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Immunology

Long-term repopulation of hematolymphoid cells with only a few hemopoietic stem cells in mice

(supplementary repopulating strategy/5-fluorouracil/hematopoietic stem cell)

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ABSTRACT **A PCR-based assay has been devised for the detection and semiquantitation of cells originating from a few donor hematopoietic stem cells (HSCs) in a background of recipient cells. Upon sequencing a segment of murine Y chromosome contained in the plasmid pY2, oligonucleotide primers were designed for specific amplification of the Y chromosome-restricted segment. The HSCs were isolated from the bone marrow of mice on day 4 following a single i.v. injection of 5-fluorouracil and were readily distinguished from other bone marrow elements by the characteristics of low density, absence of lineage-specific surface markers, lack of expression of transferrin receptor, and a high expression of major histocompatibility complex class I antigen. Injection of as few as four such HSCs was shown to produce donor-derived cells (including lymphoid cells) for at least 8 months after transplantation into syngeneic female recipients. Retransplantation, employing 10^6 bone marrow cells from the initial recipients, also yielded clear evidence of repopulation with detectable levels of male donor cells. On statistical grounds, it is clear that long-term repopulation *in vivo* may result from even a single HSC having the characteristics defined herein.**

Hematopoietic stem cells (HSCs) are closely identified with cells that exhibit capacity for long-term lymphohemopoietic reconstitution after bone marrow transplantation (BMT) (1). Recently, Spangrude *et al.* (2, 3) have purified early precursors achieving a 2000-fold enrichment of HSCs. However, Smith *et al.* (4) calculated that 1 of 13 such cells generates progeny comprising >1% of peripheral white blood cells 3–7 weeks after injection. Since it has been estimated that no more than one HSC exists per 100,000 adult bone marrow cells (BMCs) (1), most of the constituent cells in the 2000-fold-enriched HSC population must be nondescript elements. Therefore, it seems likely that hematopoietic reconstitution, in this instance, results from HSCs that comprise but a minority population of the $Lin^-/Thy\text{-}1^{low}/Sca\text{-}1^+$ fraction (5). To purify and characterize such a minor population, further progress in defining the HSC is needed.

The first step should be to establish an *in vivo* assay designed to be sufficiently sensitive to detect hemopoietic repopulation from only a few or even from a single HSC. For late hematological reconstitution, HSCs must be transplanted with early engrafting cells, distinguishable from donor test cells, which permit the lethally irradiated host to survive early hematopoietic and lymphopoietic aplasia (6). To avoid this problem, supplementary repopulating strategy involves transplanting a limited number of test cells into lethally irradiated recipients together with recipient syngeneic BMCs from donors whose marrow repopulating ability had been compromised by two previous cycles of BMT (7–11). When using syngeneic mice, the donor cells have commonly been detected using Southern blot hybridization with the Y chromosome-specific probe, pY2 (7, 8, 12). Even though the detection of HSCs by this strategy is highly selective for totipotent and reconstituting HSCs, there is difficulty in identifying minor populations of donor-derived cells in recipient mice. To improve the detection of transplanted HSCs, we have developed a PCR-based assay. This assay, which utilizes newly designed oligonucleotide primers derived from a segment of murine Y chromosome contained in the plasmid pY2, can detect rare donor-derived male progeny that originate from only a small number of transplanted HSCs in a background of female recipient cells ($<1:10^5$ cells).

The second requirement is to achieve >10,000-fold enrichment of HSCs that exhibit long-term hematopoietic repopulating capacity. Since HSCs can be closely identified with cells that are resistant to treatment with S-phase-specific cytotoxic drugs, we developed a protocol that employs a single i.v. injection of 5-fluorouracil (5-FU) as the first step toward HSC enrichment (7, 11, 13–22). The 5-FU treatment allowed for a 1000-fold enrichment of HSCs that are phenotypically Lin^- and low density (LD). However, soon after 5-FU injection, dormant HSCs begin to cycle in response to the sharp decline of absolute total cell number of BMCs (23). Therefore, elapse of a proper time interval following 5-FU treatment before harvesting BMCs and a method to distinguish the HSCs from other cycling BMC elements were necessary. Based on the rationale that HSCs display high levels of major histocompatibility complex class I surface antigens ($H\text{-}2K^b$) (8, 11, 24) and that proliferating cells possess a higher frequency of transferrin receptor (CD71) than quiescent cells (25–28), these two markers were used for further definition of the desired HSC population. We show here that as few as four HSCs thus purified not only produce donor-derived cells in primary recipients over an 8-month period after transplantation but also yield repopulation with detectable levels of primary donor-derived cells in secondary recipients employing 10^6 BMCs from the first recipients.

MATERIALS AND METHODS

Mice. $(C57BL/6 \times C3H/He)F_1$ (BCF_1) mice at the age of 6–8 weeks were purchased from The Jackson Laboratory and kept under specific pathogen-free conditions.

Antibodies. Monoclonal antibodies (mAbs) used were conjugated directly to phycoerythrin (PE) [anti-CD71 (C2)] or

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Abbreviations: HSC, hematopoietic stem cell; BMC, bone marrow cell; BMT, bone marrow transplantation; 5-FU, 5-fluorouracil; mAb, monoclonal antibody; LD, low density; IB, immunomagnetic beads; BSA, bovine serum albumin; MPC, magnetic particle concentrator; PE, phycoerythrin; FITC, fluorescein isothiocyanate; SRS, supplementary repopulating strategy; CFU-S, colony-forming units, spleen.

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fluorescein isothiocyanate (FITC) [anti-H-2K^b (AF6-88.5)]. mAbs to CD4 (GK1.5, IgG2b), CD8 (53-6.72, IgG2a), B220 (14.8, IgG2b), Mac-1 or CD11b (M1/70, IgG2b), and Gr-1 (RB6-8C5, IgG2b) were prepared for negative immunomagnetic beads (IB; Dynal, Oslo) cell separation.

DNA Sequencing of Murine Y Chromosome-Restricted Segment and Primer Design. The plasmid pY2, containing a segment of the murine Y chromosome, was kindly provided by E. Palmer (Basel Institute for Immunology, Basel) (12). Double-stranded DNA sequencing of the plasmid was carried out by Sanger's dideoxy chain-termination method using the Sequenase Version 2.0 kit produced by United States Biochemical (29). Since the murine male-specific fragment of pY2 had been previously cloned into the *Bam*HI site of pBR322 (12), sequencing primers flanking the *Bam*HI site were synthesized and used to sequence the 5′ and 3′ ends of pY2. Only the first 238 and last 222 bp of pY2 were sequenced.

The DNA sequence information obtained from the 5′ and 3′ ends of pY2 was used to design oligonucleotide primers for specific amplification of the Y chromosome-restricted segment. A pair of 21-bp oligonucleotide primers with the sequences 5′-GCATTTGCCTGTCAGAGAGAG-3′ (sense strand) and 5′-TAGTTGGAAAGCAGCAGCAGT-3′ (antisense strand) were constructed using an Applied Biosystems 392 DNA/RNA synthesizer. This primer pair was created to amplify a 411-bp region contained within the murine Y chromosome.

Murine DNA Isolation and PCR Amplification. Genomic DNA was isolated from a mixture of 10^7 spleen cells that contained serial 10-fold dilutions of male cells in a background of female cells. The DNA was isolated using the rapid genomic DNA purification kit, TurboGen (Invitrogen), and was added to a 100-μl PCR mixture containing 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide 5′-triphosphate, and 600 ng of each primer. The reaction mixture (minus the *Taq* DNA polymerase) was heated to 95°C for 5 min and then cooled to 80°C. Two and one-half units of *Taq* DNA polymerase (Perkin–Elmer) was then added. The PCR consisted of 20–30 cycles of denaturation for 1 min at 95°C, annealing for 2 min at 62°C, and polymerization for 3 min at 72°C in a DNA thermal cycler (Perkin–Elmer).

PCR amplification of the pY2 region from 1 μg of murine DNA for 30 cycles provided high sensitivity and selectivity for detection of male DNA such that 0.001% male cells could be detected in a mixture of male and female cells. To maintain the relationship between the percentage of male cells and the amount of PCR-amplified products, several doses of DNA and various numbers of cycles of PCR were performed as preliminary experiments. Two hundred nanograms of DNA in a PCR for 26 cycles could readily maintain the quantitative relationship between the percentage of male cells and the amount of PCR-amplified products when male cells represented 0.1–100% of the population. DNA from female cells containing serial 10-fold dilutions of male cells was simultaneously amplified to provide PCR products for standard optical density to which a specific curve for the exponential rise to maximum was designed.

Preparation of HSC-Enriched Population. BMCs were collected from the mice after a single injection of 5-FU (150 mg/kg, i.v.; Roche Laboratories, Nutley, NJ). LD BMCs ($1.060 \leq \rho < 1.074$ g/cm^3) were isolated by discontinuous density gradient centrifugation using Percoll (Pharmacia) (21, 24, 29) and resuspended at 5×10^7 cells per ml by adding equal volumes of buffer [phosphate-buffered saline/bovine serum albumin (PBS/BSA): 0.2% deionized BSA contained in PBS] and a mAb cocktail (mixture of L3T4, Ly-2, B220, Gr-1, and Mac-1). After 30 min on ice, the primary Ab-labeled cells were washed twice and then incubated with sheep anti-rat IgG-conjugated IB at 4°C for 30 min with gentle agitation at a 3:1 beads/cell ratio. IB-rosetted cells were removed using a magnetic particle concentrator (MPC; Dynal) (30). Unrosetted cells were recovered and reincubated with the same number of beads mentioned above, achieving a 40:1 beads/target cell ratio, since most of the target cells had been removed. The remaining unrosetted cells were considered Lin$^-$.

Flow Cytometric Analysis and Sorting. Two-color fluorescence (FL) and forward and side scatter were analyzed with an EPICS Elite flow cytometer (Coulter). The cells were gated with respect to light scatter properties of blast cells as follows: intermediate to high FL scatter (channels 18–61), intermediate side scatter (channels 6–31) (8). The thresholds were set to discriminate between H-2K$^{b\ high}$/CD71$^-$ and H-2K$^{b\ low}$/CD71^{low} populations using day 4 samples because of the clearly discernible demarcation between these two populations of cells that was not evident in the samples from days 0–3, 5, and 6.

PCR-Based Assay for HSCs. All female recipients were administered 950 cGy (76 cGy/min, ^{137}Cs) total body irradiation (TBI) and, 12 hr following irradiation, recipients were injected i.v. with male test cells and 2×10^5 BMCs from female mice that had been compromised in long-term repopulating ability by previously having been subjected to two cycles of marrow regeneration (7–10). At selected time points in the posttransplant period, DNA was isolated from 50 μl of whole blood of the recipient mice using the small-scale genomic DNA isolation kit, Micro-TurboGen, provided by Invitrogen, and was used for PCR amplification.

Eight months after BMT, surviving recipients were sacrificed and their bone marrow, thymus, and spleen cells were harvested. Lymphoid and myeloid cells (T lymphocytes, B lymphocytes, and granulocytes) from these cell suspensions were collected using IB. Each mAb to Thy1.2, B220, and Gr-1 was bound to IB as follows: IB were incubated with each mAb (10 μg per 2×10^8 beads per ml) at 4°C for 12 hr using rotation for mixing. Following incubation the IB were collected using the MPC and incubated at 4°C for 30 min, each in PBS/BSA four times to completely eliminate unconjugated mAbs. The sample cells were suspended in 200 μl of PBS/BSA and mixed with 100 μl of primary Ab-coated IB, prepared as above, in order to achieve a beads/target cell ratio range of 2:1 to 5:1.

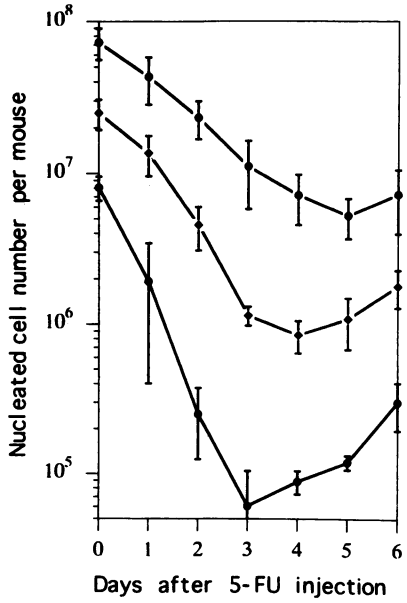

FIG. 1. Effect of a single 5-FU injection on the nucleated cell numbers. The femoral and tibial BMCs of mice after a single 5-FU injection (150 mg/kg, i.v.) on day 0 were harvested every day for 6 days. Uninjected mice served as controls (day 0). The numbers of total cells (●), LD cells (◆), and Lin$^-$ cells (○) are shown. These data were pooled from three independent experiments (mean ± SD, n = 6–24 mice per point).

After 3–5 min of gentle agitation at 4°C, IB-rosetted cells were collected with the MPC and were washed twice for 5 min to eliminate residual unbound cells. DNA was isolated from the separated cells and was used for PCR amplification.

Secondary Transplantation Assay. The secondary recipients following 950 cGy TBI were transplanted with 10^6 BMCs obtained from the first recipients 8 months after the first transplantation. Male cells originating from the primary donor-derived HSCs in the peripheral blood of secondary recipients were detected 60 days following the retransplantation.

RESULTS

The numbers of nucleated cells in unfractionated, LD, or LD/Lin$^-$ fractions are plotted against time after treatment in Fig. 1. The total number of nucleated cells decreased to 1 in 20 5 days after 5-FU treatment when compared with those of untreated control mice. A marked drop in the number of LD/Lin$^-$ cells to a nadir of 1 in 130 was observed within the first 3 days as compared with the initial untreated LD/Lin$^-$ population. The number of LD/Lin$^-$ cells was found to reach a minimum 3 logarithms less than the number of total nucleated BMCs prior to 5-FU treatment [mean ± SD per mouse (n = 6–24 mice per point); total BMCs prior to 5-FU i.v. 7.3 ± 1.7 × 10^7 to LD/Lin$^-$ cells following 5-FU i.v. 6.1 ± 4.4 × 10^4 on day 3 and 8.9 ± 1.6 × 10^4 on day 4]. Since HSCs in the G_0 phase are resistant to 5-FU, the treatment enriched the number of cells in the HSC population in the LD/Lin$^-$ fraction >2 logarithms. In addition, a >10-fold enrichment was achieved by obtaining LD/Lin$^-$ cells from the original BMC population. Thus, we could conclude that this methodology (LD/Lin$^-$ BMCs after treatment with 5-FU *in vivo*) yields a >1000-fold enrichment of the HSCs.

From adult male BMCs 4 days following i.v. administration of 5-FU, ≈500 LD/Lin$^-$ cells enriched for HSCs were transplanted along with compromised cells into five lethally irradiated recipient mice as a initial experiment. All mice (five of five) survived for >8 months after the transplantation. The percentage of donor-derived cells continuously increased to 30% over a 60-day period after BMT and then gradually declined to 10%. Even at 180 days after BMT, donor-derived male DNA could be detected in all female recipients. Donor-derived male cells were detected in all hematopoietic organs, BM, spleen, and thymus and also among the lymphoid and myeloid cell elements 240 days after BMT (data not shown).

Two-color flow cytometric analysis revealed a distinct separation between the peaks of H-2K$^{b\ low}$/CD71^{low} and of H-2K$^{b\ high}$/CD71$^-$ populations on day 4 following 5-FU treatment (Fig. 2*A*). Using these markers, it was possible repeatedly to isolate precisely the putative HSC fraction (Fig. 2*B*). Approximately 10–20% of LD/Lin$^-$ cells, 0.05–0.1% of the starting day 4 marrow cells (0.005–0.01% of the unfractionated marrow cells prior to 5-FU treatment), were found in the windows defined by these gates.

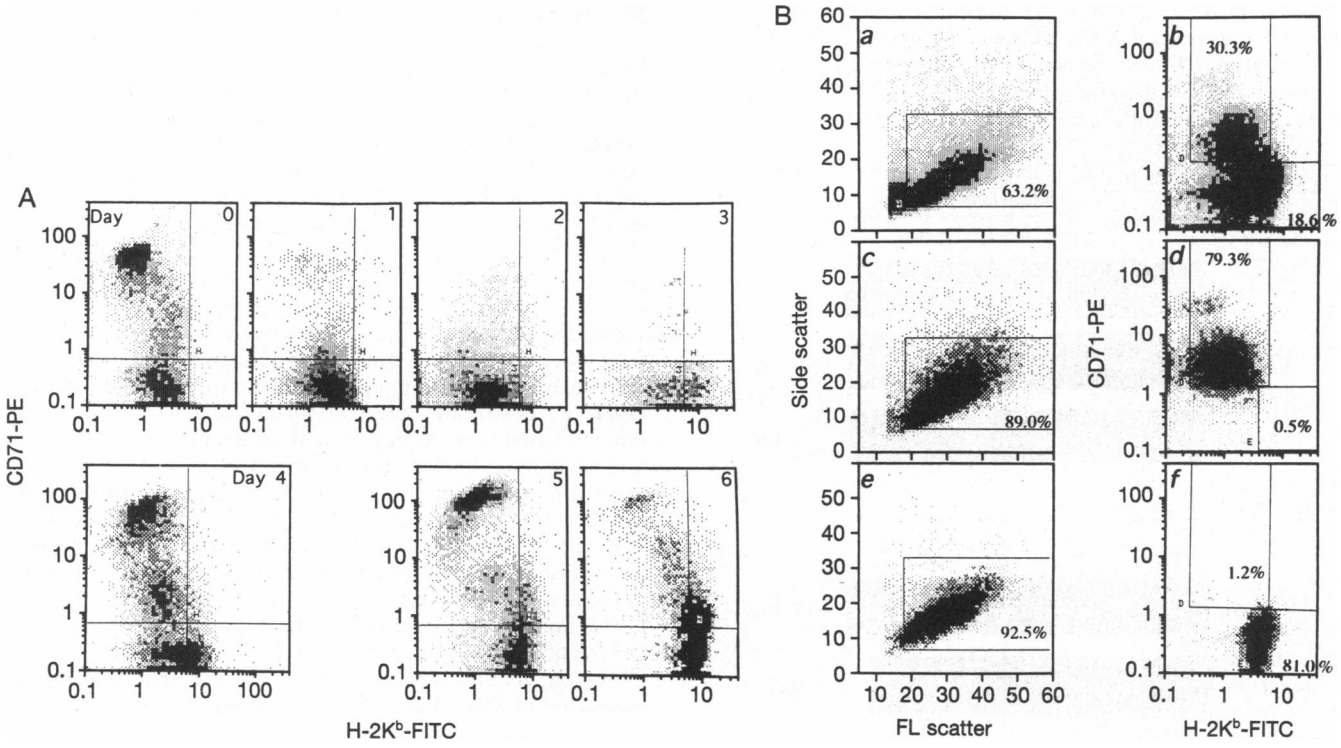

FIG. 2. Effect of a single 5-FU injection on expression of H-2K^b and CD71 by LD/Lin$^-$ cells and selection of H-2K$^{b\ high}$/CD71$^-$ cells and H-2K$^{b\ low}$/CD71^{low} cells by flow cytometry. (*A*) Coexpression of H-2K^b and CD71 on blast cell populations was analyzed by flow cytometry. The resultant green and red fluorescent profiles of LD/Lin$^-$ fractions prepared in Fig. 1 stained with H-2K^b-FITC and CD71-PE are shown. CD71^{high} and CD71^{low} populations that were dominant and that reflect the numbers of cells in the cycling phase on day 0 (63.7%) vanished from the blast gate immediately after 5-FU treatment (18.5% on day 1), reappeared on day 3 (39.9%), and recovered quickly (61.9% on day 4). On the other hand, the H-2K$^{b\ high}$ population (considered to be highly enriched for HSCs) comprised only 2.8% of the cell population obtainable on day 0, even though the population being analyzed had already been enriched for Lin$^-$ cells. However, these H-2K$^{b\ high}$ cells were dramatically concentrated and appeared abruptly in the blast gate 3 days after 5-FU treatment (47.4%). Two-color staining with H-2K^b-FITC and CD71-PE revealed negative (29.0%) and weak (18.4%) expression of CD71 on the H-2K$^{b\ high}$ population of cells. On day 4 the cells that expressed H-2K$^{b\ high}$ and CD71^{low} decreased to 3.7% and a distinctive drop was observed between the peaks of H-2K$^{b\ low}$/CD71^{low} and of H-2K$^{b\ high}$/CD71$^-$ populations. The H-2K$^{b\ high}$/CD71^{low} population reappeared and expanded rapidly after 5 days following 5-FU treatment (21.2%). This population was continuously distributed in the blast gate with no dip from the H-2K$^{b\ high}$/CD71$^-$ population. (*B*) Typical two-dimensional dot plot of the distribution of presorted cells (LD/Lin$^-$ cells 4 days following 5-FU i.v.) relative to the sorting windows are shown in *a* and *b*. Postsorted cells were always reanalyzed to confirm the distribution by the same window sets used for sorting (*c–f*).

Lethally irradiated female mice were transplanted with 100 male donor cells that were either H-2K$^{b\ high}$/CD71^{-} or H-2K$^{b\ low}$/CD71^{low} and their peripheral blood was analyzed 20, 40, and 60 days later. Only mice receiving H-2K$^{b\ high}$/CD71^{-} donor cells were found to contain detectable levels of nucleated male cells (Fig. 3*A*, lanes 1–5). Comparison of the samples with the standard PCR products of genomic DNA obtained from the various mixtures of male and female splenocytes (0–100%) revealed that male cell fractions in all of the recipients injected with 100 H-2K$^{b\ high}$/CD71^{-} cells had gradually recovered to ≈1% on day 20, between 1% and 10% by day 40, and ≈100% by day 60 after the transplantation. On the other hand, no donor-derived cells could be detected in the recipients injected with 100 H-2K$^{b\ low}$/CD71^{low} cells, suggesting that no HSCs existed in this population of donor cells (Fig. 3*A*, lanes 6–10). When mice injected with 500 or 2500 H-2K$^{b\ low}$/CD71^{low} cells were analyzed at similar time periods, we were unable to identify male-derived PCR products (data not shown). From these results, we conclude that isolation of LD/Lin^{-}/H-2K$^{b\ high}$/CD71^{-} BMCs, which fell within the blast gate obtained on day 4 after 5-FU treatment, greatly enriches the HSCs.

Then, to analyze the frequency of HSCs in our purified population by supplementary repopulating strategy (SRS), 20 and 4 of these cells were transplanted. All recipient female mice injected with 4 BMCs thus purified showed evidence of donor-derived hematopoietic repopulation 40 days after transplantation (Fig. 3*B*). When 12 recipient mice were analyzed, the cells in this population were found to generate colony-forming units, spleen (CFU-S), day 12 colonies (2.4 ± 1 per 10^2 cells), but no CFU-S, day 8 colonies (0 ± 0 per 10^3 cells).

Two of the six mice transplanted with four HSCs died prior to analysis at 8 months posttransplantation. However, donor-derived cells could still be detected in the BM and among both the lymphoid and myeloid elements in the spleen of all four surviving recipient mice (Fig. 4).

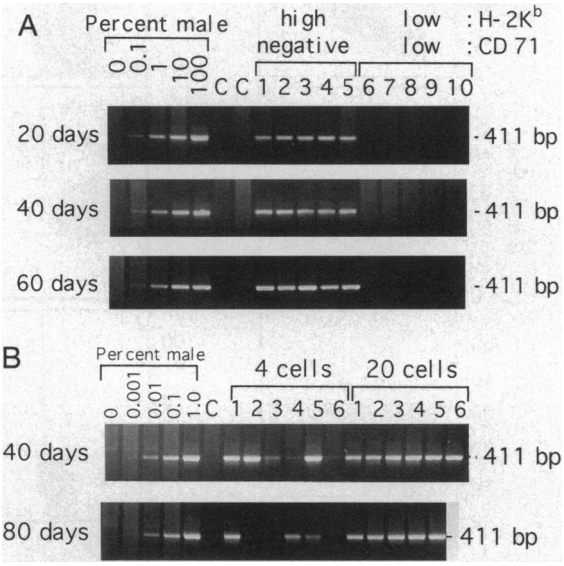

FIG. 3. Hematopoietic reconstitution of recipient peripheral blood with postsorted donor cells. Test cells were transplanted to lethally irradiated female recipients together with 2 × 10^5 compromised female cells (1, 27). (*A*) One hundred male sorted cells in each gate (Fig. 2*B*) were transplanted as test cells. PCR for 26 cycles was performed with 200 ng of DNA obtained from the peripheral blood of female recipients every 20 days after BMT to detect hematopoietic reconstitution with the male donor-derived cells. The sensitivity of the PCR analysis was 1 male cell per 10^4 nucleated cells. (*B*) The frequency of HSCs among the postsorted H-2K$^{b\ high}$/CD71^{-} cells was evaluated. Donor cells were present in 6 of 6 recipient female mice that had been injected with 20 male test cells. Even in recipient mice injected with only 4 cells, donor-derived cells were still found to generate detectable levels of nucleated male cells by 40 and 80 days (40 days, 6 of 6; 80 days, 5 of 6) posttransplantation.

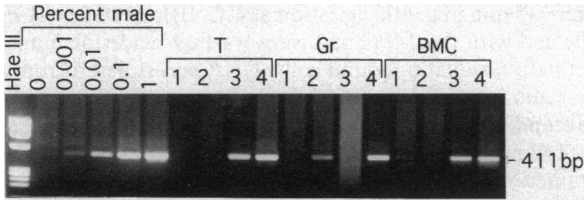

FIG. 4. Detection of long-term donor-derived cell proliferation among the lymphoid and myeloid elements. Eight months after transplantation, donor-derived cells could still be detected among the BMCs and among T cells (T) and granulocytes (Gr) in the spleen.

We also analyzed the existence of progeny that can originate from the HSCs in the peripheral blood of secondary recipients 60 days after retransplantation with 10^6 BMCs obtained from the first recipients 8 months following a first transplantation with four HSCs. Repopulation with detectable levels of the primary male donor-derived progeny was found to have occurred in peripheral blood of all five secondary female recipients (Fig. 5).

DISCUSSION

In vivo assays designed to detect repopulation from only a few HSCs or from a single HSC have been difficult to establish because of the marked fluctuation of detectable HSC progeny (30). The fluctuation of detectable HSC progeny is thought to be the result of competitive proliferation between the few donor HSCs and the recipient HSCs that have been spared from irradiation injury (31, 32). To identify a few transplanted HSCs *in vivo* for a long period, it is crucial to establish an assay system sufficiently sensitive to detect the minor cell populations that originate from those HSCs. In the system we describe herein, 2 months after HSC transplantation the percentage of male DNA, which reflects donor HSC-derived progenitors, peaked and then gradually decreased. This phenomenon may also be ascribed to competition between previously dormant recipient HSCs that had been spared from the effect of irradiation and the few transplanted donor HSCs. Even in the face of this competition, at intervals up to 8 months after the BMT, we could still detect donor-derived male DNA in all lymphohematopoietic organs and also among the lymphoid and myeloid cell elements. Harrison *et al.* (33) found that long-term repopulating ability and the ability to differentiate into myeloid and lymphoid lineages are measured directly from 2.5 to 12.5 months after transplantation, while myeloid-specific or lymphoid-specific precursors produce few or no descendants. They also suggested that most of the initially active multilineage precursors are exhausted within a short period of posttransplantation. Due to the sensitivity of the PCR, we cannot rule out the possibility that the signals generated in either the isolated lymphoid or myeloid samples

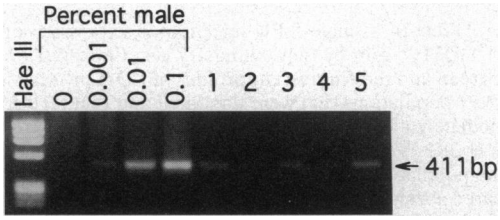

FIG. 5. Detection of primary donor-derived cells in secondary recipients. Sixty days after retransplantation, detection of male cells in peripheral blood was found in five secondary recipients (lanes 1–5) transplanted with 10^6 BMCs obtained from the first recipient (Fig. 4, lane 4) 8 months following initial transplantation with four HSCs.

cross-contaminated each other. We believe the method introduced in this manuscript to be the most effective method now available for the detection of HSCs since these signals were detected for 8 month after BMT from the four donor cells transplanted.

In this report we have used 5-FU to enrich marrow cells for HSCs. The treatment of donor mice with a single high dose of 5-FU did not inhibit the development of late-appearing spleen colonies or blast cell colonies, since the majority of HSCs are considered to be quiescent and thus to have escaped the effect of 5-FU (13–19, 34–37). Indeed, the HSCs we purified were very highly enriched for late CFU-S activity. Jones *et al.* (6) demonstrated that the fraction of cells containing 90% of the pre-CFU-S could be readily separated from the fraction of cells containing >97% of late CFU-S (CFU-S, day 12), while Pleomacher and Brons (38) found that the fraction containing cells that produce CFU-S, day 12, had a limited capacity to regenerate hematopoiesis following transplantation into irradiated mice. Taking into account (*i*) the frequency of CFU-S in BM, which is at least 100 times greater than that of HSCs, (*ii*) the wide variety of cells (on the basis of differentiation stages) capable of generating CFU-S, and (*iii*) the fact that Jones *et al.* (6) only fractionated BMCs into a few fractions using counterflow centrifugal elutriation, it is probable that HSCs were included within some of their CFU-S populations. Even if a subset of CFU-S that possess long-term repopulating ability could be separated from other CFU-S that have a limited capacity to regenerate hematopoiesis, the former CFU-S population would be hidden in the high background of other cells. Thus, the observations of Jones *et al.* (6) and our observations regarding the presence of CFU-S in the purified HSC population are not contradictory but represent interpretations of quite different experimental approaches.

As it has been previously demonstrated that staining BMCs by fluorescent dye-conjugated mAbs reduces their survival *in vivo* and decreases their capacity to regenerate hematopoieses in the marrow to approximately one-half that accomplished with unstained control cells, we calculate that injecting four stained cells is indeed equivalent to injecting only two unstained cells. Therefore, the apparent ability of only two donor cells to regenerate lymphoid and myeloid lineages in four of six recipient mice argues strongly that, on a statistical basis, long-term repopulation has been achieved with a single HSC.

The HSCs we isolated were found to be uniformly $LD/Lin^-/H\text{-}2K^{b\ high}/CD71^-$ and WGA^+, $Thy1^{low}$, $Sca\text{-}1^+$, and $c\text{-}kit^{negative\ or\ low}$. In recent studies of HSCs *in vitro*, the expression and function of c-kit on these cells has been argued (33, 38–45). In our experiments we found the HSCs with long-term hematopoietic repopulating ability *in vivo* did not possess high-level expression of c-kit (data not shown). We believe that the c-kit expression is likely to be associated with the proliferating cells rather than the dormant HSCs in adult steady-state hematopoiesis.

Since the long-term hematopoietic repopulation obtained by such HSC manipulation occurs with such impressively high frequency, it seems certain that even a single HSC, defined by the criteria we can now employ, would be capable of significant lymphohematopoietic repopulation. With this ability to isolate and purify HSCs, we can begin to investigate directly and to define more precisely the nature and the biology of this most important cell.

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