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Genetics

Combination of interleukins 3 and 6 preserves stem cell function in culture and enhances retrovirus-mediated gene transfer into hematopoietic stem cells

(growth factors)

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ABSTRACT **The effects of several hematopoietic growth factors on primitive murine bone marrow progenitor cells [colony-forming unit(s)-spleen (CFU-S)] have been investigated during culture for 2–6 days. Interleukin 3 (IL-3) was required for CFU-S survival in culture, and the combination of IL-3 and interleukin 6 (IL-6) increased the number of CFU-S in culture 10-fold over the number obtained with IL-3 alone. Stem cell function was measured by competitive repopulation; IL-3 was required, and IL-3 and IL-6 appear to act synergistically to enhance stem cell recovery from these cultures. These data appear to be relevant for retroviral-mediated gene transfer into stem and progenitor cells. Murine bone marrow cells were infected with a retrovirus containing the human β-globin gene in the presence of various growth factors. Only 2 of 17 mice reconstituted with cells infected in the presence of IL-3 alone showed long-term expression of the human β-globin gene (12 months), as opposed to 6 of 11 mice reconstituted with cells infected in the presence of IL-3 and IL-6. Medium conditioned by 5637 bladder carcinoma cells, a source of several hematopoietic growth factors, increased the frequency of infection of CFU-S but did not enhance stem cell infection or the repopulating potential of cultured bone marrow cells. Stem cells containing the human β-globin provirus from these animals were shown to be capable of reconstituting secondary recipients in which the human β-globin gene was expressed.**

All cells in the peripheral circulation are descendants of a pluripotent hematopoietic stem cell (PHSC). This cell has the capacity to self-renew or to become committed to the myeloid or lymphoid lineages (for review, see ref. 1). The introduction of new genetic material into the uncommitted stem cell provides genetic markers to study the differentiation of various cell lineages (2, 3). Perfection of this technology may allow gene replacement therapy for certain genetic disorders affecting the hematopoietic system (4). Retroviral-mediated gene transfer has been used to transfer several genes into murine bone marrow cells, including the genes for human β-globin (5–7), human adenosine deaminase (*ADA*) (8), murine interleukin (IL-3) (9), murine granulocyte–macrophage colony-stimulating factor (10), murine dihydrofolate reductase (11), and bacterial neomycin resistance (2, 3, 12, 13).

Previous studies have shown that cellular DNA replication is required for integration of murine leukemia virus DNA into the host genome of mammalian cells (14, 15). However, 97% of purified mouse stem cells are in the G_0 or G_1 phases of the cell cycle (16). Lack of stem cell DNA replication may explain the low frequency of infection of these cells in gene transfer experiments. Pretreatment of the donor marrow with 5-fluorouracil (5FUra), which increases both stem cell cycling (17, 18) and the relative number of stem cells (19), has been shown to facilitate retroviral gene transfer (3).

Several hematopoietic growth factors, including (IL-3) and interleukin 6 (IL-6), have been proposed to affect PHSC (for review, see ref. 20). IL-3 is required for the formation of multilineage blast cell colonies believed to be derived from primitive progenitor cells (21). The combination of IL-3 and IL-6 shortens the time interval between plating and the appearance of blast colonies. The authors have proposed that combination of IL-3 and IL-6 shortens the G_0 period of the blast cell colony progenitor cells and promotes entry into the cell cycle (22). Retroviral gene transfer to colony-forming unit(s)-spleen (CFU-S) is greatly enhanced in the presence of IL-3, although the frequency of stem cell infection appeared to be <10% (3). Because of the reported synergistic effects of IL-3 and IL-6 on primitive cells in culture, we have defined the effects of these growth factors on the subsequent function of PHSC and CFU-S and on retroviral-mediated gene transfer into these cells.

METHODS

Cells and Viruses. The ψβS1 cell line has been described (6). Helper virus was assayed as described (6). No helper virus was detected in supernatant from the producer cells at the time of infection or in serum from mice 5 months after transplantation.

Growth Factors. Recombinant *Escherichia coli*-derived human IL-6 (23) was provided by Steve Clark of Genetics Institute. The plasmids pCDIL-3 (24) and pCDIL-6 (25) were provided by Frank Lee of DNAX. The biological activities of murine IL-3 and IL-6 were assayed on 32D (26) and T1165 (27) cells, respectively, with known standards as controls.

Mice. $WBB6F_1$-+/+ (recipients for the CFU-S assays), $WBB6F_1$-W/W^v (recipients for long-term gene-transfer experiments), female C57BL/6J (abbreviated B6) (donors for all experiments), and female B6.C-*H-1*^{b}/*By* (HW80; donors for the competitive repopulation assay) were all purchased from The Jackson Laboratory; 4- to 6-week-old donor mice were treated with 150 mg of 5FUra (Fluka) per kg of body weight intravenously 48 hr before bone marrow harvest.

Suspension Culture. Marrow cells were harvested from the hind limbs of donor mice, washed, and resuspended at a concentration of 5×10^5 cells per ml in Dulbecco's modified Eagle's medium (DMEM; Biofluids, Rockville, MD) containing 15% (vol/vol) fetal calf serum (FCS) (HyClone) and the growth factor(s) indicated in the figure legends. Ten milliliters of this cell suspension was plated on Sarstedt plates and incubated at 37°C in 95% air/5% CO_2 for 2–6 days. The cells were recovered from the medium by centrifugation, washed, counted, assayed for viability by trypan blue exclusion, and

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Abbreviations: CFU-S, colony-forming unit(s)-spleen; 5FUra, 5-fluorouracil; IL-3, interleukin 3; IL-6, interleukin 6; PHSC, pluripotent hematopoietic stem cell.

resuspended at the appropriate concentration in phosphate-buffered saline (PBS) for injection.

CFU-S Assay. The CFU-S assay was performed essentially as described (28). Recipient mice were exposed to 900 R prior to the injection of between 2.5×10^4 to 1×10^6 cells. Spleens were harvested on day 14 after injection and fixed in formalin to facilitate counting. The [^{3}H]thymidine suicide assay was performed as described (29).

Competitive Repopulation Assay. The competitive repopulation assay was performed as described (30). After 6 days of suspension culture, HW80 cells cultured in the presence of 200 units of IL-3 per ml were mixed with an equal number of C57BL/6J cells cultured in the presence of the indicated growth factor(s) in Fig. 3. Five WBB6F$_1$-W/W^v recipients were injected with 2×10^6 cells (1×10^6 HW80 cells and 1×10^6 C57BL/6 cells) in each of five groups.

Gene Transfer Protocol. Marrow cells were harvested as above and resuspended at a concentration of 5×10^5 cells per ml in DMEM supplemented with 15% FCS and the growth factor(s) indicated in the figure legends. Ten milliliters of this cell suspension was plated on Sarstedt plates and incubated at 37°C in 95% air/5% CO_2 for 48 hr (31). The cells were recovered by centrifugation, counted, and resuspended in medium containing the same growth factors and 6 μg of Polybrene (Sigma) per ml. A total of 3×10^6 cells was added to 10-cm plates of $\psi\beta$S1 producer cells split 1:5 24 hr previously. After 48 hr of cocultivation, the cells were recovered as above and injected into WBB6F$_1$-W/W^v recipients. Each mouse was injected with 1×10^6 cells. Analysis of mouse hemoglobins, immunofluorescent analysis of human β-globin chains, DNA analysis, and RNase protection assays were carried out as described (6).

RESULTS

The Combination of IL-3 and IL-6 Increases the Number of CFU-S in Culture. To examine the effects of IL-3 and IL-6 on primitive progenitor cells, CFU-S assays were performed on suspension cultures of bone marrow cells harvested 2 days after 5FUra treatment and cultured for 6 days in the presence of no growth factor(s), IL-3, IL-6, or IL-3 plus two different concentrations of IL-6. Neither IL-6 nor COS cell-conditioned medium supported CFU-S in culture. Marrow cells cultured in the presence of 200 units of IL-3 plus 20 units of IL-6 per ml or 200 units of both IL-3 and IL-6 per ml contained 5- and 10-fold more CFU-S per 10^5 cells injected, respectively, than did marrow cells cultured in the presence of IL-3 alone (Fig. 1).

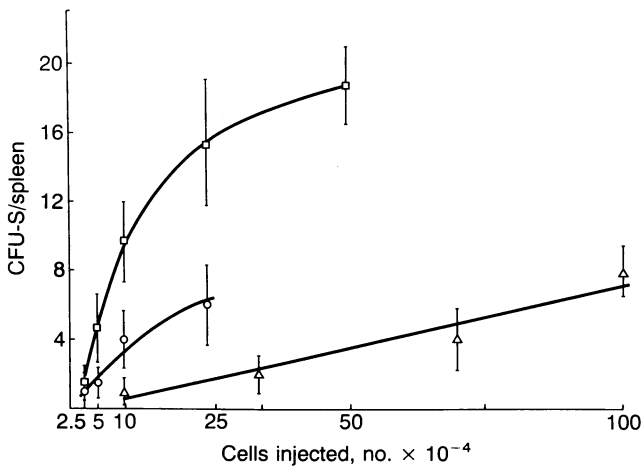

FIG. 1. The effects of IL-3 and IL-6 on the number of CFU-S in suspension culture. Lethally irradiated mice were injected with the indicated number of bone marrow cells cultured for 6 days in the presence of 200 units of IL-3 per ml (△), 200 units of IL-3 plus 20 units of IL-6 per ml (○), or 200 units of both IL-3 and IL-6 per ml (□). The points represent the average number of CFU-S (± SD) on four spleens on day 14 after injection.

We next examined the number of CFU-S in suspension culture as a function of time. Two days after 5FUra treatment bone marrow cells were cultured in the presence of either IL-3 or IL-3 and IL-6 (Fig. 2). Detectable CFU-S declined from baseline in the presence of IL-3 and IL-3 plus IL-6 by 2 days. In the presence of IL-3 plus IL-6 there was a subsequent increase on both 4 and 6 days. Consistent with this increase in CFU-S numbers was the sensitivity to [^{3}H]thymidine of 33% and 73% on days 3 and 5, respectively. The rare CFU-S in culture with IL-3 only were insensitive to [^{3}H]thymidine (Table 1).

IL-3 Is Required, and the Combination of IL-3 and IL-6 Further Increases Stem Cell Repopulating Ability in a Competitive Repopulation Assay. Bone marrow cells obtained from C57BL/6J donors [homozygous for "single" hemoglobin] 2 days after 5FUra treatment were cultured for 6 days in the presence of no added growth factor, IL-3, IL-6, or IL-3 plus IL-6. Bone marrow cells from HW80 donors, congenic with C57BL/6J but homozygous for "diffuse" hemoglobin, cultured for 6 days in the presence of IL-3 were used as a control. Equal numbers of C57BL/6J and HW80 cells were injected into W/W^v recipients. The percentage of C57BL/6J (single) and HW80 (diffuse) hemoglobin 12 weeks after injection are shown in Fig. 3*A*. C57BL/6J and HW80 marrow cultured in the presence of IL-3 competed equally well, as evidenced by the nearly identical amounts of single and diffuse hemoglobin in the recipient animals. Analysis of peptidase-3 isozymes (homozygous in donor animals, heterozygous in W/W^v recipients) demonstrated that these animals were fully reconstituted with donor cells. HW80 marrow cultured in the presence of IL-3 contributed nearly 10-fold more hemoglobin than did C57BL/6J marrow cultured in the absence of growth factors, or in presence of IL-6 alone. C57BL/6J marrow cultured in the presence of IL-3 plus IL-6 contributed >1.6-fold more hemoglobin than HW80 marrow cultured in the presence of IL-3 alone, indicating that IL-6 contributes to the repopulation potential of cultured

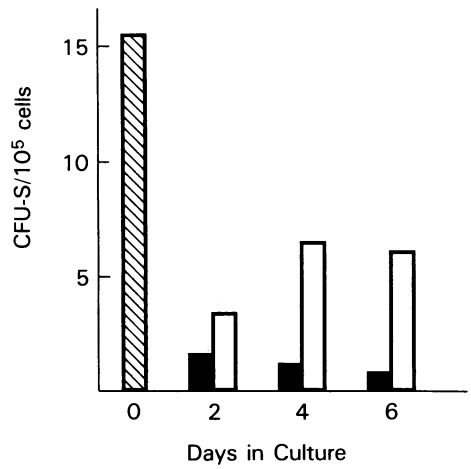

FIG. 2. Time course of the effects of IL-3 and IL-6 on the number of CFU-S in suspension culture. Lethally irradiated mice were injected with various numbers of bone marrow cells cultured in the presence of 200 units of IL-3 per ml (■) or 200 units of both IL-3 and IL-6 per ml (□). The height of the bars were determined by dividing the total number of CFU-S on 12–16 spleens by the total number of cells injected $\times 10^5$. A total of 36, 30, and 59 CFU-S was analyzed on day 2, 4, and 6 of culture with IL-3, respectively. Likewise, a total of 26, 106, and 110 CFU-S was analyzed on day 2, 4, and 6 of culture with IL-3 and IL-6, respectively. The number of CFU-S in fresh bone marrow cells 2 days after treatment with 5-FUra is represented by the hatched bar.

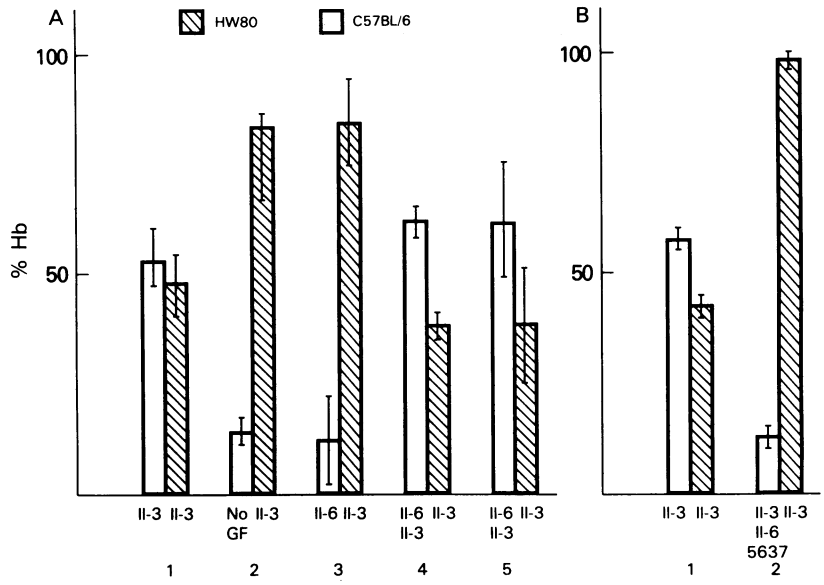

FIG. 3. Effects of IL-3 and IL-6 on stem cell repopulating ability in a competitive repopulation assay. (*A*) W/W^v recipients were injected with equal numbers of HW80 bone marrow cells cultured in the presence of 200 units of IL-3 per ml and C57BL/6J bone marrow cells cultured in the presence of 200 units of IL-3 per ml (group 1), no growth factor (group 2), 200 units of IL-6 per ml (group 3), 200 units of IL-3 plus 20 units of IL-6 per ml (group 4), or 200 units of both IL-3 and IL-6 per ml (group 5). The percent (± SD) of HW80 hemoglobin (diffuse) and C57BL/6J hemoglobin (single) is represented by the hatched and open bars, respectively. In group 5, one animal was reconstituted with 36% single and 64% diffuse hemoglobin, accounting for the large standard deviation. (*B*) W/W^v recipients were injected with equal numbers of HW80 bone marrow cells cultured in the presence of 15% WEHI-3D cell-conditioned medium and C57BL/6J bone marrow cells cultured in the presence of 15% WEHI-3D cell-conditioned medium (group 1) or 15% WEHI-3D cell-conditioned medium/15% 5637 cell-conditioned medium/200 units of recombinant human IL-6 per ml (group 2).

marrow only in the presence of IL-3. At 12 weeks posttransplantation, thymus DNA of the animals in groups 1, 2, and 5, was examined for the ratio of the β^s (single)- and β^{maj} (diffuse)-globin alleles by Southern blotting. The ratio of the β^s- to β^{maj}-globin alleles in thymus DNA was similar to the ratio of single to diffuse hemoglobin in the peripheral blood at that time, indicating multilineage reconstitution.

The Combination of IL-3 and IL-6 Increases the Frequency of Retroviral Gene Transfer to Stem Cells. We compared several variations of the original gene transfer protocol described by Dick *et al.* (2). Of 14 animals reconstituted with bone marrow cells cocultured for 48 hr in the presence of IL-3 and IL-6, no animals were DNA positive 3 months after transplantation. In contrast, preincubation of the same bone marrow cells in IL-3 and IL-6 for 48 (31) or 96 hr prior to the 48-hr cocultivation yielded 4 of 10 and 4 of 9 DNA positive animals 3 months after transplantation, respectively (Table 1). We also have compared the effects of IL-3, 5637-conditioned medium, and IL-6 on stem cell infection. Eleven animals expressed human β-globin mRNA at 3 months. Southern blot analysis of peripheral blood DNA showed that these 11 mice contained approximately 0.1 copy of the human β-globin provirus per genome. No proviral sequences were detected in 10 mice that were not expressing human β-globin mRNA. These results indicate that RNA expression is sufficient to identify positive animals. Table 1 shows that the combination of IL-3, IL-6, and 5637-conditioned medium gave the highest percentage of positive spleen foci as determined by DNA and RNA analysis. The frequency of stem cell infection was augmented by IL-3 plus IL-6 over IL-3 alone (6 of 11 vs. 2 of 17; $p < 0.05$), but 5637-conditioned medium inhibited stem cell infection. In addition, treatment of cells with 5637-conditioned medium in the presence of IL-3 plus IL-6 inhibited the repopulating potential of bone marrow cells in a competitive repopulation assay (Fig. 2*B*).

Expression of the Transduced Human β-Globin Gene in Long-Term Reconstituted Mice. The level of human β-globin mRNA in the individual mice is shown in Fig. 4 and Table 2. The overall average level of expression is ≈0.15% that of the mouse genes, although there was variation about that number. Immunofluorescent staining of peripheral red blood cells with a monoclonal antibody against human β-globin showed that

Table 1. Frequency of infection of CFU-S and PHSC cocultured in the presence of combinations of growth factors

Exp.*	Growth factor† IL-3	5637‡	IL-6§	[^{3}H]Thymidine-sensitive CFU-S, % Day 2.5	Day 4.5	CFU-S infection, no. colonies +/total (%)	Mice positive 3 mo. after transplant, no. +/total (%)
1	+	−	−	ND	ND	11/37 (30)	2/17 (12)¶
2	+	+	−	ND	ND	26/62 (42)	1/14 (7)¶
3	+	+	+	ND	ND	10/13 (77)	2/10 (20)¶
4	+	−	+	ND	ND	6/15 (40)	6/11 (55)¶
5	+	−	−	0	0	11/30 (37)	ND
6	+	−	+	33	73	24/30 (80)	ND
7	+	−	+	ND	ND	ND	4/10 (40)
8‖	+	−	+	ND	ND	ND	4/9 (44)

All experiments used bone marrow cells harvested 2 days after 5FUra treatment and prestimulated in the presence of the indicated growth factors for 48 hr prior to a 48-hr coculture with producer cells. ND, not analyzed.

*Experiments 1–4 and 7 and 8 used the same pools of donor bone marrow cells and were infected with the ψβS1 virus (titer: 2×10^6 neomycin-resistant colony forming units (neo^r cfu)/ml); experiments 5 and 6 used the same pool of donor bone marrow cells and a similar virus, Gβγ15 (titer: 2×10^6 neo^r cfu/ml).

†Experiments 1–4 used 15% WEHI-conditioned medium as a source of IL-3; experiments 5–8 used 200 units of COS cell-derived IL-3 per ml.

‡Experiments 2 and 3 used 15% 5637-conditioned medium as a source of several growth factors, including IL-1α and granulocyte colony-stimulating factor.

§Experiments 3 and 4 used 200 units of recombinant human IL-6 per ml; experiments 6–8 used 200 units of COS cell-derived murine IL-6 per ml.

¶Experiment 4 vs. experiment 1: $\chi^2 = 4.3$; $P < 0.05$; experiment 4 vs. experiments 1, 2, and 3: $\chi^2 = 7.8$; $P < 0.05$.

‖Prestimulation was for 96 hr; coculture was for 48 hr.

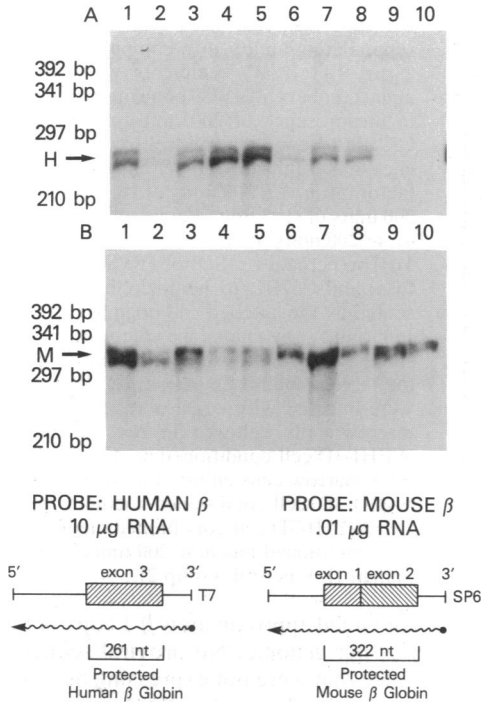

FIG. 4. Identification of human β-globin mRNA in reticulocytes of long-term gene transfer mice by RNase protection. (*A*) Reticulocyte RNA (10 μg) from eight positive mice (lanes 1–8) and two negative mice (lanes 9 and 10) were probed with the human β-globin probe described below *B*. (*B*) Reticulocyte RNA (0.1 μg) from eight positive mice (lanes 1–8) and two negative mice (lanes 9 and 10) were probed with the mouse β-globin probe described below *B*. The lane numbers do not correlate with the animal numbers in Table 2. bp, Base pairs; nt, nucleotides.

between 5% and 15% of the red cells contained human β-globin chains (data not shown), consistent with the gene copy number we observed. In agreement with our previous studies and those of others (5, 6), we calculate that the transduced human β-globin gene expressed 1–2% of the amount of mRNA as the endogenous mouse β-globin genes per gene copy.

Animal 7 was sacrificed 10 months after transplantation, and RNA was extracted from peripheral blood mononuclear cells, bone marrow, spleen, thymus, and liver. The RNA was analyzed for the presence of the transduced neomycin-resistance and human β-globin mRNAs, as well as endogenous mouse β-globin mRNA. Human and mouse β-globin mRNA was detected only in RNA from bone marrow and the spleen and not in RNA from peripheral blood mononuclear cells or thymus. No neomycin-resistance transcripts were detected in RNA from any tissue.

The Transduced Human β-Globin Gene Is Expressed in Secondary Recipients. Two animals were sacrificed 12 months after transplantation, and 10^7 bone marrow cells from these mice were used to repopulate secondary W/W^v recipients. Three months after transplantation, these mice were fully reconstituted with marrow carrying the "single" hemoglobin marker of the original C57BL/6J donor. Analysis of RNA from these secondary animals revealed the presence of human β-globin mRNA in four of five animals examined (Fig. 5).

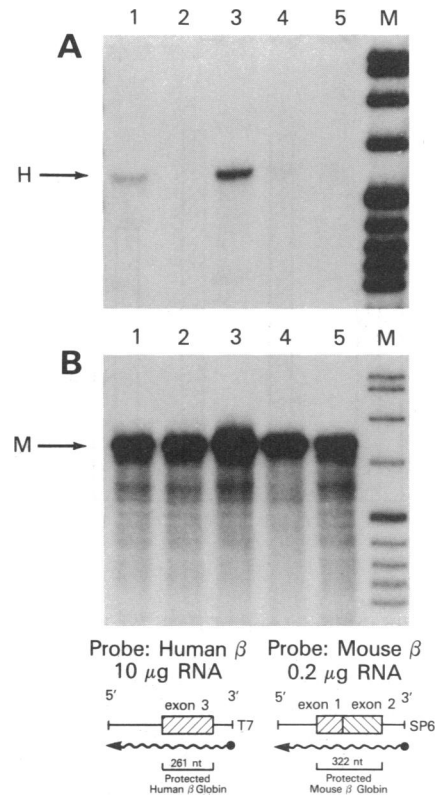

FIG. 5. Identification of human β-globin mRNA in secondary mice transplanted with cells from long-term gene transfer animals. (*A*) Reticulocyte RNA (10 μg) from five secondary transplant mice (lanes 1–5) were probed with the human β-globin probe shown below *B*. (*B*) Reticulocyte RNA (0.2 μg) from five secondary transplant mice (lanes 1–5) were probed with the mouse β-globin probe described below *B*. The markers in the lanes marked M are from top to bottom: 622, 527, 407, 309, 242/238, 217, 201, 190, and 180 bp in length. *A* and *B* were not exposed for the same length of time.

DISCUSSION

We have used *in vivo* assays to demonstrate that primitive progenitor cells (CFU-S) require IL-3 to survive in culture. These results are consistent with the observations that IL-3 maintains multipotent colony-forming cells in serum-free

Table 2. Levels of human β-globin mRNA in long-term reconstituted mice

Time,* mo	Human β-globin in RNA in mice 1–11,† % of endogenous β-globin mRNA										
	1	2	3	4	5	6	7	8	9	10	11
3	0.08	0.12	0.09	0.11	0.04	0.09	0.45	0.24	ND‡	0.02	0.30
6	0	0.08	0.13	Died	0.06	0.45	0.14	0.09	0.35	0.01	0.13
9	ND	0.11	Died	ND	0.06	0.31	0.15	0.08	0.37	0	0.08
12	ND	0.11	ND	ND	0.07	0.22	ND‡	0.14	0.22	ND	0.12

ND, not determined; mo, month.

*At each time point, all mice were treated with phenylhydrazine (0.5 mg/day) for 5 days. After 2 days of no treatment, RNA was extracted from reticulocyte-rich peripheral blood.

†Mice 1 and 2 were reconstituted with cells infected in the presence of IL-3; mouse 3, IL-3 and 5637-conditioned medium; mice 4–9, IL-3 and IL-6; and mice 10 and 11, IL-3, 5637-conditioned medium, and IL-6. The animal numbers do not correlate with the lane numbers in Fig. 4.

‡Sacrificed at 10 mo; expression level was 0.25% in bone marrow.

medium and in long-term bone marrow culture without inducing commitment (32, 33). The synergistic effects of IL-3 and IL-6 on CFU-S that we observed are analogous to those described on blast colony progenitors (22) and multipotent colony-forming cells in serum-free medium (34). Because our experiments were not done with a purified cell population, we cannot prove that the effects we observed are direct effects of the added growth factors. However, our results are analogous to those of others (21, 22), who have shown that IL-3 and IL-6 act directly on blast colony progenitor cells. The sensitivity of CFU-S treated with IL-3 and IL-6 to [^{3}H]thymidine indicates that the CFU-S number increases in culture by proliferation after an initial decline.

The competitive repopulation assay measures the number of PHSC injected and has been shown to demonstrate differences in PHSC number of <2.5-fold (30). We have used this assay to demonstrate that IL-3 is required for stem cell survival in culture and that IL-3 plus IL-6 have a synergistic effect on PHSC repopulating ability. IL-3 has been shown to act directly on highly purified mouse bone marrow stem cell populations to enhance colony formation *in vitro* (35). In addition, we have shown that stem cells infected with a retrovirus containing the murine gene for IL-3 have a growth advantage over uninfected stem cells (9). From these observations we suggest that IL-3 acts directly on PHSC. We have not examined the cycling status of PHSC in a competitive repopulation assay, but based on the increased susceptibility of PHSC treated with IL-3 and IL-6 to retroviral infection, we infer that some of these cells are in cycle.

The high frequency of CFU-S infected in the presence of medium conditioned by 5637 bladder carcinoma cells, IL-3, and IL-6 did not predict a high frequency of stem cell infection. These results are difficult to interpret in the context of complex cultures containing unfractionated bone marrow cells and multiple growth factors. However, these data suggest to us that the frequency of infection of progenitor cells is not a reliable predicter of optimal conditions for gene transfer into PHSC.

Two previous studies have examined retroviral-mediated transfer of human β-globin genes to murine PHSC. Dzierzak *et al.* (5) reported that 8 of 108 mice (7.4%) expressed the human β-globin gene and that ≈10% of the PHSC in those mice carried a retrovirus. The cells used to reconstitute these animals were infected in the presence of IL-3 only, and the stem cell infection frequency was similar to what we observed using IL-3 only. A second study had a much higher frequency of long-term mice that expressed the human β-globin gene (7). These mice were reconstituted with bone marrow cells that were cultured in G418 medium to select for stem cells that had incorporated the proviral genome. At least 3×10^7 bone marrow cells were utilized to reconstitute each mouse. This is equivalent to 1.5×10^9 cells per kg of body weight—far more than the number of marrow cells available for reconstitution of a large experimental animal or man. We conclude that the preselection protocol as currently applied is an impractical approach to human gene therapy.

Although infection in the presence of IL-3 plus IL-6 increases the frequency of gene transfer to stem cells, most stem cells are not infected. Other studies have shown that with an extremely high-titer retrovirus containing the human *ADA* gene, ≈50% of the reconstituted animals expressed the *ADA* gene for 6 months (8). As in all animals reconstituted without *in vitro* selection to date, ≈10% of the stem cells in these animals were infected. Perhaps infection in the presence of IL-3 plus IL-6 with a very high titer virus would lead to a greater efficiency of gene transfer.

The expression of the transduced human β-globin gene in mouse erythroid cells is very low. Recent experiments have shown that relatively small segments of the locus-activating region for the human β-globin gene cluster (36) can increase the level of expression of human β-globin genes in transgenic mice to near that of the endogenous mouse β-globin genes (37, 38). Incorporation of these sequences into a retrovirus to increase the level of expression of the transduced human β-globin gene following gene transfer could impact on prospects for somatic gene therapy for human hemoglobinopathies.

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