Leukemia Inhibitory Factor Improves Survival of Retroviral Vector-infected Hematopoietic Stem Cells In Vitro, Allowing Efficient Long-term Expression of Vector-encoded Human Adenosine Deaminase In Vivo

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Summary

Low recovery and poor retroviral vector infection efficiency of hematopoietic stem cells has hindered application of gene therapy for diseases affecting blood-forming tissues. Developmental restriction (or death) of stem cells during ex vivo infection has contributed to these difficulties. In these studies we report that the cytokine leukemia inhibitory factor (LIF) directly or indirectly supported the survival of hematopoietic stem cells during culture of bone marrow with vector-producing fibroblasts, resulting in efficient recovery of stem cells able to compete for engraftment in irradiated recipient animals. The infection efficiency of hematopoietic stem cells recovered from these cultures was ~80%; and all recipients (20/20) of the LIF-treated marrow were stably engrafted with the progeny of provirus-bearing stem cells. Expression of vector-encoded human adenosine deaminase (hADA) was detected in all recipients at levels averaging 15–50% of endogenous murine ADA in all their hematolymphoid tissues. Survival of stem cells in untreated cultures was ~10% of that observed from LIF-treated cultures, resulting in poor engraftment of recipient animals with transplanted cells. The infection efficiency of the few stem cells recovered from untreated cultures, however, was high (~80%), suggesting that LIF did not have an effect on infection efficiency per se, but acted at the level of stem cell survival. Consistent with the poor engraftment observed in the control animals, expression of vector-encoded ADA was only ~4–20% of the endogenous levels. These results support the postulated role of LIF as a regulator of hematopoiesis and suggest that cytokine stimulation can positively affect inefficient retroviral vector transduction in hematopoietic stem cells.

A major limiting factor in the development of retroviral vector-mediated gene transfer for treatment of diseases affecting the blood and blood-forming tissues has been the low infection efficiency of hematopoietic stem cells in vitro (1–6). Without positive selection, ~70%, or less, of murine transplant recipients can be shown to harbor proviral sequences in the differentiated progeny of donor stem cells. In these experiments, when an estimate was made, only ~10% of active stem cells were transduced (1, 2, 5). Although a 100% stem cell infection efficiency is not required to proceed from murine models to large animal or clinical models of gene therapy, the infection efficiency must be high enough such that all transplanted recipients exhibit hematopoietic repopulation with the differentiated progeny of at least one infected stem cell. While positive selection in vitro has been used successfully to enrich for infected cells in murine transplantation models (7, 8) it is not clear that this will be useful for human gene therapy. The large number of cells required for successful hematopoietic engraftment (~10^8 cells/kg body weight, reference 9) could preclude selection techniques as a substitute for efficient stem cell infection.

It has been suggested that the low observed infection efficiency is a result of stem cell quiescence during cocultivation of bone marrow with vector-producing cell lines. Integration of proviral intermediates is sensitive to target cell DNA replication (10) and hematopoietic stem cells are predominantly quiescent, spending >97% of the cell cycle transit time in the G_0 or G_1 phase (11). It has recently been shown

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that quiescent cells can be infected by HIV and harbor labile preintegration proviral intermediates for at least 5 d (12). The efficiency of integration during subsequent replication declined as a function of time postinfection. An alternative explanation for low stem cell infection efficiency is that replicating, and therefore efficiently infected, stem cells are committed to differentiate during culture before transplantation.

A prediction of either of these models is that the provision of appropriate cytokines to bone marrow culture should enhance vector-mediated gene transfer to stem cells by stimulating self-replication, or by otherwise maintaining the stem cell proliferative and differentiative capacity. IL-3 and IL-6 have been reported to be useful for prolonging survival of stem cells in vitro, but the infection efficiency was not significantly improved (13); and only ~40% of the recipient animals were shown to harbor provirus-containing cells 4 mo post-transplantation.

We have been studying other recombinant cytokines, including the leukemia inhibitory factor (LIF)[1] to determine whether they might improve stem cell survival and retroviral vector infection in vitro. LIF preserves the developmental totipotency of embryonic stem (ES) cells in vitro (14, 15), and has allowed improved recovery and retroviral vector infection of spleen colony forming cells (CFU-S) and their progenitors (preCFU-S) after cocultivation with vector-producing fibroblasts (16). In the present study, we have investigated the effect of LIF on survival of, and gene transfer efficiency into, pluripotent hematopoietic stem cells in shortterm liquid bone marrow culture. We have developed a transgenic competitive repopulation assay that has allowed assay of cytokine effects on stem cell survival in vitro, and improved analysis of stem cell infection efficiency.

The results demonstrate that LIF directly or indirectly maintained the stem cell repopulation capacity during shortterm coculture of bone marrow with retroviral vector-producing fibroblasts, and that most of the stem cells recovered from these cultures were productively-infected with the vector. Improved recovery of infected stem cells allowed nearly complete hematopoietic reconstitution with provirus-bearing cells in all recipients, and led to efficient and long-term expression of vector-encoded human adenosine deaminase (hADA).

Materials and Methods

Cell Lines. Construction of the retroviral vector ΔN2ADA has been described (1). Development of the cell line (X1) producing this vector from the retroviral packaging cell line GP+E-86 (17) has also been described (2). The titer of the retrovirus was ~10^6 infectious U/ml was determined by immunostaining titration and was free of replication-competent virus.

Transgenic Mice. Transgenic mice were generated by DNA microinjection into one-cell stage FVB/N embryos, using standard techniques (18). The microinjected DNA was a 5.3-kb EcoRI-PstI fragment from the previously described genomic clone, gr19 (19). The fragment consisted of a truncated SV40 large T antigen transcribed from an αA-crystallin promoter (18, 19), integrated into a different genomic locus in each family (Fig. 1). Expression of the transgene was limited to the lens of the eye, causing cataract or microphthalmia, allowing identification of transgenic animals by visual inspection (20). Each family of mice, designated "A" and "B", was bred to homozygosity (with respect to the transgene) by successive brother-sister matings. The control, or A type, cells each harbored approximately 20 copies of the transgene relative to a single-copy endogenous gene. The cytokine-treated, or B-type, cells each contained two copies of the integrated transgene. A high copy number family was selected for the control cultures because it was suspected that there would be a reduction of stem cell activity in these cultures. Starting with 20 copies increased the sensitivity of detection by Southern blotting and thereby enhanced our ability to detect any residual stem cell activity from this population of cells.

Infection Protocol. Bone marrow (BM) was collected from 10- to 12-wk-old female transgenic mice. BM, flushed from the femur and tibia of each hind leg, was cocultured (1:1 ratio) on monolayers of irradiated (20 Gy total dose) fibroblasts, as described (16). B-type donor marrow cells were cultured on irradiated monolayers of GP+E-86 packaging cells producing the replication-defective retrovirus ΔN2ADA with 0, 10, or 1,000 U/ml recombinant LIF derived from transfected *COS* cells (16). The LIF preparation had an activity of 75,000 U/ml as measured on DA-1 cells. A-type donor marrow cells were cultured on monolayer of GP+E-86 without added cytokine.

After 72 h cocultivation, BM cells were harvested by collecting non-adherent and loosely-attached cells. Viable cell counts were performed by trypan blue exclusion, followed by mixing (1:1, A:B cells) and transplantation into lethally-irradiated syngeneic FVB recipients (8×10^6 cells/animal).

Transplantation Protocol. 10–12-wk-old syngeneic (FVB) female recipients were irradiated (10Gy) at least 4 h before transplant. Irradiation was performed with a ^{137}Cs source (Gammacell 1000; Atomic Energy Ltd., Kanata, Ontario, Canada) at a dose rate of 2.5 Gy/min. Recipients were injected intravenously with 8×10^6 viable bone marrow cells in culture medium. Transplantation recipients were housed in microisolator units placed on laminar-flow cage racks (Lab Products, Maywood, NJ); and fed sterilized rodent chow and sterile, acidified water.

Analysis of Hematopoietic Tissues from Primary Recipients. 6 mo post-transplant the spleen, thymus, bone marrow, blood, and thioglycolate (TG)-induced peritoneal exudate were harvested from each primary recipient. Splenic B cells were prepared by positive immunoselection with anti-mouse IgG-coated magnetic beads (Advanced Magnetics Inc., Cambridge, MA), as per the manufacturer's recommendations. The peritoneal exudate from each animal was cultured in vitro in the presence of IL-3 (10% WEHI3 conditioned medium) to enrich for adherent macrophage/mast cells. Bone marrow (5×10^4 cells) from each primary recipient animal was injected into each of three irradiated (10 Gy) secondary recipients for isolation of clonal stem cell progeny (repopulated spleen colony-forming cells, CFU-S). Serum from three animals in each group was tested for the presence of replication-competent virus by marker rescue assay. All these samples were negative for helper virus activity.

Protein extracts were prepared from each tissue and used to analyze expression of vector-encoded hADA by Western blotting, as described (2). Quantitative analysis of hADA expression was performed by video densitometry of hADA bands on Western blots, followed by comparison to standard curves developed with purified hADA. These were generated by plotting the specific optical den-

[1] *Abbreviations used in this paper:* BM, bone marrow; *dl*, downless; ES, embryonic stem; hADA, human adenosine deaminase; LIF, leukemia inhibitory factor; SOD, specific optical density.

sity (SOD) against the varying amounts of purified recombinant human ADA (kindly provided by Dr. Rod Kellems, Baylor College of Medicine) applied to each lane. SOD was calculated by subtracting the integrated density of the individual lane background from integrated density of the hADA-specific band. The specific activity of the purified recombinant hADA was 2.0×10^5 U-nM/mg/min at 25°C as determined by the spectrophotometric method (21). The standard curves were linear with respect to hADA quantity vs. SOD in the signal range of our samples (between 3.0 ng and 24 ng; $r = 0.9998$). To quantitate the human ADA enzyme in the transplant recipient tissue specimens each immunoblot was then scanned. The amount of hADA was estimated in MOLT4 (human T cell lymphoma cell line) controls and in experimental samples by interpolation based on this linear standard. A fixed amount of protein extract from MOLT4 was run on each immunoblot gel (7.2 μg; specific activity 425 U-nM/mg/min corresponding to 16.2 ng or 3.25 U hADA). This standard was used to normalize the values from each experimental sample by reference to the efficiency of detection on each blot: detection efficiency (DE) = estimated MOLT4 U hADA/3.25 U (1). The quantity of hADA in the experimental samples was calculated by the following equation: U hADA activity = (est. sample ng hADA) (specific activity rhADA)/(μg protein) (DE) (2).

High molecular weight DNA was prepared from each sample, except blood, and subjected to analysis of transgene representation and proviral integration by Southern blotting and PCR. Quantitative video densitometry of transgene band intensities on Southern blots, compared to known copy number controls, allowed estimation of competitive ability and extent of repopulation by each type of marrow; while assay of proviral integration allowed estimation of vector infection and expression efficiencies.

For transgene analysis, genomic DNA (5 μg) was digested with a restriction endonuclease that cuts only once in the transgene (BamHI), releasing unique-length DNA fragments from each marrow type (Fig. 1). Digested DNAs were electrophoresed in 0.8% agarose for 48 h, at room temperature and 30 volts. Gels were blotted onto paired (one for the upper half of the gel, fragments greater than 2K bp; and one for the lower half, less than 2K bp) nylon membranes (GeneScreen Plus; NEN Research Products, Boston, MA) as per the manufacturer's recommendations. A 650-bp HindIII-HindIII fragment of the plasmid pGr4 (19), containing parts of the transgene and mouse endogenous downless (*dl*) locus, was labeled with α-^{32}P-dCTP by random hexamer priming. The probe hybridized to both the transgenic bands and the endogenous *dl* locus, providing a convenient single-copy control. Hybridization in 5× SSPE, 1% SDS (65°C, overnight) to the membrane blotted with >2K bp fragments, followed by washing at high stringency (0.1× SSC, 1% SDS, 65°C) and autoradiography (XAR-5 film, Eastman-Kodak, Rochester, NY and Lightning Plus intensifying screen) at −70°C, allowed detection of transgenic bands. Quantitation of Southern blots by video densitometry allowed estimation of repopulation by each marrow type in each tissue. SOD was measured and calculated as described above for the immunoblot analysis. Normalization for DNA loads and hybridization efficiency for each lane was accomplished by the presence of the internal, single-copy control in each lane (endogenous *dl* locus). DNAs from each parental strain (A and B) were also included on all gels to provide a transgene copy number control. The adjusted SOD of each sample band was compared to the adjusted SOD of the control transgene bands to achieve an estimate of hematopoietic repopulation by each donor marrow. Adjusted band intensity = (SOD sample) × (SOD dl sample)/(SOD dl control) (3); and percent reconstitution = (adjusted band intensity/control transgene intensity) × 100 (4).

For analysis of proviral integration, the half of each membrane pair that contained <2K bp DNA fragments was hybridized (5× SSPE, 1% SDS, 65°C, overnight) with a ^{32}P-labeled hADA cDNA probe. Washing at 0.5× SSC at 55°C, followed by autoradiography allowed detection of a common 1.2K bp provirus band from all integration sites. Video densitometry of the provirus band in each lane compared to a known-copy-number control DNA prepared from the ΔN2ADA packaging cell line, present on each gel, allowed quantitation of proviral copy number for each tissue.

Assay of CFU-S Derived from Primary Recipients. Analysis of repopulated CFU-S derived from the bone marrow of reconstituted primary recipients allowed estimation of the percent reconstitution and clonality of transgenically-marked stem cells. By determining the percentage of CFU-S from individual animals containing each transgenic marker, an independent estimation of stem cell repopulation was derived. A primary assumption with this method was that all CFU-S derived from each primary marrow were the progeny of transplanted or endogenously repopulating stem cells. The CFU-S assay was performed as described (16), and analysis of transgene representation and proviral integration was performed as described above.

Results

Transgenic Competitive Repopulation Assay. To investigate the effect of LIF on stem cell survival in vitro, we designed a transgenic repopulation assay that compared the ability of LIF-treated bone marrow cells to compete with untreated cells during reconstitution of hematopoiesis in irradiated recipients (Fig. 1 *A*). To allow identification of the two sets of marrow, and to distinguish between endogenous repopulation and reconstitution with transplanted cells, we used two different families of inbred transgenic FVB mice as marrow donors (Fig. 1 *B*). A-type (control) bone marrow cells were cultured in the absence of added growth factor on GP+E-86 retroviral packaging cells that were not transfected with a vector construct. B-type bone marrow cells were cultured on ΔN2ADA vector-producing fibroblasts (GP+E-86 derived) to uniquely mark individual self-replicating stem cells. Recombinant murine LIF was included in cultures of B-type cells at either 0, 10, or 1,000 U/ml.

To prove equivalent reconstitution potential of each transgenic marrow, ten recipients were transplanted with equal mixtures of fresh marrow explants. Southern analysis of transgene representation in hematopoietic tissues six months post transplant demonstrated that neither marrow had a gross competitive advantage, leading to equivalent reconstitution with each transgenic marrow type in all tissues (spleen, thymus, bone marrow) and enriched cell populations (splenic B cell, macrophage) studied (Fig. 2, *A* and *B*). Southern analysis of transgene representation in clonal stem cell progeny (spleen colony-forming cells, CFU-S) derived from the bone marrow of reconstituted primary animals revealed that approximately 20% of repopulation was by endogenous stem cells in these controls (Fig. 4 *B*).

Effect of LIF on Stem Cell Survival In Vitro. To document the extent of stem cell loss during culture in the absence of added LIF, we transplanted irradiated recipient animals with cells recovered from control cultures without added cytokines.

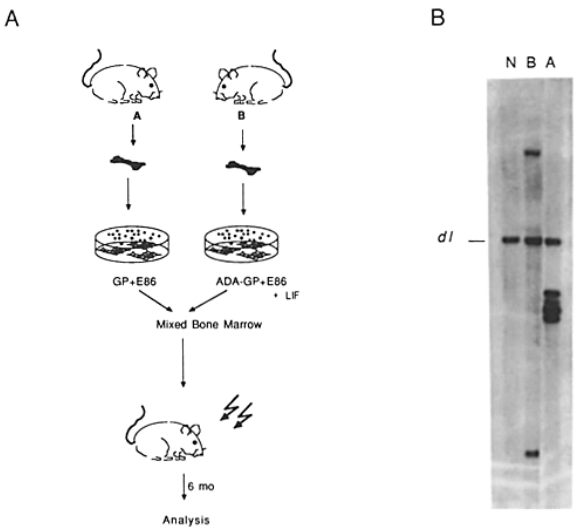


Figure 1. Competitive repopulation assay. (*A*) Inbred (FVB) transgenic donors are used as a source of genetically-marked bone marrow. Briefly, A-lineage cells were plated onto control cultures of irradiated packaging cells that did not produce the ΔN2ADA vector. B-type cells were plated onto ΔN2ADA-producing fibroblasts. No growth factor was added to the "A" cultures, but 0-1,000 U/mL recombinant LIF was added to "B" cultures. After 72 h of cocultivation, nonadherent and loosely-adherent cells were recovered from the two cultures, counted, and mixed at a 1:1 ratio. Irradiated (10 Gy) syngeneic recipient animals were injected intravenously with 8×10^6 viable cells and allowed to recover without experimental manipulation for 6 mo. At sacrifice, primary hematopoietic tissues (spleen, thymus, bone marrow, blood) and enriched cell fractions (splenic B cells, peritoneal macrophages, CFU-S) were analyzed as described in the Materials and Methods; (*B*) Families of donor mice harbor multiple copies of the transgene, consisting of a truncated SV40 large T antigen driven by an α-crystallin promoter. A-type mice harbor a twenty copy transgene array visualized as a complex multiple-band pattern on Southern blot of *Bam*HI-digested DNA, while the B-type mice harbor a two copy array visualized as nonsegregating single-copy bands on Southern blot of *Bam*HI-digested DNA. Only the lower band was used for quantitation of B-type cells on Southern blots because the upper band was often lost in the background of sheared DNA at the top of each lane. The presence of sequence from the endogenous murine downless (*dl*) locus on the plasmid fragment used as a probe for the transgene Southerns allows detection of the single-copy *dl* band as an internal control for DNA load in each lane.

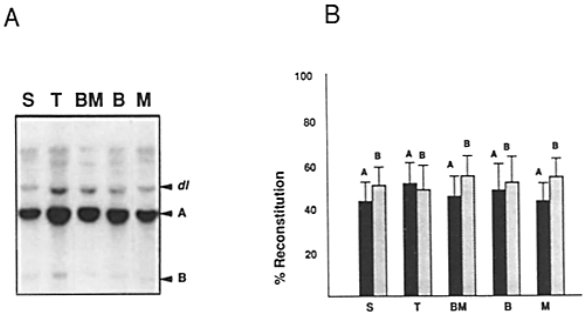


Figure 2. Control animals transplanted with fresh marrow explants. (*A*) Southern analysis of transgene repopulation in a single control animal. Genomic DNA (5 μg) from spleen (S), thymus (T), bone marrow (BM), splenic B cells (B), and peritoneal macrophages were analyzed as described in the Materials and Methods; *dl*, endogenous single-copy control. (*B*) Average repopulation with transgenically-marked cells from a group of ten recipients. The columns filled with hatched shading represent the observed repopulation with B-type cells; the solid black columns represent the percent repopulation with A-type cells. Error bars delineate limits of the standard error of the calculated mean (SEM).

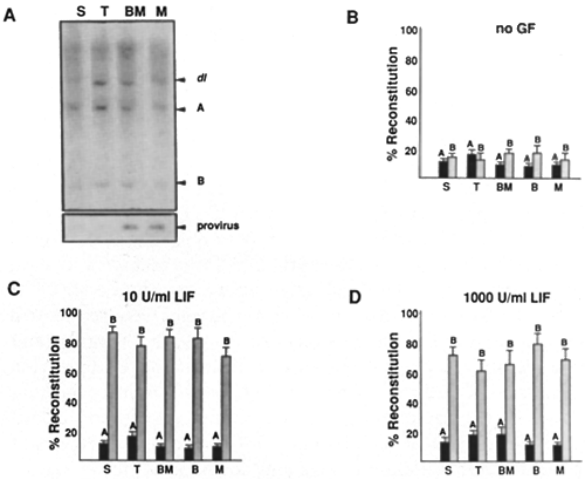


Figure 3. Effect of LIF on hematopoietic repopulation. (*A*) Southern analysis of transgene repopulation in a single recipient, animal 24.6. DNA was analyzed as described in the legend to Fig. 2 and in the Materials and Methods; *dl*, single-copy control. Abbreviations are as in the legend to Fig. 2. Genomic DNA from these tissues was also studied with an hADA cDNA probe to measure provirus representation. (*B*) B-type cells cultured without added cytokines. (*C*) B-type cells cultured with 10 U/mL LIF. (*D*) B-type cells with 1,000 U/mL LIF. The figures represent average repopulation with transgenically-marked cells from groups of ten recipients. The columns filled with hatched shading show the observed repopulation with B-type cells; the solid black columns represent the percent repopulation with A-type cells. Error bars delineate limits of the standard error of the calculated mean (SEM). Abbreviations are as in the legend to Fig. 2.

Culture of either transgenically marked bone marrow in the absence of LIF resulted in loss of the stem cell repopulating activity, as demonstrated by quantitative reduction in copy number of the transgenic markers on Southern blots of DNA from hematopoietic tissues harvested six months posttransplant. Differentiated progeny of transgenically-marked stem cells accounted for ~30%, on average, of total hematopoietic cells at sacrifice (Fig. 3 *B*). Analysis of repopulated CFU-S from these animals confirmed loss of the stem cells during culture (Fig. 4 *B*), with an average of 37% of total CFU-S arising from transgenic donor cells. Repopulation by endogenous stem cells accounted for the balance of CFU-S and, therefore, differentiated progeny of hematopoietic stem cells in these control animals.

Culture of B-type cells with 10 U/ml LIF, followed by competitive repopulation with untreated cells of the A-type, resulted in an average 79% (± 6%) reconstitution with progeny of LIF-treated stem cells in all (10/10) recipient animals

(Fig. 3 *C*). Untreated A type cells were represented at an average of 11% (± 2%), consistent with the A- and B-type controls cultured without added LIF. Analysis of transgene representation in CFU-S derived from bone marrow of these recipients correlated with Southern blot analysis of primary hematopoietic tissues, with an average 88% (139/157 total colonies from 10 animals) of spleen colonies derived from LIF-treated (B-type) stem cells. Ten percent (16/158) of spleen colonies were derived from A-type cells and 3% (4/157) of colonies were derived from endogenous stem cells that survived irradiation (Fig. 4 *B*).

At 1,000 U/ml LIF, a result similar to the 10 U/ml condition was obtained in the competitive repopulation assay, with progeny of LIF-treated stem cells representing 71% (± 6%), on average, of hematopoietic cells present in the reconstituted recipients (Fig. 3 *D*). A-type cells represented an average of 15 ± 3% of differentiated progeny in these animals. Analysis of repopulated CFU-S confirmed the finding in primary recipients, with an average of 78% (102/131) of total spleen colonies derived from LIF-treated stem cells, 16% (21/131) from A-type cells, and 6% (8/131) from endogenous repopulation (Fig. 4 *B*).

Retroviral Gene Transfer to Stem Cells. Proviral sequences were not detectable by Southern blotting in primary hematopoietic tissues of recipients transplanted with marrow cultured in the absence of LIF. Southern blotting and PCR analysis of proviral representation in the B-type CFU-S from these animals, however, revealed that 79% (15 provirus positive of 19 total) harbored the provirus.

All (20/20) recipients of LIF-treated marrow had proviral integration that could be detected by Southern blotting in hematopoietic tissues following reconstitution. At 10 U/ml LIF, two copies of provirus, on average, were detected per B-type cell (Table 1). Analysis of repopulated CFU-S from these animals revealed that, of the B-type colonies present, an average of 70% (98/139) were infected (Fig. 4 *B*). Provirus-positive CFU-S were recovered from all (10/10) 10 U/ml LIF primary animals, representing from 8% to 100% of B-type colonies per animal (Table 1). Proviral copy number detected in the bone marrow of each primary recipient was consistent with the percentage of provirus-positive CFU-S generated from that bone marrow.

At 1,000 U/ml LIF, the average proviral copy number was 0.7 per B-type cell (data not shown). Analysis of CFU-S from these animals showed that of the B-type colonies present, 69% (75/102) had detectable provirus. Provirus-positive CFU-S were recovered from 9/10 1,000 μ/ml LIF animals, representing 25–100% of B-type spleen colonies per animal (data not shown). Again, proviral copy number measured in the bone marrows of primary recipients was consistent with the percentage of provirus-positive CFU-S generated from each marrow.

Analysis of proviral junctions in repopulated CFU-S revealed oligoclonal reconstitution in primary animals receiving LIF-treated marrow (Fig. 4 *A*). Production of CFU-S from a single infected stem cell predominated in most animals, however, with additional stem cells either quiescent or contributing very low numbers of differentiated hematopoietic cells.

Expression of Vector-Encoded Human Adenosine Deaminase. Expression of vector-encoded hADA was examined in hematopoiesis tissues of recipient animals at the time of sacrifice (6 mo posttransplantation). Western blots of protein extracts were developed with a hADA-specific antibody, revealing increased levels of hADA protein in animals receiving LIF-treated marrow, compared to controls. To estimate the amount of human ADA expressed by vector transduced cells, immunoblots were analyzed by quantitative video-imaging densitometry. Human ADA-specific bands on individual nitrocellulose filters were corrected for variances in transfer and development by comparison to a positive control (Molt4) on

Table 1. *Recovery and Infection Efficiency of CFU-S Derived from Primary Transplant Recipients*

Animal	Endogenous colonies	A-type colonies	B-type colonies	Infection efficiency
	%	%	%	%
24.1	0/13 (0)	2/13 (15)	11/13 (85)	7/11 (64)
24.2	0/12 (0)	2/12 (17)	10/12 (83)	8/10 (80)
24.3	0/15 (0)	3/15 (20)	12/15 (80)	8/12 (67)
24.4	0/19 (0)	2/19 (9)	17/19 (91)	16/17 (94)
24.5	1/13 (5)	2/13 (15)	10/13 (80)	6/10 (60)
24.11	3/17 (18)	1/17 (6)	13/17 (76)	9/13 (69)
24.12	0/14 (0)	2/14 (14)	12/14 (86)	1/12 (8)
24.13	0/21 (0)	0/21 (0)	21/21 (100)	10/21 (48)
24.14	0/9 (0)	0/9 (0)	9/9 (100)	9/9 (100)
24.15	0/24 (0)	0/24 (0)	24/24 (100)	24/24 (100)

B-type marrow was exposed to 10 U/ml LIF during 72-h cocultivation with the retroviral vector producing cell line.

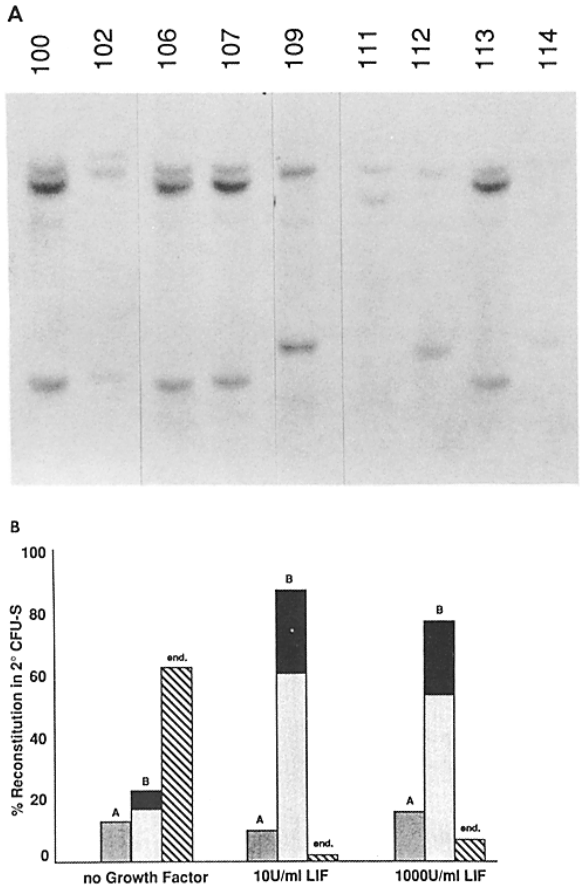


Figure 4. Analysis of repopulated CFU-S. (*A*) Southern blot of unique proviral integrations in B-type repopulated CFU-S. Shown are colonies from a single animal, 24.6; analyzed as described in the Materials and Methods. Bands from each of the transgene markers and the integrated provirus are indicated. (*B*) Average transgene representation in clonal CFU-S derived from primary bone marrow of each animal. Columns represent average number of colonies with each type of transgenic marker (A - A-type; B - B-type; end - endogenous nontransgenic) divided by the total number of colonies. To control for statistical effects of differing numbers of colonies derived from separate animals, averages were calculated on a per animal basis, rather than as a percentage of total colonies. The shaded area in the B column represents the average percentage of CFU-S bearing the ADA vector provirus.

each filter. The hADA concentration was predicted from a standard curve based on purified recombinant human ADA and expressed as nM/min/mg. The level of hADA protein was variable between animals in each treatment group and within tissues from single recipients (Table 2).

We also compared the quantity of hADA protein to various control groups. Endogenous ADA expression was measured spectrophotometrically both in normal non-transplanted mice and a group transplanted with fresh marrow not infected by the hADA retrovirus. The results of control ratios are depicted in Table 3. In the first transplant control group the level of endogenous ADA ranges from 1.2-fold to 2.5-fold the nontransplant group in all tissues except the thymus. The explanation for the very low thymus ADA enzyme activity in these transplant controls is unclear but may represent relative depletion of the cells expressing the highest levels of the enzyme. Further comparison of hADA levels with endogenous mouse is also contained in Table 3. The estimated levels of hADA in the mice transplanted with the LIF-treated marrow ranged from 18 to 130% in the various tissues. The levels of hADA were substantially lower in the non-LIF treated retrovirally-infected marrow group ranging from 5 to 25% of the control. A further comparison was made between the mean levels of hADA expression in the LIF treated group vs. the no-LIF control group. The levels of hADA in the 10 U/ml LIF group were 2.2–12-fold the no-LIF control. Likewise the 1,000 U/ml LIF group demonstrated levels of hADA that ranged from 1.5 to 8.4-fold the no-LIF control. Quantitative analysis of hADA levels revealed no absolute correlation between proviral copy number and expression, when measured either within individual animals or across tissue groups. This suggests that integration position effects play an important role in the level of expression from the individual stem cell clones.

Discussion

Recovery of infected hematopoietic stem cells from coculture with vector-producing fibroblasts has been a limiting factor in developing models for human gene therapy. We have observed that LIF directly or indirectly promoted survival of hematopoietic stem cells in vitro, and that stem cells recovered from LIF-treated cultures exhibited a significant competitive advantage over untreated cells in repopulating the hematopoietic system of irradiated transplant recipients. Moreover, the retroviral vector infection efficiency of LIF-treated stem cells was high, resulting in hematopoietic reconstitution of all recipients with progeny of at least one infected stem cell. Oligoclonal hematopoiesis was observed in the transplanted animals, but a single infected clone predominated in each animal. The observation of predominant clones in these animals precludes direct estimation of stem cell infection efficiency because of the skewing of relative activity between different stem cells. Since most of the CFU-S from individual primary recipients analyzed were derived from a few stem cell clones, the 70% infection efficiency of repopulated CFU-S only roughly corresponds to the absolute stem cell infection efficiency. Nearly complete hematopoietic reconstitution with infected stem cells enabled expression of vector-encoded hADA at physiologically relevant levels in the important lymphoid compartments for at least 6 mo in all recipient animals.

The mechanism by which LIF stimulation improved stem cell survival is unknown, but could include a direct effect of LIF alone or in combination with other cytokines on stem cells, and/or an indirect effect on marrow accessory cells or the vector-producing fibroblasts. Because the source of the LIF in these experiments was a Cos cell derived conditioned medium, a contribution of cytokine activity from the Cos cells cannot be excluded. In previous experiments (16) no effect of the Cos conditioned media alone was observed on primary

Table 2. *Quantitative Analysis of hADA Protein in Tissues of Recipient Animals*

Animal	Growth factor	Spleen	Thymus	Bone marrow	B Cell	MΦ	Blood
4.1	None	4.1	0.8	12.0	6.9	ND	0
4.2		4.3	0.5	6.7	19.1	ND	0.2
4.3		5.4	0.1	2.4	4.9	ND	0
4.4		2.4	0	4.5	2.4	0.4	0
4.5		2.1	0.9	2.5	13.8	2.6	0
4.6		4.9	2.6	9.6	20.3	11.9	0.7
4.7		ND	ND	ND	ND	ND	ND
4.8		0	0	3.1	0	0	0
Average		3.3	0.7	5.8	9.6	3.7	0.1
24.1	10 U/ml LIF	22.1	10.9	45.8	25.1	7.7	2.2
24.2		7.9	7.8	8.4	43.3	42.4	1.8
24.3		ND	ND	5.1	29.2	10.7	0.4
24.4		9.2	22.7	9.1	29.9	16.2	6.0
24.5		12.2	5.8	13.4	31.3	1.8	0.5
24.11		7.4	10.4	5.3	12.7	8.1	0.3
24.12		9.5	6.1	9.1	11.9	8.3	0.1
24.13		4.8	4.2	4.6	8.1	20.7	0.2
24.14		6.7	4.8	17.9	14.3	8.3	0.1
24.15		6.2	4.4	11.4	9.9	8.7	0.5
Average		9.6	8.6	13.0	21.6	13.3	1.2

hADA levels are reported in units of nanomoles ADA activity/minute/milligram total protein. Protein extracts were prepared and hADA quantitated as described in the Materials and Methods. Indicated growth factors were added to cultures of B-type cells prior to competitive repopulation with untreated A-type cells. There was no significant difference in hADA protein levels between animals in the 10 U/mL and 1,000 U/ml groups.

CFU-S survival or retroviral vector infection efficiency. Synergistic interaction of LIF with cytokine(s) present at subthreshold levels in the Cos media are a possibility. We have generated preliminary evidence (not shown) suggesting that FACS-sorted stem cells (WGA^+, $CD4^-$, $CD8^-$, $B220^-$, $Mac\text{-}1^-$; ~200-fold CFU-S purification) are infected at high efficiency (80%) during coculture with vector-producing fibroblasts in the presence of LIF, thus possibly removing the majority of marrow accessory cells from consideration as LIF targets in this system. It is possible that LIF-treatment induced secondary cytokine production by, or acted in combination with factors normally secreted from the vector-producing fibroblasts present in our system. We were unable to detect the production of either IL-6 or IL-3 bioactivity by whole bone marrow or vector-producing fibroblasts in response to LIF stimulation (data not shown), suggesting that the induction of these factors did not produce the present results. Production of the recently identified stem cell factor (MGF) by fibroblasts in vitro (22) was interesting in this regard, and we have begun investigating a possible interaction between LIF and MGF on the survival and proliferation of purified stem cells in vitro.

Although LIF can improve the recovery of pluripotent stem cells and preCFU-S from liquid bone marrow culture, there is no evidence that LIF has a direct proliferative effect on these cell populations. Purified (WGA^+, lin^-, $Rhodamine\text{-}123^{dull}$) stem cells are not induced to proliferate in liquid culture by LIF treatment, and proliferative index (by [^{3}H]thymidine in-

Table 3. *Comparison of Relative ADA Expression in Transplant Recipients*

Group	Spleen	Thymus	Marrow	B Cell	Blood
Control*	1.3	0.03	1.7	1.2	2.5
0 U/mL LIF‡	0.18	0.05	0.11	0.25	0.11
10 U/mL LIF	0.54	0.60	0.25	0.56	1.3
Ratio§	2.9	12.0	2.2	~1.5	12.0

* Control: Five mice were transplanted with fresh marrow not infected with the human ADA retrovirus. Endogenous ADA levels were determined spectrophotometrically and are expressed as the ratio of transplant control to normal nontransplant control.

‡ Human ADA expression in the different experimental groups compared to the level of endogenous ADA expression in normal nontransplanted FVB mice. The results are expressed as the ratio of transplant group to normal adult control. ADA levels from infected transplant groups and controls are listed in Table 2.

§ Ratio of the mean levels of hADA in the LIF treated transplant group vs. the no growth factor group. The ratios for cultured macrophages was 3.6. The mean values from each group are in Table 2.

corporation) is not improved by addition of LIF to other defined cytokines, including MGF (data not shown). We suggest, rather, that the primary effect of LIF in our system is the delay or prevention of stem cell commitment to differentiation, an effect similar to the LIF action on ES cells.

An unexpected finding from these studies was that the few stem cells recovered from untreated cultures also were infected at high efficiency, suggesting that there is no inherent block to retroviral infection of stem cells in the absence of added cytokine, a result in agreement with our hypothesis of LIF action. We speculate that this high infection efficiency results from the very high titer of ADA vector produced from the packaging cell and the apparent requirement for very high multiplicity of infection in the stem cell pool. In addition, intrinsic variability of the vector packaging cell lines in their interaction with the marrow cells may influence the apparent infection efficiency. When cocultivation of the marrow cells with the vector packaging fibroblasts is used these cells may behave as a stromal microenvironment. The documented loss of stem cells during coculture (23) is suggested to have been responsible for the previously-observed low proviral copy number in transplanted recipients, not low stem cell infection efficiency per se. The growth and differentiation of marrow cells in culture might also affect the apparent infection efficiency. For example, efficient infection of precursors that differentiated to more mature cells would appear to be low in these bioassays if the differentiation process led to loss of clonogenic or repopulating activity among those cells infected with the vector. We hypothesize that the apparent increase in infection efficiency of primary CFU-S after LIF stimulation (16) reflects, at least in part, a loss of infected CFU-S from cultures without cytokine support.

In conclusion, these results suggest that LIF directly or indirectly maintains the hematopoietic stem cell phenotype in shortterm culture, thereby facilitating recovery of stem cells. Stem cells recovered from LIF-treated cultures are efficiently infected and can repopulate the hematopoietic system of all transplanted animals, leading to longterm expression of vector-encoded hADA at significant levels. The ability, demonstrated here, to repopulate all bone marrow transplant recipients with efficiently-infected stem cells significantly advances gene transfer methodology in the murine model. Future studies on the effect of LIF in human hematopoietic precursor cultures should indicate whether similar protocols will allow efficient transduction of human stem cells for the purpose of gene therapy.

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