chemotherapeutic” model agent for subsequent studies. To
examine the effect of rH IL-2 administration on leukopenia induced by CTX, it was injected IP at 200 mg/kg to normal mice and rH IL-2 was administered at various doses 24 hours later. Figure 5 shows that a single injection of at least $5 \times 10^4$ U/kg rH IL-2 can significantly increase the CFU-C activity to levels equal to or greater than those observed in normal animals. There was no increase in bone marrow cellularity at day 4, but the increase in total CFU-C number was again associated with CFU-C frequency. However, the increase in CFU-C activity per femur was greater following two injections of rH IL-2 on days 1 and 3 as compared with a single injection on day 1 (Fig 6). The increase in CFU-C total number on day 8 was increased by two injections of $5 \times 10^5$ U/kg rH IL-2 per animal, whereas a dosage dependency for CFU-C response developed with a single injection. There were no differences in cellularity after either one or two injections of rH IL-2.

Effects of timing of rH IL-2 administration on recovery from CTX treatment. The effect of the schedule of rH IL-2 administration on recovery from CTX-induced myelosuppression is shown in Fig 7. Animals received 200-mg/kg IP injections of CTX. rH IL-2 was administered either before or after CTX as a single injection or multiple injections at $2.5 \times 10^6$ U/kg, and marrow CFU-C activity was measured on day 4. Multiple injections of rH IL-2 beginning 24 hours after CTX produced an increase in bone marrow CFU-C frequency and total number per femur, as did a single injection 48 hours after CTX treatment. In this study, a single injection 20 hours before or after CTX administration did not increase CFU-C frequency as compared with administration beginning 48 hours after CTX injection. Thus, multiple injections of rH IL-2 and/or injections beginning 48 hours after CTX administration appear to produce the greatest degree of recovery from CTX-induced myelosuppression.

Effect of IL-2 on survival after toxic doses of CTX. To examine the biologic effect of rH IL-2–induced bone marrow stimulation, mice received lethal doses of CTX, and the ability of rH IL-2 to prolong survival was examined. Figure 8 shows that daily injection of $2.5 \times 10^6$ U/kg rH IL-2 per animal for seven days significantly prolonged survival after an LD$_{50}$ (P = .02) or LD$_{90}$ (P = .04) dose of CTX was administered. In this study and other studies, rH IL-2 significantly prolonged survival and increased the number of animals surviving CTX administration.

Effect of rH IL-2 after lethal irradiation. Additional studies were undertaken to examine the ability of rH IL-2 to prolong survival of mice receiving lethal doses of $\gamma$-irradiation (Fig 9). Eight-week-old C57BL/6 mice, housed in a specific pathogen-free facility, received a lethal dose of $\gamma$-irradiation from a cesium source (950 rad). rH IL-2 ($2.5 \times 10^6$ U/kg) was administered either 20 hours before irradiation or daily for three days beginning two hours after irradiation. On day 7 after irradiation, a cohort of animals was removed, and their CFU-C frequency and bone marrow cellularity were determined (results not shown). rH IL-2 injections on days 0 to 3 (day 0 was more than two hours after irradiation) significantly prolonged survival (P = .05), whereas the preradiation injection (–20 hours) did not (P = .12). This observation agrees with our finding that postirradiation rH IL-2 injections significantly increased the number of CFU-C per femur. There was, however, minimal effect on bone marrow cellularity at day 7. Statistical analysis revealed a significant correlation between survival and total number of CFU-C (P = .0014), bone marrow cellularity (P = .0007), and CFU-C frequency (P = .0000). Based on these findings and observation of a greater increase in CFU-C frequency after multiple injections of rH IL-2, we repeated the study and gave daily IP injections (five days per week) of rH IL-2 at $2.5 \times 10^6$ U/kg or rM GM-CSF at 50 U/kg for 2 weeks (Fig 10). A significant prolongation of survival was observed after rH IL-2 (P = .0006) or rM GM-CSF (P = .0000) administration. The combination of rM GM-CSF and rH IL-2 (P = .0000) resulted in a greater prolongation of survival as compared with rH IL-2 (P = .005) or rM GM-CSF (P = .017) alone. Although survival was prolonged as compared with the previous studies, all animals did eventually die. Therefore, to achieve long-term survival after lethal irradiation, administration for
Fig 7. Mice received an IP injection of CTX at 300 mg/kg and then a single injection of rH IL-2 at $2.5 \times 10^9$ U/kg at various times. On day 4 after CTX administration, bone marrow cellularity ($\times 10^6$), PBL number ($\times 10^6$), CFU-C frequency ($\times 10^{-6}$), and number of CFU-C per femur ($\times 10^6$) were determined.

>ten days may be necessary. On day 10 after lethal irradiation, a cohort of animals was examined and bone marrow cellularity, CFU-C activity, and PBL number was determined. In addition, PBL number was examined three days after lethal irradiation. Cellularity, CFU-C frequency, and total CFU-C per femur increased significantly in all groups, which correlated with prolongation of survival according to Pearson’s correlation analysis ($P = .001$ for bone marrow cellularity, $P = .005$ for CFU-C frequency, and $P = .001$ for CFU-C per femur). PBL number correlated with survival on day 10 ($P = .005$) but not on day 3 ($P = .274$).

**DISCUSSION**

Since the cloning of the gene for rH IL-2, much has been learned about the immunomodulatory and immunotherapeutic properties of this cytokine. IL-2 was originally...
100

Cumulative Proportion Surviving

Survival (days)

Survival (days)

Fig 9. Mice received a lethal dose of \( \gamma \)-irradiation from a cesium source (950 rad) on day 0. Either 20 hours before or beginning two hours after irradiation, the mice were given rH IL-2 at \( 2.5 \times 10^8 \) U/kg by IP injection. Animals receiving rH IL-2 beginning two hours after irradiation also received injections on days 1 through 3. Statistics are compared with those for saline control animals, and survival is shown as a Kaplan-Meier plot (\( n = 10 \)).

Fig 10. Mice received a lethal dose of \( \gamma \)-irradiation from a cesium source on day 0 and were injected IP with rH IL-2 at \( 5 \times 10^8 \) U/kg on days 0 and 10, rM GM-CSF at 50 \( \mu \)g/kg on days 0 through 10 by IV injection, or a combination of rH IL-2 and rM GM-CSF after irradiation of the abovementioned schedules. Survival was plotted by the Kaplan-Meier method (\( n = 10 \)).

reported to act largely on T cells\(^{3,7,11,14,15} \) expressing the TAC receptor.\(^{64-69} \) In addition, the ability to use recombinant proteins has resulted in demonstration of therapeutic activity with rH IL-2 alone\(^ {16-21,53-56,58-67} \) or in combination with lymphokine-activated killer cells or T cells cultured in vitro.\(^ {22-31,68-73} \) This activity has been demonstrated in preclinical studies as well as clinical trials. We report a new IL-2-associated property, the ability to increase CFU-C activity and to facilitate recovery from drug- or irradiation-induced myelosuppression.

rH IL-2 increases mitosis of thymocytes and bone marrow cells in vivo\(^ \)\(^7\)\(^6\) and, in the present studies, elevated bone marrow CFU-C activity in a dose-dependent manner. rH IL-2 appears to increase CFU-C frequency by upregulating production of CSF and IL-3. Preliminary studies in which we used molecular probes to study RNA levels of IL-3 and GM-CSF after rH IL-2 treatment suggested that their induction in bone marrow cells may be the mechanism of activity. rH IL-2 was inactive in nude mice, suggesting that T cells are important for its myelostimulatory activity and may be the source of the CSF/IL-3 activity. In addition, rH IL-2 can facilitate host recovery from the myelosuppression induced by cytotoxic agents such as CTX and \( \gamma \)-irradiation. Indeed, it can prolong survival and, in some cases, "rescue" animals receiving lethal doses of CTX or irradiation. The greatest biologic activity, as measured by survival, is
observed when rH IL-2 is injected immediately after irradiation or as soon as the cytotoxic agent has cleared from the circulation (≥48 hours for CTX in mice). Although CTX has been administered before irradiation, in addition, we noted a protective effect when IL-2 was administered chronically after lethal doses of irradiation. In summary, rH IL-2 dramatically affects CFU-C frequency in normal animals as well as in animals receiving myelosuppressive doses of cytotoxic agents or irradiation. However, the effects of rH IL-2, rM IFN-γ, and rH IL-1 appear to depend on the presence of T cells, since these compounds are inactive in nude mice. This suggests that either (a) nude mice do not produce GM-CSF or IL-3 in response to these cytokines; or (b) as we have observed, nude mice do not respond to rM GM-CSF or rH G-CSF; or (c) an additional signal or cytokine produced by T cells is required for a myeloid response to a single cytokine. Nonetheless, nude mice do have myeloid stem cells that proliferate; therefore, they do produce and respond to growth factors, suggesting that there may be additional novel cytokines important to myeloid regulation. The additive or synergistic therapeutic activity of rH IL-2 with alkylating agents such as CTX appear in part to relate to its myelorestorative activity. Therefore, the mechanism of therapeutic activity associated with CTX and rH IL-2 is not limited to reduction of suppressor cell activity and augmentation of effector cells but may also relate to recovery from myelosuppression. However, a combination of these mechanisms of activity most likely is responsible for increased therapeutic activity.

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Myelostimulatory activity of recombinant human interleukin-2 in mice

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