Gene Transfer to Primary Normal and Malignant Human Hemopoietic Progenitors Using Recombinant Retroviruses

By Donna E. Hogge and R. Keith Humphries

To study the feasibility of using retroviruses for gene transfer into human hemopoietic cells, various cell types were exposed to virus carrying the gene for neomycin resistance (neo'). In preliminary studies using K562 cells as targets, we found that high viral titer and co-cultivation with viral producer cells rather than incubation in medium exposed to viral producer cells were important variables for achieving high frequencies of G418 resistant (G418') colonies. The maximum frequency of G418' K562 colonies after co-cultivation with cells producing a neo' virus titer of $4 \times 10^8$ cfu/mL was 60%. When primary human progenitors from normal marrow, fetal liver, or chronic myelogenous leukemia blood were exposed to high titer viral stocks, both with and without helper virus, under conditions optimized for K562 cells, maximum frequencies of G418' colonies were 3% to 16% for granulocyte macrophage progenitors and 2% to 6% for primitive erythroid progenitors. The presence of the neo' gene in both G418' K562 and primary hemopoietic colonies was verified by Southern blot. Expression of the neo' gene was shown by RNA spot blot. These data demonstrate efficient transfer and expression of the neo' gene in both K562 cells and primary human hemopoietic cells from normal and leukemic individuals.

THE DELIVERY of exogenous genetic material into various target cells is important for many experimental and clinical goals. Recombinant retroviruses provide an attractive vehicle for gene transfer for a number of reasons. These include the high efficiency with which they are able to enter cells and integrate into host DNA, their wide target cell range, and their lack of toxicity.

Mammalian bone marrow is a convenient source of primitive cells with high proliferative and self-renewal capacity on which to test the feasibility of using a gene transfer technique to primary cells. A number of workers have already shown that it is possible to introduce a variety of foreign genes into the bone marrow stem cells of mice using recombinant retroviruses. Two of these authors have shown a high efficiency of gene transfer, and the transferred gene has remained stably integrated through serial marrow transplants. Although some expression of the transferred genes has been demonstrated, difficulties in obtaining satisfactory levels of the products of certain genes such as human adenosine deaminase have been encountered. In addition, the long-term stability of gene expression has been questioned. Nevertheless, it is clear that retroviruses are capable of delivering functional genes into murine hemopoietic progenitors.

Retroviruses have also been used for gene transfer to humans, although the available data are less extensive than for murine targets. The enzyme deficiency in hypoxanthine phosphoribosyl transferase (HPRT) negative human fibroblasts and B lymphoblasts has been corrected by infection with recombinant virus containing the HPRT gene. Similar results have been reported for experiments in which a virus containing the human adenosine deaminase (ADA) gene was used to infect human ADA-deficient B lymphocytes. Some data exist for cells of the hemopoietic system from experiments in which long-term human marrow cultures were infected with virus containing oncogenes or the human HPRT gene. Viral replication in the hemopoietic cells in these cultures was documented, but the extent to which the gene of interest had been successfully transferred and expressed by primitive blood cell progenitors was not studied. Recently more direct quantitative data have been published by Hock and Miller demonstrating retroviral-mediated gene transfer to primary human marrow progenitors with recombinant viruses carrying the neo' or the DHFR* gene.

The present study was undertaken to investigate and identify variables that may influence the frequency of successful gene transfer to human hemopoietic progenitors. A number of the available viral packaging lines and retroviral vectors have been evaluated using a spectrum of target cell types including an established cell line as well as fresh progenitors of both normal and leukemic origin.

MATERIALS AND METHODS

Cells and culture conditions. Cell lines were cultured in Dulbecco's modified Eagle medium with high glucose (4.5 g/L) and 10% heat-inactivated calf serum (for 6AM or 62 cells) or 10% fetal calf serum for all other cell types in 5% CO2 atmosphere at 37°C. The amphotropic retrovirus packaging lines, 6AM, PA12 and PA317, and the ecotropic packaging line, 62, have been previously described.

Primary human cells were obtained either from consenting adults (CML peripheral blood or normal bone marrow) or from second trimester abortions (fetal liver cells) after approval from the Clinical Screening Committee for Research Involving Human Subjects of the University of British Columbia. Blood and bone marrow cells were passed over a percoll density gradient (density 1.063) and light

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density cells collected and used in subsequent experiments. Fetal liver was minced with scissors and incubated for three hours at 37 °C in a medium with 20% fetal calf serum and collagenase 1 mg/mL (Sigma, St. Louis). The cells were then passed through a succession of needles of decreasing gauge, washed, and used for the experiments described.

Primary human cells were grown in a medium supplemented with 20% fetal calf serum and 10% agar-stimulated leukocyte-conditioned medium while in suspension culture and in methylcellulose assays as previously described for assessment for hemopoietic progenitors.

**Virus production and assay.** The general strategy for generating high-titer retroviral-producer cell lines was as outlined by Miller et al. The retrovirus packaging line, PA2, was transfected with viral plasmid DNA (pZipNeo or pN2). These plasmids are both MoMuLV-based vectors that contain the gene for neomycin phosphotransferase (neo'). After 48 hours the medium from the transfected 2 cells that contained neo' virus (v-neo) was removed, and aliquots were used to infect the amphotropic packaging lines, PA12, or PA317. After 48 hours amphotropic neo'-producing clones were selected in medium containing the neomycin analogue G418 at 1 mg/mL (Gibco Laboratories, Chagrin Falls, OH). G418 was dissolved in distilled water and added to growth medium to achieve the desired final concentration in total mg/mL. The effective drug concentration was approximately 50% of that value for the two lots of G418 used. Colonies were isolated by cloning rings, expanded, and examined for neo' titer on 3T3 cells and for amphotropic helper virus using the S + L' assay.

**Viral infection.** The K562 human leukemic cell line or primary cells were infected with v-neo' by either co-cultivation with amphotropic viral producer cells that had received 1,500 R irradiation or incubation in medium previously exposed to virus-producing cells for various periods of time. After the infection period they were maintained in suspension culture for 24 to 48 hours before plating in methylcellulose assay with or without G418. Control cultures that were not exposed to virus were grown in suspension culture and plated with or without G418 at the same time as the infected cells. All infections and control suspension cultures were done with polybrene, 8 µg/mL. Colonies were scored after plating in methylcellulose on days 5 to 7 for K562 and days 10 to 14 for granulocyte macrophage colonies (from CFU-GM) and days 18 to 21 for large erythroid colonies (from BFU-E) for primary progenitor assays. Colonies were not scored unless they contained at least 30 cells and, in the case of BFU-E, had at least three clusters and were clearly hemoglobinized.

The infectious center assay was done by picking individual G418 resistant (G418') granulocyte-macrophage colonies from methylcellulose assay and placing the dispersed cells from one colony in a 2 cm² tissue culture well containing 10⁴ NH-3T3 cells in medium with 8 µg/mL polybrene. After overnight incubation the medium was replaced with fresh medium containing G418 1 mg/mL. Seven days later the assay was scored for the presence of G418' 3T3 cells.

Cell and viral manipulations and cultures were performed under level C containment following Medical Research Council of Canada guidelines for handling retroviruses and human samples.

**DNA and RNA studies.** High molecular weight DNA was harvested from expanded clones of K562 cells and pooled hemopoietic colonies by proteinase K digestion followed by phenol and chloroform extractions. Southern blotting of K562 and primary hemopoietic colony DNA to nitrocellulose filters was performed by standard methods.

Total cellular RNA was harvested by the method of Meinkoth and Wah14 from K562 or from primary hemopoietic colonies that had been individually plucked and pooled from methylcellulose assays. The RNA was diluted to various concentrations and applied to nitrocellulose filters using a spot blot manifold according to established methods (Schleicher and Schuell, Keene, NH).

To detect neo' sequences, a 2.3 kb neo'-specific Bam H1, Hind III fragment isolated from RSV-neo plasmid DNA was ³²P-labeled by nick translation kit (Bethesda Research Laboratories, Gaithersburg, MD) for use as a probe. Filters were prehybridized, hybridized, washed, and autoradiographed using standard techniques.

**RESULTS**

**Viral producer cells.** Many attempts were made to obtain the highest possible v-neo' titer from various combinations of packaging lines and either the ZipNeo or the N2 vector. For the combination of PA12 cells and pZipNeo, the highest titer of helper-free virus obtained after screening 61 clones was 2 x 10⁶ CFU/mL. With PA12 and pZipNeo, several of the 21 clones studied produced approximately 10⁵ CFU/mL, but these were found to produce helper virus as well. With the combination of PA12 and the N2 vector, higher titers of v-neo' (4 x 10⁶ CFU/mL) were obtained, but these also contained helper virus. The highest titers of helper-free v-neo' (5 x 10⁶ CFU/mL) were obtained from the PA317 packaging cell line containing the N2 proviral DNA.

**K562 experiments.** To optimize conditions for high-frequency retroviral infection of human hemopoietic progenitors, we used K562 cells as a convenient model for experiments in which a number of variables were explored. Initially the best ratio of target K562 cells to irradiated viral producer cells was studied over a range of 10⁴:1 to 1:10. The transformation of K562 to G418' increased in frequency as the proportion of v-neo' producer cells increased up to a cell ratio of 1:1 and then plateaued with higher numbers of producer cells. A total of 10⁵ K562 cells were then incubated for two hours with 3 mL of medium exposed to v-neo' producing cells, either undiluted or diluted to concentrations as low as 1:10⁵. There was a direct correlation between the concentration of v-neo'-containing medium and the frequency of G418' K562 cells for medium from all the viral producer lines tested. For the highest titer virus (4 x 10⁶ CFU/mL) the frequency plateaued above concentrations of 1:10.

Figure 1 shows the frequency of G418' K562 cells obtained by infection with undiluted v-neo'-containing medium or co-cultivation with confluent cells of various types. The cell lines selected were those producing the highest v-neo' titer for the various packaging line/neo' plasmid combinations. At viral titers ≤10⁴ CFU/mL the maximum frequency of G418' K562 cells was approximately 2% after co-cultivation with producer cells. Frequencies obtained after infection with medium exposed to the same cell lines was at least fourfold lower than that obtained by co-cultivation. At the highest titer, 4 x 10⁶ CFU/mL, the difference in efficiency between co-cultivation and cell-free medium disappeared. The highest frequencies of G418' K562 colonies were obtained with the PA12/N2 producer cells, approximately 60% for both co-cultivation with producer cells and incubation with medium.

A number of other variables that were investigated did not
appear to affect the frequency of K562 cell transformation to G418'. These included extending the duration of co-cultivation from six to 48 hours or extending the duration of exposure to medium from producer cell lines from two to 48 hours.

The neo' gene was demonstrated in G418' K562 cell DNA by expanding individual colonies in liquid culture and extracting cellular DNA for Southern blots. Figure 2A demonstrates the 4kb neo'-specific XbaI fragment found in four G418' K562 colonies but not found in control K562 DNA. Figure 2B shows DNA from the same four G418' K562 colonies and two uninfected control K562 colonies cut with enzymes that cut only once within the neo' proviral DNA. The neo'-specific fragments demonstrated in the G418' colonies are single and unique to each colony, indicating one random site of integration for the provirus in the cellular DNA.

Infections of primary hemopoietic progenitors. A total of four normal marrow, six fetal liver, and six CML blood samples were infected with v-neo' from a variety of packaging cell line.neo' plasmid combinations. The infection procedure did not appear to be toxic to the target cells as shown by a lack of any effect on cell recovery or plating efficiency. (Values for infected cells were 80% to 100% of control values.) A G418 concentration of 2 mg/mL (effective drug concentration 1 mg/mL) completely inhibited all colony growth in assays of uninfected primary progenitors. No difference in G418 sensitivity between the various target cell sources or between progenitors of different lineages (ie, BFU-E and CFU-GM) could be demonstrated.

Figure 3A shows the frequency of G418 CFU-GM seen after infection by v-neo' producer cells at various viral titers. Each point represents a different experiment combining target cells of a certain type with a specific viral producer cell line. Although there was a correlation between viral titer and frequency of G418', considerable variation in transformation efficiency at any given titer was also found; for example, from 2.4% to 15.7% G418' CFU-GM at a titer of 4 x 10^6 CFU/mL. There was also a low (0.045% to 0.4%) but reproducible incidence of G418' colonies after infection with cells producing viral titers as low as 10 CFU/mL.

Figure 3B shows similar data for BFU-E. Infections with cells producing a v-neo' titer of 1 x 10^3 CFU/mL yielded a
frequency of large G418' erythroid colonies of less than 1%, while frequencies up to 5.6% were seen after infections done
d with cell lines producing higher titers. However, the correla-
tion of G418' frequency with titer was less clear, and the
variation in G418' frequency at any given titer was similar to
that seen for CFU-GM. From these data (Figs 3A and B) it
appears that BFU-E may be less readily transformed by
v-neo' than CFU-GM. Although this may be true, it is
possible that the apparent difference is the result of experi-
mental variables that particularly affect erythroid colony
growth. For example, it was found that in general G418'
erythroid colonies were not as large or as red as those
obtained from infected BFU-E plated in the absence of
G418. It is possible that impurities in the G418 affect the
growth of erythroid colonies containing the neo' gene. Alter-
natively, the neo' gene may be expressed at a lower level in
eythroid cells as they differentiate. Therefore it is likely that
the frequencies recorded here for G418' BFU-E represent
minimum estimates of successful gene transfer.

The maximum frequencies of G418' progenitors from
these experiments are summarized in Table 1. The highest
frequency was usually observed after infection with the cell
line producing the highest viral titer, PA12/N2 cells produc-
ing v-neo' at 4 x 10^6 CFU/mL, in addition to helper virus.
The ability of medium exposed to v-neo'-producing cells,
as compared to co-cultivation with producer cells of various
types, to transform primary progenitors to G418' is com-
pared in Fig 4. At a titer of 6 x 10^5 CFU/mL produced by
vAM/ZipNeo cells, v-neo'-containing medium was unable
to produce a significant incidence of G418' colonies while
cocultivation yielded a frequency of approximately 0.1%. At
titers of 5 x 10^5 or 4 x 10^5 CFU/mL, medium with virus was
effective at transforming both BFU-E and CFU-GM. How-
ever, the frequency was still two- to 50-fold higher using
cocultivation. Nevertheless, the frequency of G418' colonies
was higher after infection with medium at high titers (10.6% for
CFU-GM at 4 x 10^5 CFU/mL) than with cocultivation at low titers (0.13% CFU-GM at 6 x 10^5 CFU/mL).
The frequencies of transformation for primary progenitors
were not as high as those obtained for K562 cells. To try to
increase the efficiency, target cells were allowed to remain on
viral producer cells for as long as seven days without any
increase in the efficiency of transformation.

The ability of G418' CFU-GM that had been infected with helper-containing v-neo' to infect 3T3 cells was tested
by infectious center assay. In two experiments, five of 22 and
24 of 24 individual CFU-GM tested transformed 3T3 cells to
G418 resistance. The production of G418' 3T3 cells in this
assay requires that the infecting cells be producing v-neo'. To
do so they themselves must have been infected by both v-neo'

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**Table 1. Maximum Frequency of G418' Hemopoietic Colonies After Co-cultivation With Various v-neo' Producer Cells**

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Viral Producer Cells</th>
<th>v-neo' Titer (CFU/mL)</th>
<th>G418' Colonies/Colonies Without G418 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CFU-GM + Virus</td>
</tr>
<tr>
<td>Normal Marrow</td>
<td>vAM/ZipNeo</td>
<td>3 x 10^6</td>
<td>26/2775 (0.94)</td>
</tr>
<tr>
<td></td>
<td>PA317/N2</td>
<td>5 x 10^6</td>
<td>78/1860 (4.2)</td>
</tr>
<tr>
<td></td>
<td>PA12/N2*</td>
<td>4 x 10^6</td>
<td>127/1800 (7.1)</td>
</tr>
<tr>
<td>Fetal Liver</td>
<td>vAM/ZipNeo</td>
<td>3 x 10^6</td>
<td>24/2960 (0.81)</td>
</tr>
<tr>
<td></td>
<td>PA317/N2</td>
<td>5 x 10^6</td>
<td>2/158 (13.1)</td>
</tr>
<tr>
<td></td>
<td>PA12/N2*</td>
<td>4 x 10^6</td>
<td>13/414 (3.1)</td>
</tr>
<tr>
<td>CML Blood</td>
<td>vAM/ZipNeo</td>
<td>6 x 10^6</td>
<td>48/9900 (0.48)</td>
</tr>
<tr>
<td></td>
<td>PA317/N2</td>
<td>5 x 10^6</td>
<td>66/3120 (2.1)</td>
</tr>
<tr>
<td></td>
<td>PA12/N2*</td>
<td>4 x 10^6</td>
<td>656/4170 (15.7)</td>
</tr>
</tbody>
</table>

* + Helper virus.
† Control — Cells from the same sample plated in methylcellulose ± G418 without exposure to v-neo' producer cells.
and helper virus, which may occur relatively infrequently. This would account for less than 100% of G418' CFU-GM being positive in the infectious center assay.

Figure 2C is a Southern blot of DNA harvested from pooled hemopoietic colonies grown from CML blood cells that were either uninfected or exposed to viral stocks containing v-neo'. After digestion with EcoR1, the neo'-specific probe identified the expected 1.5 kb neo' specific fragment in G418' cells (lane 3). A less intense signal is seen in lane 2 where the same cells were exposed to medium containing v-neo' but not selected in G418, indicating that some of the cells did not contain the neo' gene (8% of the CFU-GM in this sample were G418'). In lane 5 the DNA from cells exposed to viral producer cells but not selected in G418 shows a strong signal. In colony assays 15.7% of the cells in this sample contained the neo' gene. DNA from cells from the same sample that were not exposed to virus in lanes 1, 6, and 7 show no signal.

Expression of the neo' gene by v-neo' infected, pooled primary progenitors or K562 cells was shown by RNA spot blot (Fig 5). Total cellular RNA was hybridized with a neo' specific probe. Uninfected, control cells show no evidence of neo' hybridization, while the same number of infected cells show a strong signal. The signal is strongest for cells that were selected in G418 (lanes b, CFU-GM and all infected K562 cells) but is also seen in cells that were infected but not selected (lanes a, CFU-GM).

DISCUSSION

These data demonstrate efficient transfer and expression of the neo' gene to both the K562 human leukemic cell line and a variety of normal and malignant primary human hemopoietic progenitors using recombinant retroviruses.

A number of variables were investigated to try to achieve the highest possible efficiencies of gene transfer. Co-cultivation with viral producer cells rather than infection with medium exposed to viral producer cells and use of high titer virus were both identified as important parameters, but viral titer clearly had the greatest influence on the frequency of transformation to G418 resistance. Considerable effort was
devoted to studying viral producer cells generated by various packaging line/neot-containing plasmid combinations. The choice of these variables had a major impact on the titer of both v-neo and helper virus. We were unable to generate high titer v-neo from the PA12 packaging line that was not associated with helper virus. The use of the N2 vector rather than ZipNeo raised the v-neo titer but did not eliminate the problem of helper virus. Although the PA12 line has been constructed to generate helper-free recombinant virus, it appears that with the vectors used in this study sufficient genetic recombination occurs between the defective packaging and recombinant neo' viral sequences to frequently generate significant helper virus titers. Helper-free v-neo' generated by the ψAM packaging line containing pZipNeo DNA was always of relatively low titer (~10^4 CFU/mL). The newer generation of retroviral packaging lines, such as the PA317 developed by Miller and Buttimore, may solve the problem of obtaining high-titer recombinant virus without associated helper.

Although both ZipNeo and N2 contain the same neo' gene under the control of the promoter in a Moloney leukemia virus LTR, the two vectors are not identical in structure.6,19 Although the differences appear subtle, they seem to affect the viral titers obtained from producer cells and might also cause different levels of expression of the transferred foreign gene in infected hemopoietic cells. For this reason we elected to test virus derived from both the N2 and ZipNeo vectors on our target cells. Although our data are not definitive, some of our results (eg, Fig 1 where K562 were the target cells and a fivefold increase in viral titer led to a 20-fold increase in frequency of G418' colonies) may be explained if neo' was expressed more efficiently in cells infected with virus derived from N2 rather than ZipNeo.

In experiments done with helper-free virus from a producer line derived from PA317 cells and the N2 plasmid, we were successful in demonstrating gene transfer at levels only slightly lower than those achieved with helper containing virus of significantly higher v-neo' titer. No obvious effect of helper virus on the frequency of gene transfer was detected.

In addition to normal bone marrow we evaluated two alternative sources of primary human progenitors for retroviral infections. These were fetal liver and CML blood. In both cases a high proportion of the primitive progenitors in these tissues are in the active part of the cell cycle as compared to their counterparts in normal marrow, which are largely quiescent.22 Cell cycling status has been suggested as an important variable affecting the success of retroviral infection and integration into host DNA.23 Although some of our highest frequencies of G418' colonies were seen in the CML blood cultures, there was sufficient variability from experiment to experiment that no overall difference was evident. However, there was clearly no dramatic improvement in the frequency of G418' progenitors in experiments using either of the primary human targets that were presumably cycling optimally.

There are many possible reasons for the variability in the frequency of successful gene transfer and expression in primary progenitors, as illustrated in Fig 3. Although the viral titer from our v-neo' producer cell lines was quite stable over long periods of time, some differences did occur. For example, after the producer cells reach confluence the viral titer begins to drop significantly. Similarly, the ability of target cells not only to become infected but to integrate and express the viral genome is probably affected by a number of factors, including their level of differentiation, cell cycle status, and perhaps subtle metabolic factors that may be altered by the unavoidable day-to-day changes in complex culture conditions. Although care was taken to ensure that both viral producer and target cells were in optimal condition at the time of infection, some variability undoubtedly occurred and may account for changes in frequency of G418' colonies. For example, the fetal liver samples underwent considerable manipulation, and the plating efficiency of uninfected cells in progenitor assays was highly variable. Changes affecting the ability of hemopoietic progenitors to form colonies in culture might even more profoundly reduce the frequency of successful gene transfer. If so, this would account for data such as the low numbers of G418' BFU-E after infection of fetal liver cells with virus from PA12/N2 producer cells (Table 1).

Although encouraging, the frequency of gene transfer demonstrated to primary progenitors by these data is still much lower than the theoretical 100% that should be obtainable with retroviruses.1 Our frequencies are reasonably close to those reported by Hock and Miller for gene transfer to primary normal human hemopoietic progenitors from bone marrow24 but are much lower than the 75% to 100% frequency reported by Eglitis et al for murine CFU-S.6 However, it is notable that the latter authors and others have found the level of expression of G418 resistance or neo' gene product to be highly variable among different CFU-S containing the neo' gene.6,4 The frequencies of G418 resistance in murine CFU-GM infected and selected in vitro have been approximately 10% to 30%,4 which is also lower than one might have predicted and consistent with our results on human progenitors.

A large number of techniques exist for transporting genetic material into cells. A number of these have been applied to primary hemopoietic progenitors, including calcium phosphate co-precipitation and electroporation.24-26 Although initial results using the former technique to confer methotrexate resistance on murine bone marrow cells appeared promising, similar results have not been forthcoming for other genes or for human cells. Electroporation is a relatively new technique that has not been widely applied to gene transfer into primary cells. Initial results with human bone marrow progenitors demonstrate low levels of transfer and expression of the xanthine-guanine phosphoribosyl transferase gene in CFU-GM.26 The full potential of this technique remains to be explored. At the present time the bulk of evidence would indicate that the highest efficiencies of gene transfer are those mediated by retroviruses. Where high frequencies of transformation are necessary and appropriate vectors are available, these viruses appear to be the current method of choice for transporting genes into cells.

The knowledge gained from the present studies should facilitate the successful use of the technique of retroviral-mediated gene transfer to study genetic factors important in
normal and malignant hemopoiesis. In addition, there are a number of inherited human hematologic disorders that are known to have a molecular genetic etiology. Some of these may ultimately be curable by gene replacement therapy, possibly with retroviruses providing the means of transporting the normal gene into primitive hemopoietic progenitors.27

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