High-Efficiency Gene Transfer to Human Hematopoietic Cells Maintained in Long-Term Marrow Culture

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We used a helper-free recombinant retrovirus carrying the neomycin resistance (neo') gene to investigate methods for improving gene transfer efficiencies to clonogenic hematopoietic progenitor cells of human origin and to assess the possibility of gene transfer to the more primitive cells from which clonogenic cells are derived after several weeks in long-term human marrow cultures. The proportion of neo' CFU-GM in methylcellulose assays of infected fresh marrow was increased by six- to eightfold (mean 37.4%) by the addition of extra GM colony-stimulating factor and interleukin-1β or medium conditioned by a human marrow "stromal" cell line to medium conditioned by agar-stimulated human leukocytes both during the infection and the colony growth period. Similar increases were also noted in the proportion of neo' BFU-E, although the efficiencies overall were somewhat lower (up to 25.7%, mean 16.3%).

GENE TRANSFER using retroviruses is ideally suited for lineage analysis during development and for investigations of the molecular mechanisms that regulate normal and abnormal cell growth and differentiation. The hematopoietic system is an attractive model for such studies because of its well-documented hierarchical structure and the recent identification of some of the genes that regulate hematopoiesis. These include genes for growth factors and growth factor receptors as well as several protooncogenes. In addition, genetic disorders of the hematopoietic system are likely to serve as the first clinical targets for somatic cell gene therapy.

Studies in the murine system have confirmed the utility of retroviral vectors.14 Efficient gene transfer to primitive hematopoietic cells including CFU-S and cells capable of reconstituting lethally irradiated mice has been accomplished, although expression of the introduced genes remains variable. Experience with human marrow cells is less extensive, but transfer to human hematopoietic colony-forming cells has been documented for a number of genes, including neo', HPRT, and β globin.15

In an effort to improve and characterize the efficiency of gene transfer to the most primitive hematopoietic cells detectable in human marrow, we have begun to explore its application to the cells that initiate hematopoiesis in long-term human marrow cultures.11 In these cultures, hematopoiesis can be routinely maintained for 2 to 3 months, and the initial cell responsible for this is believed to be more primitive than the progenitor cells routinely detected in colony assays.12-16 Consistent with this are recent results demonstrating the feasibility of using cells derived from long-term human marrow cultures for autologous bone marrow transplantation.15-17

We now report modifications to an earlier infection protocol involving hematopoietic growth factor supplementation during the first 48 hours that allow high-efficiency gene transfer to normal human marrow progenitors to be consistently obtained. Moreover, this high efficiency applies not only to colony-forming cells but also to the cells capable of initiating the sustained hematopoiesis that is observed in long-term marrow cultures.

MATERIALS AND METHODS

Cell lines. The amphotropic retrovirus packaging cell line PA317 and the ecotropic packaging line ϕ2 were described previously.18,19 These were cultured in Dulbecco's modified Eagle's medium with high glucose (4.5 g/L) and 10% heat-inactivated calf serum (for ϕ2 cells) or 10% fetal calf serum (for PA317 and NIH-3T3) in a 5% CO2 atmosphere at 37°C.

Retroviral vectors. Tk-neo' is a murine leukemia virus-based vector containing the gene for neomycin phosphotransferase (neo') linked to the herpes simplex thymidine kinase (Tk) promoter in the reverse orientation relative to hybrid Moloney and Harvey murine leukemia virus long terminal repeat regions. It was derived from MMCV-neo',20 a gift from Dr B. Vennstrom, by removal of myc sequences and reinsertion of the Tk neo' sequence in the 3' to 5' orientation. In preliminary experiments, the N2 vector containing the neo' gene expressed off the viral LTR was also used.

Virus production. The general strategy used to generate high-efficiency gene transfer to human hematopoietic cells maintained in long-term marrow culture.
titer retrovirus-producer cell lines was the same as that outlined by Miller et al. Clones that were helper virus-free (helper titer < 1 ffu/mL) were selected and expanded; cells. After 48 hours, v-neo'-producing PA317 clones were selected containing neo'.

Clones that were helper virus-free (helper titer < 1 ffu/mL) and had titers of 5 x 10^8/mL were then selected for use in the present study.

Viral infection of marrow cells. Leftover human bone marrow aspirate cells, obtained with informed consent as part of routine hematologic assessment procedures or from bone marrow transplant harvests, were passed over a Percoll (Pharmacia Canada; Baie d'Orfe, Quebec, Ontario, Canada) density gradient (density = 1.066), and light-density cells were collected and used for subsequent infections. Most experiments were performed with marrow from normal donors, although in some cases “uninvolved” marrow from newly diagnosed or treated cancer patients was also used. Control infection conditions were as previously described. Dishes (60 mm) of near-confluent viral producer cells were irradiated with 1.5 Gy (250-kVp x-rays), then marrow cells (5 x 10^6) in α-medium with 20% fetal calf serum and 10% agar-stimulated retrovirus-producer cell lines was the same as that outlined by Miller et al.2 Retroviral plasmid DNA was transfected by calcium phosphate precipitation into 2'1'2 cells and, after 48 hours, medium was aspirated and had titers of 5 x 10^8/mL. Parallel long-term cultures containing control (uninfected) cells were similarly maintained and assayed. In some experiments the adherent cell fraction was also harvested and assayed. Cell and viral manipulations were performed under level C containment following Medical Research Council of Canada guidelines.

DNA analysis. Genomic DNA was extracted from normal marrow, adherent and non-adherent fractions of long-term marrow cultures, or from pooled colonies plucked from methylcellulose cultures by standard techniques. Southern blot analysis was performed by standard methods with DNA transfer to nylon membrane (Zetaprobe, BioRad Laboratories, Mississauga, Ontario, Canada) under alkaline conditions and hybridization to 32P-oligolabeled probes made from the purified EcoRI-Smal fragment of the neo' gene or SacI-EcorI fragment from the human GM-CSF gene. For analysis by polymerase chain reaction (PCR),28 oligomers were synthesized at the University of British Columbia Department of Biochemistry and purified with Sep-Pak cartridges (Waters Associates, Wilford, MA). The sequence of each was as follows: neo-L, 5'-GACAATCGGCTGCTCTGATG-3', and neo-R, 3'- CTGGTGAGCTGATGCCGTC-5'. The predicted size of the PCR product is 350 base pairs. Approximately 1 µg genomic DNA was analyzed using PCR in a reaction mixture (modified from that of New England Biolabs, Beverly, MA) of 16.6 mM/L (NH4)SO4, 67 mM/L Tris-HCl, pH 8.8, 6.7 mM/L MgCl2, 170 µg/mL bovine serum albumin, 10 mM/L 2-mercaptoethanol. 10% dimethyl sulfoxide, 250 mM/L of each deoxynucleoside triphosphate (dNTP), 1 µmol/L of each primer, and 2 U Taq polymerase (New England Biolabs) in a final volume of 100 µL. Before test DNA was added, the reaction mixture was treated with HhaI (10 U for 2 hours at 37°C) to minimize the possibility of contamination (Hughes PFD, Humphries RK, manuscript in preparation). The HhaI was then inactivated by incubation at 95°C for 10 minutes. DNA samples were amplified with a programmable heating block (Eriocorp, San Diego, CA) set for a cycle of 15 seconds at 94°C (denaturing), 60 seconds at 55°C (annealing), and 90 seconds at 72°C (synthesis). The actual time spent at each temperature was slightly longer than set; each cycle took 6 minutes. The samples were amplified for 30 cycles, including a final 5 minutes at 72°C. Fifty microliters of each of the reaction products was ethanol precipitated, resuspended, and electrophoresed on a 3% agarose gel. Transfer to nylon membranes and hybridization to a 32P-labeled probe for neo' were as described above.

RESULTS

Effects of growth factors on gene transfer efficiencies. We initially elected to use the Tk-neo 19 vector because of reports of the beneficial effect of the internal TK promoter on expression of genes transferred to primitive murine hematopoietic cells; however, in initial experiments, in which the N2 and Tk-neo viral vectors were compared for gene transfer efficiencies to clonogenic progenitors using established infection conditions, no significant difference was observed (data not shown). We elected to use Tk-neo 19
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in the subsequent experiments since it was at least as good as N₂ and may still confer an advantage in gene transfer to the primitive human cells. To investigate the possible effects of various conditions of growth factor stimulation, human bone marrow cells were exposed to Tk-neo 19 viral producer cells by a 24-hour cocultivation technique. Recombinant human GM-CSF and recombinant human IL-1β were used together to supplement agar-LCM during the infection phase, and recombinant GM-CSF was used as a supplement during selection and assay of clonogenic progenitors in methylcellulose (Fig 1). When G418 was incorporated into the methylcellulose assay at a dose of 1,000 µg/mL (effective drug), standard infection conditions (agar-LCM only) yielded 1.2% ± 0.1% neo' CFU-GM (mean ± SEM, four experiments). This frequency was increased sixfold to 7.6% ± 0.6% (mean ± SEM, six experiments, P < .001, Student's t test) when GM-CSF and IL-1β were added to both the initial infection and subsequent assay cultures. When these factors were present either during the selection or the infection period, but not both, a slight but not statistically significant improvement was obtained. When conditioned medium from a human marrow-derived adherent cell line (ST-16) was added in place of recombinant GM-CSF and IL-1β, a similar significant improvement in recovery of neo' progenitors was also observed (data not shown).

To establish the most appropriate quantitative endpoint for measuring the proportion of neo' progenitors obtained with different infection protocols, we compared full G418 dose-response curves for each condition. Examples for CFU-GM from uninfected marrow and marrow infected under standard conditions or with growth factor supplements as described above are shown in Fig 2. In these experiments, the uninfected cells were subjected to the same manipulations and assayed using the same conditions as the growth factor stimulated-infected cells (ie, using added GM-CSF and IL-1). Although the addition of these growth factors had no effect on the G418 dose-response of control cells, a significant effect was observed in the infected population as predicted by the data in Fig 1. At a G418 dose of 800 µg/mL (effective) uninfected cells produced no colonies; whereas

![Fig 2. Effect of increasing G418 concentration on the plating efficiency of CFU-GM from normal human marrow infected with neo' virus in medium supplemented with GM-CSF and IL-1 (B), uninfected (incubated as for infection with GM-CSF and IL-1 (C); infected under standard conditions (A). Results are the mean ± SEM of three experiments.](image)

37.0% ± 0.6% of CFU-GM infected and grown under growth factor-supplemented conditions versus 4.9% ± 0.7% of CFU-GM infected and grown under standard conditions were resistant to this concentration. The slopes of all curves were linear and parallel, demonstrating that the difference in the percentage of CFU-GM detected at any concentration of G418 between 400 and 800 µg/mL is constant and can thus be used to obtain a consistent measure of the proportion of G418' progenitors. For all subsequent assessments of neo' progenitors, a concentration of 800 µg/mL G418 was used.

Table 1 compares the effect of modifying the growth factor content of infection or selection cultures or both on gene transfer efficiency to CFU-GM. The lower dose of G418 used resulted in generally higher efficiencies as compared with Fig 2, but this effect applied to all conditions tested and is not significant in itself. What was apparent was that the growth factor supplements in the form of purified recombinant growth factor (experiments 1 and 2), when present both during the infection and selection phase, had a highly significant enhancing effect on gene transfer efficiency and this is similar in magnitude to that shown in Figs 1 and 2. In addition, a separate contribution of growth factor supplementation to both the infection and the selection phases was evident (experiments 1 and 2, Table 1). Enhancement to a similar degree was also observed with growth factor supplementation in the form of conditioned medium from a marrow-derived adherent cell line (experiment 3, Table 1).

This effect was also noted on BFU-E. In two experiments, growth factor supplementations led to efficiencies of 25.7% and 19.8%, increases of approximately eightfold over standard conditions. Subsequent analyses of BFU-E in long-term culture experiments (described below) yielded somewhat lower efficiencies; the overall mean of five experiments was 16.3%.

To obtain more direct evidence of neo' gene transfer to BFU-E and CFU-GM, erythroid and granulopoietic colonies grown without G418 were plucked, and DNA from pooled colonies was then analyzed for neo' sequences by
Table 1. Numbers of CFU-GM Colonies (Both G418\(^{+}\) and Total) and Gene Transfer Efficiencies Measured in Three Experiments in Which Different Conditions of Infection and Selection Were Used

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Colonies*</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment</td>
<td>G418(^{+})</td>
</tr>
<tr>
<td>Standard</td>
<td>1</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF + IL-1</td>
<td>1</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Standard</td>
<td>1</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF + IL-1</td>
<td>1</td>
<td>771</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard ST-16</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>GM-CSF + IL-1</td>
<td>3</td>
<td>244</td>
</tr>
<tr>
<td>Standard ST-16</td>
<td>3</td>
<td>93</td>
</tr>
<tr>
<td>ST-16</td>
<td>3</td>
<td>58</td>
</tr>
</tbody>
</table>

*G418 concentration was 800 μg/mL effective. Control, uninfected cells were also tested for G418 resistance. Totals observed were experiment 1, 0 in 2,140; experiment 2, 0 in 832; experiment 3, 0 in 1,112.
†Efficiencies under these three conditions were significantly different (P < .01, Student’s t test) from that under standard/standard conditions.

Southern blotting (Fig 3). Transfer efficiencies to both types of clonogenic progenitors appeared to be similar, as assessed by digestion with KpnI to release intact provirus. Comparison of the intensity of the neo\(^{+}\) signal between both CFU-GM-derived and BFU-E-derived DNA and the control K562-neo\(^{+}\) DNA that contains a single copy of the provirus indicated a level of gene transfer of approximately 30%. Use of a single-copy gene probe (GM-CSF) on the same blot revealed that approximately twice as much K562-neo\(^{+}\) DNA was loaded than colony DNA, suggesting that the actual transfer efficiency could be as high as 60% (data not shown). The absence of bands in the lanes digested with HindIII, which cuts only once within the provirus sequence, indicated that provirus had integrated (ie, not present episomally) and that at least several different integration sites were present in this pool of DNA. The absence of detectable integration specific bands is consistent with the large number of colonies pooled for analysis (about 1,500 CFU-GM colonies and 1,000 BFU-E colonies).

Long-term cultures. We then investigated whether gene transfer was possible to cells that initiate hematopoiesis in long-term marrow cultures. To maximize efficiencies, marrow was infected using the same growth factor supplementation (GM-CSF + IL-1\(β\)) during the infection phase described above. An aliquot of cells was removed for assessment of gene transfer efficiency to clonogenic progenitors present in the initial marrow. The remaining cells were placed on preestablished irradiated marrow feeder layers and then maintained as long-term cultures as described in the Materials and Methods section. Figure 4 shows the total nucleated cell, total CFU-GM and neo\(^{+}\) CFU-GM, and total BFU-E and neo\(^{+}\) BFU-E in the nonadherent fraction of cultures from a typical experiment. No differences were observed between infected and control cultures in the production of nonadherent cells, indicating that the infection was sufficient to achieve significant gene transfer in vivo.
the proportion of neo1 fraction in two of three experiments. Analysis of BFU-E efficiencies in CFU-GM ranged from 26.5% to 38.7%, and in the nonadherent fraction revealed the same pattern, with this high level was maintained over 6 weeks in the nonadherent fraction of six separate long-term culture experiments (six different marrows) is summarized in Fig 5. Five of the six experiments showed neo1 transfer efficiencies similar to those of clonogenic progenitors initially present (about 30%). One was somewhat lower. The proportion of neo1 CFU-GM remained at a high level in five of these six experiments; in one case, the proportion of neo1 CFU-GM decreased and no neo1 CFU-GM were detectable after 6 weeks.

These encouraging results were extended in a series of three new experiments, in which both the adherent and nonadherent fractions were assayed for both CFU-GM and BFU-E (Table 2). As in the previous experiments, initial efficiencies in CFU-GM ranged from 26.5% to 38.7%, and this high level was maintained over 6 weeks in the nonadherent fraction in two of three experiments. Analysis of BFU-E in the nonadherent fraction revealed the same pattern, with the proportion of neo1 BFU-E present at 6 weeks similar to that in the initial marrow inoculum in two of three experiments. Assays of the adherent fraction, believed to contain the most primitive hematopoietic progenitors, also show that the initial gene transfer is maintained in long-term culture. In all three experiments, neo1 BFU-GM and neo1 BFU-E were present at 6 weeks; the proportion of neo1 BFU-E was similar to the initial values, and that of CFU-GM was slightly lower than it was initially. In one of these experiments, G418' progenitors were not detected in the corresponding nonadherent fraction despite normal numbers of clonogenic progenitors in this fraction and the presence of approximately 20% of G418' progenitors in the adherent layer.

Attempts to detect the transferred neo gene by Southern blotting of long-term culture nonadherent cell DNA proved largely unsuccessful, both because of inadequate cell numbers and because of other unexplained factors. Although enough cells were present in the initial inoculum to allow DNA analysis, by 6 weeks these were generally fewer than 10^6 cells per culture (Fig 4). However, the neo gene was easily detectable in long-term culture-derived cells by the polymerase chain reaction technique27 (Fig 6). Neo-specific bands were present in amplified DNA derived from cells of both the nonadherent and adherent fractions, as well as from pooled colonies of either CFU-GM or BFU-E origin, taken from long-term cultures at 6 weeks. This analysis serves to confirm the functional analysis of clonogenic cells using G418 resistance. The viral producer cell line used for these infections was helper virus-free, but in addition supernatants from long-term cultures were removed at 2 and 5 weeks, and testing for helper virus by SL assay showed them to be negative (<1 ffu/mL).

**DISCUSSION**

Although retroviruses currently offer the highest gene transfer efficiencies to hematopoietic cells, these are still not optimal in terms of the expression and stability exhibited by the transferred genes. These are important considerations both for analysis of hematopoietic stem cell function and for applications of gene transfer for therapeutic benefit. In this experiment, the proportion of G418' progenitors in the adherent layer remained constant and at about the same level as was observed initially in the population of clonogenic progenitors present at the time of infection.

The protocol itself was not toxic. The number of nonadherent progenitors decreased slowly after 3 weeks, as typically occurs in these cultures.11 However, throughout the experiment, the proportion of G418' clonogenic progenitors remained constant and at about the same level as was observed initially in the population of clonogenic progenitors present at the time of infection.
Table 2. Progenitor Numbers (Both G418' and Total) and Gene Transfer Efficiency Measured By Assessment of Clonogenic Cells Present in Infected Marrows Initially and After 6 Weeks in Long-Term Culture

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial G418'/Total</th>
<th>Efficiency (%)</th>
<th>Nonadherent 6 wk G418'/Total</th>
<th>Efficiency (%)</th>
<th>Adherent 6 wk G418'/Total</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CFU-GM 216/558</td>
<td>38.7</td>
<td>38/126</td>
<td>30.1</td>
<td>27/144</td>
<td>18.7</td>
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<tr>
<td></td>
<td>BFU-E 29/202</td>
<td>14.3</td>
<td>5/38</td>
<td>19.1</td>
<td>10/62</td>
<td>16.1</td>
</tr>
<tr>
<td>2</td>
<td>CFU-GM 154/496</td>
<td>31.0</td>
<td>0/180</td>
<td>0.0</td>
<td>21/110</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td>BFU-E 25/208</td>
<td>12.0</td>
<td>0/62</td>
<td>0.0</td>
<td>8/38</td>
<td>21.0</td>
</tr>
<tr>
<td>3</td>
<td>CFU-GM 154/580</td>
<td>26.5</td>
<td>63/216</td>
<td>29.2</td>
<td>14/107</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>BFU-E 20/204</td>
<td>9.8</td>
<td>4/48</td>
<td>8.3</td>
<td>6/53</td>
<td>11.3</td>
</tr>
</tbody>
</table>

G418' was assessed at 800 μg/mL effective drug concentration. No colony formation was observed at this concentration from control uninfected cells either initially or after long-term culture.

In this study we analyzed parameters important in effecting gene transfer to primitive hematopoietic cells in human bone marrow. For this we used a population enriched approximately eightfold in these cells obtained by density separation and used a cocultivation technique. To optimize our ability to assess the efficiency of neo transfer and expression quantitatively, we used no preselection with G418 and determined resistance to neo by comparing plating efficiencies of clonogenic progenitors with or without 800 μg/mL G418 because complete dose-response curve analyses showed this to identify a constant proportion of cells with a significantly increased resistance of G418 (ability versus inability to generate a scorable colony). Gene transfer efficiencies measured in this way were significantly influenced by the concentration of hematopoietic growth factors present both during the infection and selection (colony assay) phases. Specifically, raising the levels of both GM-CSF and IL-1 considerably above those present in 10% agar-LCM (which contains ≤ 1

Fig 6. Polymerase chain reaction analysis of DNA from long-term cultures. (A) Ethidium bromide-stained agarose gel of PCR products. (B) Autoradiograph of Southern blot of the same gel probed with a neo-specific probe. Source of DNA: lanes 1 and 2, normal human genomic DNA; lanes 3 through 7, cells from a single long-term culture experiment (experiment 3 in Table 2); lane 3, nonadherent cells from week 1; lane 4, nonadherent cells from week 6; lane 5, adherent cells from week 6; lane 6, pooled CFU-GM–derived colonies from week-6 assays; lane 7, pooled BFU-E–derived colonies from week-6 assays; lanes 8 through 11, nonadherent cells from week-6 long-term cultures from four different experiments: 1 μg DNA was analyzed in each case. Molecular-weight markers were provided by a BglII/Hind I plus BglI digest of pTZ 18R DNA. The position of the 350 base pair neo-specific band is shown.
The effect of IL-1 appears to be either synergistic or difference in gene transfer efficiencies to clonogenic cells that differ in their cycling characteristics. One possibility may be that hyperstimulation of cells with certain growth factors close to the time of their infection facilitates integration of the provirus into areas of the genome where expression is more likely, regardless of the initial cycling status of the infected cell.

GM-CSF and IL-1β were initially chosen because of their suggested role in stimulation of early progenitors and stem cells. The effect of IL-1 appears to be either synergistic or indirect. In one experiment, IL-1α alone had no effect on gene transfer efficiency to clonogenic cells although extra GM-CSF alone did, and the combination of GM-CSF and IL-1 had a significantly greater effect.

The ability to achieve efficient transfer and expression of the neo gene reproducibly in clonogenic human hematopoietic progenitors prompted us to extend our analyses to the cells that initiate hematopoiesis in the long-term human marrow culture system. Several lines of evidence indicate that these cells represent a distinct population of primitive cells that differ by a variety of physical, surface marker, and biochemical properties from most if not all of the clonogenic cells to which they give rise after 5 to 8 weeks in culture. Recently, the long-term culture initiating cell was partially purified based on these differences. This cell population is deficient in clonogenic progenitors, but when used to initiate long-term cultures can produce normal numbers of clonogenic progenitors when assayed after 5 weeks. Similarly, treatment of human marrow with 4-hydroperoxycyclophosphamide eradicates all clonogenic progenitors, but marrow so treated is able to form normal numbers of progenitors after 2 to 4 weeks in culture. Because cells that initiate long-term murine marrow cultures give rise to CFU-S for many weeks and because cells from long-term human marrow cultures also appear capable of engraftment in vivo, the long-term culture initiating cell in human marrow probably belongs to a primitive population. For all these reasons, the progenitors at present 6 weeks in long-term cultures probably represent the progeny of more primitive cells, rather than arising from dormant progenitors present since the beginning.

Successful infection of cells present in long-term human marrow cultures with retroviruses was first demonstrated by Rothstein et al., who used a replication-competent Kirsten or Harvey murine sarcoma virus (with an amphotropic coat) and showed efficient viral replication in the long-term cultures obtained. In the present study defective replication-incompetent viruses were used. These are no longer infective once integrated, a feature that is clearly necessary for studies of the developmental potential of individual stem cells or for any clinical application of gene transfer. We showed that replicator-incompetent retroviruses can efficiently infect both clonogenic cells and the cells from which they can be derived after more than 1 month in culture in the absence of any selection. This was demonstrated both functionally, using G418 resistance as a marker for expression of the transferred gene, and molecularly, directly assessing the presence of the neo gene by the polymerase chain reaction.

Our results indicate that the infection protocol used results in the same high level of gene transfer to both of these primitive cell populations, with maintenance of expression of the transferred gene through several generations of their progeny. These results, together with the recent work of Bordinon et al., are of interest because they indicate that the long-term culture system is well suited for investigations using retrovirus-mediated gene transfer as a method for tracking or modifying the behavior of human hematopoietic stem cells. The present results also provide optimism for the likelihood of achieving gene transfer to autologous human marrow transplants suitable for gene therapy.

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