Cytokine combinations were tested for their ability to expand murine bone marrow (BM) progenitors in short-term suspension cultures (Δ-cultures) with the aim of providing an enriched source of progenitors for BM transplantation (BMT). In a comparison of the efficacy of the combinations interleukin-1 (IL-1) + IL-3, IL-1 + kit-ligand (KL), and IL-1 + IL-6 + KL, BMT with IL-1 + KL expanded progenitors was found to be most effective in accelerating the recovery of peripheral blood leukocytes, platelets, and erythrocytes in lethally irradiated mice. The ex vivo expansion of BM in IL-1 + KL-stimulated Δ-cultures also greatly reduced the number of transplanted cells needed to provide radioprotection. All mice survived at least 30 days when receiving 5 × 10^3 Δ-cultured d1 5-fluorouracil (5-FU) BM cells (BM cells harvested 1 day after 5-FU administration), whereas complete survival of mice receiving fresh d1 5-FU BM required BMT with a 200-fold greater number of cells. BMT with expanded BM lead to predominantly donor-derived hematopoietic reconstitution for 280 days postprimary BMT and another 71 days after secondary BMT. The expansion of BM did not adversely affect the proliferative capacity and lineage potential of the stem cell compartment.

A NUMBER OF cytokines active in regulating hematopoiesis have been discovered and produced in large quantities through the use of recombinant DNA technology. These purified growth factors make the in vitro and in vivo manipulation of hematopoiesis possible. Insight into the growth factor requirements of hematopoietic progenitors, including the most primitive progenitors, is aiding in the development of systems for more effective maintenance and/or expansion of these cells in vitro. The in vitro expansion of early progenitor cells with a high proliferative capacity could provide a rich source of transplantable bone marrow (BM). In vitro culture conditions that optimize the proliferation of progenitors while minimizing stimuli for the terminal differentiation of these progenitors are being sought. In particular, the maintenance of stem cell totipotentiality and proliferative capacity during in vitro culture is necessary for protocols, such as gene therapy, that involve the ex vivo manipulation of BM.

In a previous study, we investigated the in vitro expansion of murine BM by the synergistic combination of interleukin-1/β (IL-1) and IL-3. Mice received transplants consisting of BM, enriched for early progenitors by the in vivo administration of 5-fluorouracil (5-FU) 1 day before harvesting (d1 5-FU BM) or d1 5-FU BM that had been expanded in vitro for 7 days in suspension cultures (Δ-cultures) stimulated with IL-1 + IL-3. Lethally irradiated mice receiving transplants of Δ-cultured BM showed an accelerated recovery of peripheral blood cells, required fewer transplanted BM cells for survival, and had a comparable hematopoietic proliferative capacity up to 10 weeks after BM transplantation (BMT) relative to control mice receiving fresh d1 5-FU BM.

The identification of the ligand for the c-kit receptor, kit-ligand (KL), has uncovered an important regulator of hematopoiesis. Alone, KL (also known as mast cell growth factor, stem cell factor, or Steel factor) is a strong stimulator of murine mast cell growth, but has only a limited ability to stimulate myelopoiesis. However, KL has powerful synergistic activity in combination with hematopoietic growth factors. Synergism between KL with erythropoietin (Epo), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-3 in stimulating human hematopoietic progenitors in clonal cultures has been reported. The ability of KL to stimulate populations of primitive hematopoietic progenitors is supported by the increased size and recloning potential of colonies stimulated by combinations of growth factors and KL. In assays of murine BM progenitors, KL was shown to synergize with Epo, G-CSF, macrophage colony-stimulating factor (M-CSF), GM-CSF, IL-1, IL-3, IL-6, and IL-7 in combinations of two or more growth factors. Work in our laboratory has shown the combinations IL-1 + KL or IL-1 + IL-6 + KL to be synergistic in the stimulation of murine d1 5-FU BM. Stimulation of Δ-cultures with IL-1 + KL or IL-1 + IL-6 + KL resulted in over 1,000-fold expansions in the number of low proliferative potential–colony-forming cells (LPP-CFC) and over 100-fold expansions in the number of high proliferative potential–colony-forming cells (HPP-CFC). Δ-Cultures stimulated with IL-1 + KL were also capable of stimulating over a 100-fold increase in the number of colony-forming units–spleen (CFU-S) during 7 days of in vitro incubation. In comparison to the combination IL-1 + IL-3, the combinations IL-1 + KL or IL-1 + IL-6 + KL were more effective in stimulating the expansion of d1 5-FU BM in Δ-cultures.

Herein we investigated the efficacy of the cytokine combinations IL-1 + IL-3, IL-1 + KL, and IL-1 + IL-6 + KL in stimulating the expansion of murine BM for purposes of...
BMT, BMT with d1 5-FU BM expanded in Δ-cultures by the stimulus IL-1 + KL resulted in the swiftest recovery of peripheral blood cell numbers in lethally irradiated mice. Expansion of BM by the combination IL-1 + KL also drastically reduced the number of BM cells required to rescue lethally irradiated mice. We show long-term (40 weeks) engraftment of host mice by in vitro-expanded donor BM. The short-term growth of BM in vitro did not appear to damage the lineage potentiality or proliferative capacity of the early hematopoietic progenitor compartment.

MATERIALS AND METHODS

Mice. Male and female (C57BL/6 × DBA/2)F1(B6D2Fr) mice (The Jackson Laboratory, Bar Harbor, ME) were at least 8 weeks old at the onset of each experiment. All mice were maintained in cages receiving filtered air and were provided acidified and/or autoclaved drinking water. Sentinel mice, housed along with experimental mice, were routinely shown to be free of specific pathogens.

BM. BM was obtained from the femora and sometimes the tibia of at least 3 mice per donor group. Donor mice purged with 5-FU received an intravenous dose of 150 mg/kg 5-FU 1 or 8 days before harvesting the BM. Analysis of the hematopoietic tissues of transplanted mice was performed 1 day after 5-FU administration by intraperitoneal injection. The total mouse BM cellularity and progenitor cell content was estimated by assuming that 1 femur contains 7% of a mouse's total BM.14

Cytokines. Purified recombinant human IL-1β (IL-1; specific activity, 1.32 × 105 U/mg; Syntax Laboratories, Inc, Palo Alto, CA) was used at 100 U/mL. The partially purified concentrated conditioned medium from the WEHI 3B D cell line was used as a source of murine IL-3; IL-3 was used at a concentration equivalent to 20 ng/mL purified recombinant murine IL-3, which achieved the maximum growth of BM progenitors from B6D2Fr mice. Purified recombinant human IL-6 (Immunex Corporation, Seattle, WA) was used at 20 ng/mL. Purified recombinant mouse KL (Immunex Corporation) was also used at 20 ng/mL.

In vitro BM expansion. BM was expanded in cytokine-stimulated or unstimulated Δ-cultures as previously described.2 Normal BM (NBM) or d1 5-FU BM was grown in culture medium containing Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY), 20% fetal bovine serum (FBS; Hyclone Laboratories Inc, Logan, UT), and 0.05 mg/mL gentamicin (Gibco) in 75 cm2 tissue culture flasks for 7 days at 37°C in air supplemented with 5% CO2. BM expansion was initiated with 1 × 106 d1 5-FU BM cells in 40 mL of culture medium/flask. However, in the experiment described in Fig 2, Δ-cultures were initiated with the equivalent of 1 femur of d1 5-FU BM or 1/8 of a femur of NBM per flask.

BMT. Host mice received a split dose of radiation from a dual 137Cs γ-ray source totaling 12.5 Gy. The radiation was administered as an 8.0-Gy dose followed by a 4.5-Gy dose 3 hours later. BM cells were transplanted by tail vein injection in a solution of Earl's Balanced Salt Solution (EBSS) supplemented with 10 mmol/L HEPES (pH 7.2) (Gibco), 0.05 mg/mL gentamicin, and 100 mg/mL bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO), 2 to 3 hours after the final irradiation.

Enumeration of peripheral blood cells. Peripheral blood cell counts were measured in the recovering BMT recipients as previously described.2

BM progenitor cell assays. The numbers of HPP-CFC and LPP-CFC were determined in clonal colony assays as previously described.14,15 Clonal cultures contained IL-1 + IL-3 as a stimulus for the growth of hematopoietic progenitors. Highly cellular colonies with diameters of at least 0.5 mm were scored as HPP-CFC; smaller colonies with at least 50 cells were scored as LPP-CFC.19

The proliferative capacity of d1 5-FU BM from mice receiving transplants or control mice was analyzed using the Δ-assay as previously described.2,17 BM was stimulated with IL-1 + KL during the suspension phase of the assay. After 7 days of suspension culture, secondary HPP-CFC and LPP-CFC were grown in clonal cultures as described above.

Southern blot analysis of host mouse hematopoietic tissues. Genomic DNA was prepared from hematopoietic tissues using the method of Telco.20 Y-chromosome content was measured using Southern blot analysis.21 Genomic DNA samples were digested with the restriction endonuclease Pvu II (Boehringer Mannheim Corp, Indianapolis, IN) and electrophoresed on a 1.0% agarose gel. DNA samples were subsequently transferred to a nylon membrane (ICN Biotrans, Irvine, CA) by electroblotting (Transphor Electrobetter; Hoefer Scientific Instruments, San Francisco, CA). The nylon membranes were baked for 2 hours at 80°C. The blotted membranes were prehybridized (15 to 30 minutes) and hybridized with a 32P-labeled probe (approximately 24 hours) in a solution of 1 mmol/L EDTA, 7% sodium dodecyl sulfate (SDS), 0.5 mol/L Na2PO4, pH 7.2, at 65°C. A 720-bp fragment of the plasmid pY2, containing murine Y-chromosome repetitive DNA, was used as a probe.22 Hybridized blots were washed twice for 30 minutes at room temperature in 2× SSC, 0.1% SDS and once at 70°C for 40 minutes in 0.2× SSC, 0.1% SDS. The presence of bound radiolabeled probe was determined after 12 to 24 hours of radiography in the presence of intensifying screens.

Isolation of splenic T cells for Southern blot analysis. T cells from mice receiving transplants 280 days before with Δ-cultural BM were isolated from spleens harvested one day after 5-FU administration. Splenic cells were incubated in EBSS/BSA for 30 minutes at 4°C with 50 μg of biotinylated anti-L3T4 (GK1.5) and anti-Lyt-1 (53-7.313) antibodies and then washed free of any unbound antibodies. Antibody-coated cells were then isolated by binding to streptavidin-coated paramagnetic particles at a ratio of 5 particles per cell for 15 minutes, followed by 15 minutes of magnetic separation. DNA was then isolated from the paramagnetic particle-coated lymphocytes as described above.

T cells from non-5-FU-treated mice were magnetically isolated from spleen cells 210 days after BMT using a method similar to that described above. However, before coating the spleen cells with antibody, light-density nonadherent cells were recovered from a 1.090 g/mL Nycodenz gradient (Accurate Chemical and Scientific Corp, Westbury, NY) followed by a 4-hour adherence depletion at 37°C in tissue culture flasks. Splenic T cells were isolated using a biotinylated monoclonal antibody against the α subunit of the T-cell receptor (PharMingen, San Diego, CA).

Statistics. Results are presented as the mean or mean plus standard error (SE). Significance was determined using the two-tailed paired Student's t-test. Results were considered significant if P < .05.

RESULTS

Expansion of BM in cytokine-stimulated Δ-cultures accelerates the recovery of peripheral blood cells after BMT. The recovery of peripheral blood cell counts was accelerated in lethally irradiated mice receiving transplants of expanded BM relative to control mice receiving transplants of fresh d1 5-FU BM (Fig 1). The in vitro expansion of 1 × 106 d1 5-FU BM in IL-1 + IL-3, IL-1 + KL, or IL-1 + IL-6 + KL before BMT resulted in early myeloid, erythroid, and platelet recoveries. Peripheral blood neutrophils were
the first lineage to recover in mice transplanted with Δ-cultured cells. The most rapid recovery in neutrophils was observed in mice receiving transplants of BM expanded in IL-1 + KL, resulting in significantly greater numbers of circulating neutrophils, during the period 4 to 10 days post-BMT, relative to mice transplanted with fresh d1 5-FU BM (P < .05 on all 7 days). During this same period, neutrophil numbers in mice receiving transplants of BM expanded in Δ-cultures stimulated by IL-1 + IL-3 or IL-1 + IL-6 + KL were significantly higher (P < .05) for 4 or 5 of the 7 days, respectively, relative to mice receiving transplants of fresh d1 5-FU BM. Although mean neutrophil numbers were observed to recover the fastest in mice receiving transplants of IL-1 + KL-expanded BM, there was no significant difference between the number of neutrophils in mice receiving transplants of IL-1 + KL- and IL-1 + IL-6 + KL-expanded BM, thus suggesting that the addition of IL-6 to IL-1 + KL-stimulated Δ-cultures does not notably retard or improve the ability of the expanded BM to reconstitute peripheral blood cell numbers after BMT. Neutrophil numbers in mice receiving transplants of IL-1 + KL-expanded BM were, however, significantly higher on days 4 and 6 post-BMT than in mice receiving transplants of IL-1 + IL-3-expanded BM (P < .03), suggesting, therefore, that IL-1 + KL is more effective in stimulating the ex vivo expansion of BM than IL-1 + IL-3. The increased number of total white blood cells observed from 4 to 10 days post-BMT in mice receiving transplants of expanded BM populations was caused in most part by the accelerated recovery of neutrophils during the early phase of engraftment. Platelet recovery was improved during the period of 6 to 12 days post-BMT in mice receiving transplants of Δ-cultured BM as compared with those receiving transplants of d1 5-FU BM. Platelet numbers during days 8 to 10 post-BMT were 2.1- to 5.5-fold higher in recipients of Δ-cultured BM relative to control mice. These increases in platelet numbers were significantly higher (P < .05) in all three groups of mice receiving transplants of expanded BM on days 9 and 10 post-BMT relative to mice receiving transplants of fresh d1 5-FU BM. Hematocrits were normal at 10 days post-BMT in recipients of ex vivo–expanded BM, whereas d1 5-FU BM–transplanted mice had a mean hematocrit of 21.7% (P < .001 for all Δ-cultured BM recipients vs d1 5-FU BM recipients). The BM expansion protocol using IL-1 + KL was overall the most successful at accelerating the recovery of peripheral blood cell numbers and was therefore used in all further experiments.

The leukocyte recovery patterns of mice receiving transplants of six BM populations differing in progenitor cell content were investigated (Fig 2). All lethally irradiated mice received transplants of an equivalent proportion (0.1 femur) of total donor BM. Because it has been shown that a single dose of 5-FU does not reduce the numbers of long-term repopulating stem cells, it was reasoned that all the donor BM populations in this experiment began with an equivalent number of stem cells.23 However, the ex vivo culturing of these cells could affect the numbers of stem cells. The kinetics of neutrophil reconstitution of the three non-Δ-cultured BM populations (NBM, d1 5-FU BM, and d8 5-FU BM) was varied. The slowest recovery was shown in mice receiving transplants of d1 5-FU BM; neutrophil numbers began to increase between days 9 and 11 after BMT (Fig 2). Both NBM– and d8 5-FU BM–transplanted mice showed neutrophil recoveries that started 5 to 6 days post-BMT. Mice receiving d8 5-FU BM did exhibit a more rapid increase in neutrophil numbers, beginning between days 8 and 9, once recovery had begun relative to hosts receiving transplants of NBM. Mice receiving BM expanded in IL-1 + KL showed the earliest recovery of leukocyte numbers (Fig 2). The recovery of these mice began 4 to 5 days after BMT. These results show that the Δ-culturing of both NBM and d1 5-FU BM in IL-1 + KL can result in an
accelerated recovery of peripheral blood leukocytes relative to hosts receiving transplants of fresh BM populations. However, BMT of Δ-cultured d1 5-FU BM cells resulted in a faster reconstitution of neutrophil numbers than was observed with the transplantation of Δ-cultured NBM. The accelerated recovery of peripheral blood cells in mice receiving transplants of Δ-cultured BM was clearly dependent on the addition of cytokines to the Δ-cultures. Mice receiving d1 5-FU BM Δ-cultured in the absence of added cytokines had a leukocyte recovery pattern similar to mice receiving transplants of fresh d1 5-FU BM.

Long-term survival and peripheral blood cell reconstitution of mice receiving transplants of IL-1 + KL-expanded BM. The long-term repopulating potential of limiting numbers of Δ-culture-expanded BM cells was compared with the repopulating potential of 1 × 10⁶ d1 5-FU BM cells. The survival of recipients of expanded BM or d1 5-FU BM transplants was observed for up to 280 days. All untreated donor mice as well as mice receiving transplants of either 1 × 10⁶ d1 5-FU BM or 1 × 10⁶ IL-1 + KL-expanded BM cells survived for 280 days after BMT. Deaths did occur in mice receiving transplants of smaller doses of Δ-cultured BM. All recipients of 1 × 10⁴ and 5 × 10³ IL-1 + KL-expanded BM cells survived the first 3 weeks after BMT. However, only 7 of 15 mice receiving transplants of 1 × 10³ Δ-cultured BM cells survived the first 3 weeks after lethal irradiation. The survivors of transplants of 1 × 10³ IL-1 + KL-expanded BM cells were reconstituted with a remarkable 0.016% of a donor's femur or 0.0011% of the total donor's BM. Our earlier results showed that mice receiving transplants of 1 × 10⁶ d1 5-FU BM cells did not survive past 14 days post-BMT. These results show that in vitro BM expansion stimulated by IL-1 + KL can dramatically reduce the number of fresh BM cells required for radioprotection during the early phase of hematopoietic reconstitution after a lethal dose of radiation.

The peripheral blood leukocyte and platelet numbers were assessed 280 days post-BMT with fresh or expanded d1 5-FU BM. With the exception of surviving hosts receiving transplants of 1 × 10³ Δ-cultured BM cells, recipients of both d1 5-FU BM and Δ-cultured BM showed similar numbers of peripheral blood cells as compared with donor mice not receiving transplants. Two of five mice that survived 280 days after BMT with 1 × 10³ expanded BM cells showed decreased numbers of circulating neutrophils (41% of control) and platelets (65% of control) relative to donor mice not receiving transplants.

Male donor hematopoietic cells were distinguished from female host cells by Southern blot analysis of genomic DNA isolated from the hematopoietic tissues of mice 280 days (40 weeks) after BMT. DNA was prepared from the peripheral blood, BM, and spleen of mice receiving transplants of d1 5-FU BM or Δ-culture-expanded BM. When Southern blots of host mouse hematopoietic tissues were probed with...
a Y-chromosome-specific repetitive DNA fragment, host mice were shown to be predominantly reconstituted with donor-derived male cells. Only a few mice who received transplants of low numbers of Δ-cultured BM cells showed any chimerism of male and female DNA (data not shown). Furthermore, when secondary female hosts received transplants of the d1 5-FU BM from mice receiving transplants of Δ-cultured BM 280 days earlier, the secondary hosts were also reconstituted with male cells 71 days after secondary BMT (Fig 3). Splenic T cells were isolated using monoclonal antibodies and paramagnetic particle separation. T cells isolated in this manner 210 or 280 days after primary BMT were shown to be derived from Δ-cultured donor BM cells (Fig 4).

The in vitro expansion of BM in IL-1 + KL does not decrease the long-term proliferative capacity of stem cells. In an earlier study, we showed that the numbers of primary HPP-CFC and LPP-CFC, present in the d1 5-FU BM of mice receiving transplants 5 and 10 weeks earlier, were diminished relative to animals not receiving transplants. This loss of hematopoietic potential was accentuated in the numbers of secondary HPP-CFC and LPP-CFC generated by the growth of the 5-FU-purged BM in Δ-cultures stimulated with IL-1 + IL-3.17 In this study, the greater proliferative stimulus of IL-1 + KL was substituted for the stimulus IL-1 + IL-3 in the suspension culture phase of the Δ-assay.17

Primary HPP-CFC and LPP-CFC, as well as Δ-culture-generated secondary HPP-CFC and LPP-CFC, were measured from the d1 5-FU BM pooled from 3 mice/group 5 and 10 weeks after BMT (Fig 5). The numbers of primary and secondary colony-forming progenitors were most abundant in control animals not receiving transplants relative to all groups of mice that received transplants at both 5 and 10 weeks post-BMT (P ≤ .05). The numbers of primary LPP-CFC were nearly identical in mice receiving transplants of d1 5-FU BM or of Δ-culture-expanded BM. However, primary HPP-CFC numbers did differ among the transplant recipients. At 5 weeks post-BMT, there was no significant difference between the numbers of primary HPP-CFC in recipients of d1 5-FU BM and recipients of Δ-cultured BM, whereas 10 weeks after BMT, mice receiving fresh d1 5-FU BM had approximately 3.5-fold more high proliferative progenitors relative to the recipients of Δ-cultured BM cells (P ≤ .05). The pattern observed in the assay of primary HPP-CFC was also observed in the numbers of secondary HPP-CFC and LPP-CFC. The most dramatic difference among mice receiving transplants 5 weeks earlier was seen in the low number of secondary HPP-CFC found in the recipients of BM cells expanded in IL-1 + IL-3 as compared with mice receiving transplants of fresh d1 5-FU BM (P = .02). However, the numbers of secondary HPP-CFC did show some recovery by 10 weeks after BMT in the mice receiving IL-1 + IL-3 Δ-cultured BM. There were no significant differences observed between the numbers of primary or secondary colony-forming progenitors in mice receiving transplants of Δ-cultured cells expanded in IL-1 + KL and IL-1 + IL-6 + KL (P > .05), thus suggesting that the addition of IL-6 to Δ-cultures stimulated with IL-1 + KL had no beneficial effect on the numbers and proliferative capacity of early hematopoietic progenitors/stem cells.

Although peripheral blood cell numbers and hematopoietic tissue cellularity have returned to normal values 5 to 10 weeks after BMT, the early progenitor cell compartments may still be regenerating (Fig 5 and Muench and Moore). We therefore analyzed the hematopoietic tissues of individual mice receiving transplants 280 days earlier of either 1 × 10⁶ d1 5-FU BM cells or limiting numbers, 1 × 10⁴ to 1 × 10⁶, of d1 5-FU BM cells expanded in IL-1 + KL-stimulated Δ-cultures. The numbers of total BM cells were similar in all groups of transplant recipients; however, these values were 24% to 50% lower than in animals not receiving transplants (data not shown). Splenic tissue cellularity was more varied with the lowest values, representing a 56% reduction in cellularity relative to mice not receiving transplants, measured in mice receiving transplants of 1 × 10³ Δ-cultured BM cells. The number of cells measured in the BM and spleen were not significantly different between mice receiving transplants of either 1 × 10⁶ d1 5-FU BM cells or the equivalent number of cells expanded in IL-1 + KL-stimulated Δ-cultures (P > .05).

The numbers of primary HPP-CFC and LPP-CFC pres-
en in mice receiving transplants of fresh d1 5-FU BM or
limiting numbers of d1 5-FU BM cells expanded in IL-1 +
KL-stimulated Δ-cultures were measured 40 weeks after
BMT (Fig 6A and B). The number of progenitors detected
in the BM of mice receiving transplants of fresh d1 5-FU
BM or 1 × 10^6 Δ-cultured cells were both significantly re-
duced as compared with control mice not receiving trans-
plants (P < .01). The fewest HPP-CFC and LPP-CFC were
measured in mice receiving transplants of 1 × 10^3 Δ-cul-
tured BM cells. Relative to mice not receiving transplants,
the number of HPP-CFC present in mice receiving trans-
plants were up to 45-fold fewer splenic
LPP-CFC and up to 25-fold fewer BM LPP-CFC than mice
not receiving transplants. However, the numbers of CFU-C
measured in the d1 5-FU BM and spleen of mice receiving trans-
plants of 1 × 10^3 d1 5-FU BM cells or the equivalent
number of d1 5-FU BM cells expanded in Δ-culture were
not significantly different (P > .05), suggesting therefore
that the expansion of BM in Δ-cultures does not decrease
the long-term proliferative capacity of stem cells.

Forty weeks after BMT, the numbers of secondary HPP-
CFC and LPP-CFC generated from the BM of mice receiv-
ing transplants of 1 × 10^6 IL-1 + KL–expanded d1 5-FU
BM and mice receiving transplants of 1 × 10^6 fresh d1 5-FU
BM were equivalent (P > .05) (Fig 6C). However, the ability
of BM from mice receiving transplants to generate second-
ary CFU-C in Δ-cultures did not recover to normal levels
after 40 weeks post-BMT. The numbers of secondary HPP-
CFC were reduced 13- to 180-fold in mice receiving trans-
plants relative to mice not receiving transplants (P = .001 to
.003). The numbers of secondary LPP-CFC generated from
the BM of mice receiving transplants were reduced 8.1- to
46-fold as compared mice to not receiving transplants (P =
.0003 to .07). The fewest secondary CFU-C were generated
in Δ-cultures of BM obtained from mice receiving trans-
plants of 1 × 10^3 Δ-cultured cells.

The ability of d1 5-FU BM from BM transplant recipients
to further reconstitute secondary lethally irradiated hosts
was assessed (Table I). Primary hosts received transplants of
limiting numbers of d1 5-FU BM expanded in IL-1 + KL–
stimulated Δ-cultures or of fresh d1 5-FU BM. Forty weeks
after primary BMT, 0.25 femur of d1 5-FU BM was trans-
planted into secondary hosts. Six of eight mice receiving trans-
plants of BM from mice not receiving transplants, who
were age-matched to the original donors of the primary
transplant, survived for 71 days postsecondary BMT. Notice-
ably, few mice receiving transplants of BM from pri-
mary BM transplant recipients survived past 2 weeks post-
BMT. No mice receiving transplants of BM from primary
recipient mice of fresh d1 5-FU BM survived past 14 days post-
secondary BMT. Six of 32 mice receiving transplants of BM
from Δ-cultured BM recipients survived for 71 days post-
secondary BMT. Secondary BM transplant recipients were reconsti-
tuted with donor BM, as indicated in Fig 3. The diminished
ability of previously transplanted BM to reconstitute second-
ary hosts is consistent with the decreased number of CFU-
C– and Δ-culture–generated secondary CFU-C measured
from these mice relative to mice not receiving transplants
(Fig 6). The in vitro expansion of BM before primary BMT
did not decrease the ability of this BM to further reconsti-
tute secondary hosts.

**DISCUSSION**

The ex vivo expansion of BM progenitors accelerates the
recovery of peripheral blood cell numbers after BMT and
reduces the number of BM cells required for radioprotec-
tion. This study shows that the expansion of BM progeni-
tors in short-term suspension cultures stimulated by IL-1 +
IL-3, IL-1 + KL, or IL-1 + IL-6 + KL before BMT leads to
an accelerated recovery of peripheral blood cell counts (Figs
The expansion of progenitors capable of reconstituting multiple blood cell lineages was shown in the accelerated recovery of peripheral blood leukocytes, platelets, and erythrocytes. In a direct comparison of the efficacy of IL-1 + IL-3, IL-1 + KL, or IL-1 + IL-6 + KL. Progenitors were measured in IL-1 + IL-3–stimulated clonal cultures of pooled d1 5-FU BM obtained from 3 mice/group. Secondary CFU-C were generated in IL-1 + KL–stimulated Δ-cultures. BM was harvested 5 and 10 weeks post-BMT. The results are presented as the mean + SE of CFU-C numbers from 3 culture dishes. Significant differences (P ≤ .05) between mice receiving transplants of fresh d1 5-FU BM and mice receiving transplants of Δ-cultured BM are indicated by an asterisk. Note that the numbers of CFU-C are on a logarithmic scale. Refer to Fig 1 for further details of the mice used in this experiment.
al further showed that serial BMT resulted in a decrease in the number of CFU-S and presumably stem cells while maintaining the number of committed CFU-C. Upon BMT with this serially transplanted BM, deficient in early progenitors, the initial phase of hematopoietic reconstitution was intact; however, hematopoiesis was impaired beyond 3 weeks post-BMT. These results therefore showed that hematopoietic stem cells were not responsible for the initial reconstitution of the peripheral blood after BMT. In humans, increased numbers of committed CFU-C present in BM transplants and peripheral blood stem cell transplants have also been linked with an accelerated rate of hematopoietic reconstitution after transplantation. The importance of sufficient CFU-C numbers to the rapid recovery of peripheral blood leukocytes was further emphasized by the observation by Douay et al that reconstitution required a threshold number of CFU-C below which leukocyte recovery did not correlate with the number of CFU-C transplanted. The ex vivo expansion of human hematopoietic progenitors could thus lead to significantly accelerated peripheral blood cell recoveries by increasing the compartment of committed progenitors that can most quickly regenerate mature blood cell pools.

The expansion of d1 5-FU BM cells in IL-1 + KL-stimu-

![Graph A](imageA)

![Graph B](imageB)

![Graph C](imageC)
TRANSPLANTATION WITH IN VITRO-EXPANDED MARROW

Table 1. Survival of Secondary BM Transplanted Mice Receiving Transplants of d1 5-FU BM From Mice Previously Receiving Transplants of Limiting Numbers of Expanded BM

<table>
<thead>
<tr>
<th>1° BMT Group</th>
<th>No. of Survivors for 71 d</th>
<th>Day of Death Post-2° BMT*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor control†</td>
<td>6/8</td>
<td>9.5 ± 0.5</td>
</tr>
<tr>
<td>1 × 10^6 d1 5-FU BM</td>
<td>0/8</td>
<td>11.9 ± 0.7</td>
</tr>
<tr>
<td>1 × 10^6 ΔL-1 + KL</td>
<td>1/8</td>
<td>10.7 ± 0.8</td>
</tr>
<tr>
<td>1 × 10^6 ΔL-1 + KL</td>
<td>2/8</td>
<td>11.8 ± 1.0</td>
</tr>
<tr>
<td>5 × 10^3 ΔL-1 + KL</td>
<td>3/8</td>
<td>11.4 ± 0.9</td>
</tr>
<tr>
<td>1 × 10^3 ΔL-1 + KL</td>
<td>0/8</td>
<td>11.1 ± 0.7</td>
</tr>
</tbody>
</table>

Mice received transplants of d1 5-FU BM obtained from mice receiving transplants 280 days before. See the legend of Fig 6 for further details of the primary BMT recipients.

† The results are presented as the mean ± SE of the number of days until death post-BMT.

‡ The survival of 8 female B6D2F1 mice receiving transplants of male d1 5-FU BM from donors not receiving transplants was observed for 71 days in parallel with the secondary BM transplants. The donor mice used for the control transplants were born on the same day as the donor mice used for the primary Δ-cultured BM transplants.

lated Δ-cultures drastically reduces the number of BM cells required to provide radioprotection after lethal irradiation. We have previously shown that 1 × 10^6 d1 5-FU BM cells were incapable of rescuing mice after a dose of radiation totaling 12.5 Gy, whereas BMT with a 10-fold higher number of d1 5-FU BM cells resulted in nearly complete host survival. The expansion of BM cells in Δ-cultures reduced the number of BM cells required for engraftment by at least 200-fold. When mice received transplants of limiting numbers of IL-1 + KL-expanded BM cells, all mice receiving transplants of 5 × 10^7 d1 5-FU BM cells expanded ex vivo survived the early phase of recovery. Approximately one-third of the mice receiving transplants of 1 × 10^8 d1 5-FU BM cells expanded in Δ-cultures survived for greater than 3 weeks after BMT. However, the number of mice surviving for 40 weeks after BMT was decreased by transplantation of fewer than 1 × 10^6 BM cells.

The ex vivo expansion of BM progenitors does not impair the lineage potential and proliferative capacity of the hematopoietic stem cell compartment. Mice receiving transplants of ex vivo expanded d1 5-FU BM cells showed normal numbers of circulating peripheral blood cells 40 weeks after BMT, although a slight decrease in cell numbers was observed in recipients of the fewest number of Δ-cultured BM cells. One day after 5-FU administration, the hematopoietic tissue cellularity of mice receiving transplants 40 weeks previously were decreased relative to control BM recipients of BM transplants of the equivalent number of fresh d1 5-FU BM cells. The hematopoietic tissues of female hosts receiving transplants were predominantly donor derived as assessed through Southern blot analysis using a probe specific for host Y-chromosome repetitive DNA (Fig 3). Southern blot analysis also showed reconstitution of the T-cell lineage with Δ-cultured BM, suggesting the ex vivo growth of BM does not deplete the multilineage potential of stem cells (Fig 4).

Many studies have shown that BMT damages the hematopoietic stem cell compartment. Serial BMT has been shown to lead to an eventual loss of the transplant's BM reconstituting capacity. Harrison et al have also shown a decrease in the ability of previously transplanted BM to provide long-term BM reconstitution when compared with the repopulating ability of fresh BM. Although mice receiving BM transplants have been shown to have normal numbers of mature progenitors, a loss in early progenitors, CFU-S and HPP-CFC, has been detected in these mice. We have previously shown that the growth of BM from mice receiving transplants in Δ-cultures provides a sensitive assay for the detection of the loss in proliferative capacity of early progenitors, as measured by the number of secondary HPP-CFC and LPP-CFC generated in the suspension cultures. The use of the Δ-assay in probing the early progenitor compartment may also be of use in studies of primate and human BM because assays of HPP-CFC and CFU-S are not reliable or possible for these species.

No significant decrease in the proliferative capacity of d1 5-FU BM harvested from mice receiving transplants of Δ-cultured BM was observed relative to mice receiving transplants of an equivalent number of fresh d1 5-FU BM cells (Figs 5 and 6). In comparing the proliferative capacity of mice receiving transplants of BM expanded in IL-1 + IL-3, IL-1 + KL, or IL-1 + IL-6 + KL stimulated Δ-cultures, it was observed that those mice receiving transplants of BM from KL-containing cultures had slightly greater numbers of primary and Δ-culture-generated secondary HPP-CFC and LPP-CFC relative to recipients of IL-1 + IL-3-expanded BM (Fig 5). The addition of IL-6 to the expansion protocol did not improve the proliferative capacity relative to mice receiving transplants of IL-1 + KL-expanded BM. However, these results do not rule out an effect of IL-6 in the ex vivo growth of BM because low levels of IL-6 activity, and most likely several other growth factors, have been detected in the supernatants of unstimulated and IL-1-stimulated Δ-cultures. Although peripheral blood cell reconstitution had occurred by 5 weeks post-BMT, the number of early progenitor cells may still have been in flux, and thus the ability of transplanted BM cells to provide long-term hematopoietic reconstitution may not have been accurately gauged at these early time points. A comparison of the numbers of primary and secondary HPP-CFC and LPP-CFC measured in d1 5-FU BM and Δ-cultured BM recipients 40 weeks post-BMT showed that the hematopoietic proliferative capacity of these two groups of mice was equivalent (Fig 6). Furthermore, the survival of secondary transplanted mice, receiving transplants of the d1 5-FU BM donated from d1 5-FU BM transplanted mice or Δ-cultured BM transplanted mice, further showed that the expansion of BM in IL-1 + KL-stimulated Δ-cultures resulted in a population of hematopoietic stem cells comparable in hematopoietic reconstituting capacity to fresh d1 5-FU BM cells (Table 1). These results show that the growth of BM cells in short-term suspension cultures in the presence of IL-1 + KL
does not significantly affect the proliferative capacity of the hematopoietic stem cell compartment.

Mauch et al.\textsuperscript{15,16} have shown that the impairment in early progenitor proliferative capacity in mice receiving transplants is related to the dose of BM transplanted. The results from this study also show that the impairment in hematopoietic tissues was greatest in those mice receiving transplants of the fewest number of A-cultured BM cells. This impairment was observed in the survival of mice, the number of circulating peripheral blood cells, the total hematopoietic tissue cellularity, the numbers of primary and secondary CFU-C (Fig 6), and the survival of secondary transplanted mice (Table 1). These results show that the BMT of limiting numbers of cells is more damaging than the short-term ex vivo expansion of progenitors to the early hematopoietic progenitor compartment.

The ability to manipulate BM progenitors in vitro without sacrificing the proliferative capacity of the stem cell compartment has potential clinical relevance if these results can be extended to the human system. The expansion of progenitors could be used in protocols for autologous BMT or peripheral blood stem cell transplantation as part of a treatment for iatrogenic cytopenias. Gene therapy protocols involving hematopoietic stem cells also require the ex vivo manipulation of BM progenitors. Furthermore, BM expansion may also have use in ex vivo BM purging protocols used in the treatment of cancer, as has been recently shown with the use of a murine model system.\textsuperscript{37}

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