# *In Vivo* Biosafety Model to Assess the Risk of Adverse Events From Retroviral and Lentiviral Vectors

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Serious adverse events in some human gene therapy clinical trials have raised safety concerns when retroviral or lentiviral vectors are used for gene transfer. We evaluated the potential for generating replication-competent retrovirus (RCR) and assessed the risk of occurrence of adverse events in an *in vivo* system. Human hematopoietic stem and progenitor cells (HSCs) and mesenchymal stem cells (MSCs) transduced with two different Moloney murine leukemia virus (MoMuLV)-based vectors were cotransplanted into a total of 481 immune-deficient mice (that are unable to reject cells that become transformed), and the animals were monitored for 18 months. Animals with any signs of illness were immediately killed, autopsied, and subjected to a range of biosafety studies. There was no detectable evidence of insertional mutagenesis leading to human leukemias or solid tumors in the 18 months during which the animals were studied. In 117 serum samples analyzed by vector rescue assay there was no detectable RCR. An additional 149 mice received HSCs transduced with lentiviral vectors, and were followed for 2–6 months. No vector-associated adverse events were observed, and none of the mice had detectable human immunodeficiency virus (HIV) p24 antigen in their sera. Our *in vivo* system, therefore, helps to provide an assessment of the risks involved when retroviral or lentiviral vectors are considered for use in clinical gene therapy applications.

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## INTRODUCTION

In recent years remarkable progress has been achieved toward providing seriously ill patients with clinical benefit by applying hematpoietic stem cell–directed gene therapy. However, serious adverse events in some clinical trials have highlighted safety concerns when retroviral or lentiviral vectors are used for gene transfer.[1–4] It is therefore important to investigate the *in vivo* biosafety profile of retroviral Moloney murine leukemia virus (MoMuLV)-based and lentiviral [human immunodeficiency virus-1 (HIV-1)]-based vectors that are under consideration for clinical trials. In order to develop an *in vivo* system to examine vector biosafety, we chose a long-term mouse xenograft model, consisting of immunodeficient beige/nude/xid (bnx) mice with a life span of 2 years,[5–7] with transplants of transduced human cells. Given that vector-transduced cells are present for up to 18 months in this system, we hypothesized that it would be possible to detect not only recombinants that were present in the initial batch of vector supernatant used for transducing human cells, but also recombinants that could potentially arise through RNA rearrangements *in vivo*. In addition, the effects of insertional mutagenesis could potentially be detected, because the transduced human cells would be followed *in vivo* for up to 18 months, the longest period possible in human-to-mouse xenotransplantation experiments. The immune-deficient mice used in our experiments are excellent models for vector safety studies because they lack B, T, and natural killer cell function. Therefore murine or human solid tumors and leukemias that might arise in the mice from insertional mutagenesis cannot be rejected by the animals,[5–7] and consequently become quickly noticeable in terms of the animals' rapid decline in health. Bnx mice have no fur, so any skin discoloration, enlarged lymph nodes, or solid tumors that may develop are readily visible.

Our initial experiments were designed to demonstrate the sensitivity of the system, and showed that replication-competent viruses derived from an N2 vector, earlier shown to generate recombinants *in vivo*[8] could be detected with high sensitivity. Next, studies were performed using transduced human hematopoietic stem and progenitor cells (HSCs) and mesenchymal stem cells (MSCs) cotransplanted into a total of 630 immune-deficient mice. The study animals were observed daily for up to 18 months after the transplantation in order to detect adverse events. If any sign of illness was observed (detailed in the later text) the mice were killed and subjected to a series of biosafety analyses (**Figure 1**). If the animals remained event-free, they were allowed to carry the transduced human HSC and MSC for up to 18 months

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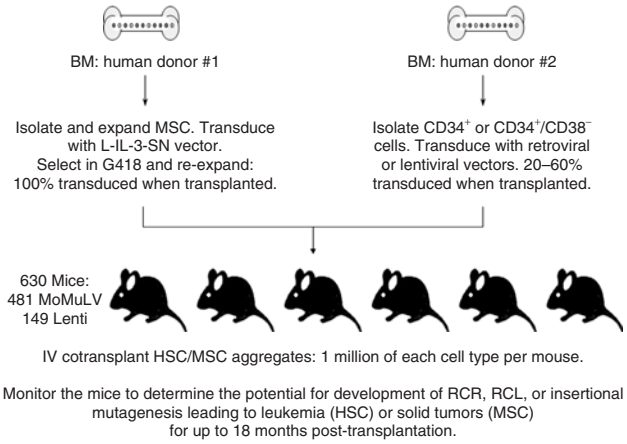


**Figure 1 Experimental schema.** Mesenchymal stem cells (MSCs) were isolated, expanded, and transduced with a Moloney murine leukemia virus (MoMuLV)-based vector that carries the human interleukin-3 (IL-3) complementary DNA. The MSCs were fully selected and then cotransplanted into the mice with human hematopoietic stem and progenitor cells (HSCs) in all the experiments so as to provide the HSCs with species-specific cytokine support. The HSCs were isolated from a second human donor and transduced by retroviral (MoMuLV-based) or lentiviral (HIV-1-based) vectors. The HSCs were not preselected before cotransplantation with the engineered MSCs. A total of 630 mice received cotransplants of the IL-3-producing MSCs plus HSCs transduced by retroviral ($N$ = 481) or lentiviral ($N$ = 149) vectors. After the transplantation, the mice were monitored daily for up to 18 months for the development of adverse events, and were subjected to full autopsy and biosafety analyses when killed. BM, bone marrow; RCL, replication-competent lentivirus; RCR, replication-competent retrovirus.

after transplantation. After the animals were killed autopsies were performed, and all the tissues were collected, banked, and analyzed for the presence of human cells, vector elements, recombinant viruses, and malignancies.

## RESULTS

### Establishing the sensitivity of the assays

It was imperative to determine that, if replication-competent virus was generated it would cause adverse events in the mice that received transplants of human cells. Before starting the sensitivity studies, eight spiking experiments were initially performed in order to establish the sensitivity of the *in vitro* assay for replication-competent retrovirus (RCR). One LN vector–producing PA317 packaging cell was added by limiting dilution into a culture of 1 million nontransduced primary human MSCs. The supernatant was collected after 24 hours and tested for virus as shown in **Figure 2**. In seven of the eight trials, the addition of the single vector-producing fibroblast to 1 million nontransduced MSCs could be detected as a positive event (namely, the generation of G418-resistant colonies in the target 3T3 cells) using the *in vitro* RCR assay as shown in **Figure 2**. These data established that the assay was sensitive enough to detect one virus-producing cell per million plated cells.

In order to establish the sensitivity for the *in vivo* system, 12 bnx mice were given transplants of 1 million human bone marrow–derived CD34$^+$ progenitors that had been transduced for 72 hours with the N2 vector. The N2 vector had been packaged by initial transfection into the Psi2 cell line, followed by transduction and selection of PA317 packaging cells. The N2 vector does not have the safety modifications implemented in the LN-based

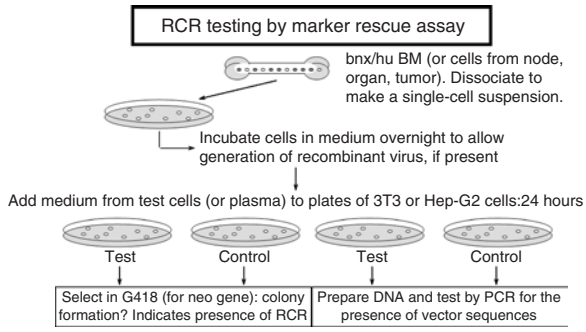


**Figure 2 Schema for analysis of replication-competent retrovirus (RCR).** Single-cell suspensions were obtained from the organs or tumor tissue of mice that had suffered adverse events. The cells were plated in minimal medium overnight to allow generation of infectious virus, if present. The medium from the test cells, or plasma isolated directly from the mice, was added in duplicate to plates of 3T3 (murine fibroblast) or Hep-G2 (human liver) cells for 24 hours. Medium alone was added to two additional control plates for each test sample. The plates were incubated for an additional 24 hours, and then one set of test and control plates was lysed for DNA preparation, while the second set was selected in G418 to determine whether the *neomycin phosphotransferase* gene had been transferred. BM, bone marrow.

vectors and in packaging systems currently used for the generation of clinical trial vector supernatants.[9] Because it has earlier been shown that the N2 vector allows recombination events that lead to adverse events in an animal model,[8] we selected this vector to determine whether similar events arising in the bnx mice could be detected. This, in turn, would allow testing of the sensitivity of the system.

Murine lymph node tumors arose in 11 of 12 mice that received N2-transduced human cells. The *neo* gene could be detected in murine cells as well as in human cells. Significant lymphoproliferation could be seen only in the murine pre-T cells. It took 5 months for murine leukemia to arise; the affected mice displayed symptoms of extreme sickness rapidly, with 5 of the 12 mice becoming moribund on exactly the same day (**Figure 3**), and 6 others becoming moribund within a 1-month period. Cotransplantation of 10 live vector-producing PA317/N2 cells with syngeneic mouse marrow cell carriers into each of 5 bnx mice had the same effect, but with an accelerated course of disease development and death at 3 months. Of the 12 mice that had received N2-transduced human cells, 11 had to be killed because they developed visibly enlarged lymph nodes and spleen, hunching, and decrease in body weight, as shown in **Figure 3**. RCR could be detected in the sera of these 11 mice, and this RCR was shown to be capable of transducing both human and murine cells *in vitro*, thereby indicating an amphotropic envelope. The 12th mouse was observed carefully for 14 months; it did not show any signs of leukemia or other adverse events, and had no abnormal tissues when it was autopsied.

DNA was removed from the marrows of bnx mice that were killed because they had developed lymphoma. The extent of vector integration in each sample was assessed by clonal analysis using inverse PCR (**Figure 4**). The level of engraftment by human CD45$^+$ cells, as assessed by fluorescence-activated cell sorting (FACS), is shown at the bottom of each lane in **Figure 4**. Mice that are extremely ill or dying of leukemia often lose the human hematopoietic graft, and this phenomenon was observed in 6

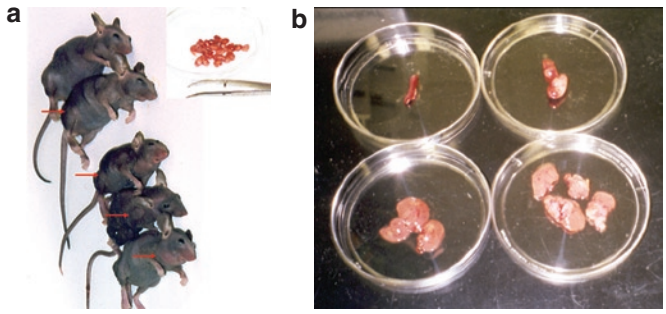


**Figure 3 Sensitivity of the bnx/hu xenograft assay for detecting recombinant vectors.** Photographs of the appearance and tissues of bnx mice that had received N2-transduced human hematopoietic cells are shown. Of the 12 mice that had received transplants, 5 were moribund on the same day (159 days after transplant) and were killed and autopsied immediately after the photo was taken. (**a**) Five anesthetized mice are shown with extremely enlarged lymph nodes, that were readily visible on daily inspection (red arrows). Excised, massively enlarged lymph nodes recovered from the same mice as shown in the inset. These nodes are ~25× normal size. (**b**) Spleen and liver from an affected mouse with heavy organ infiltration by leukemic cells (plates on the right) and a mouse with less severe organ infiltration (left).

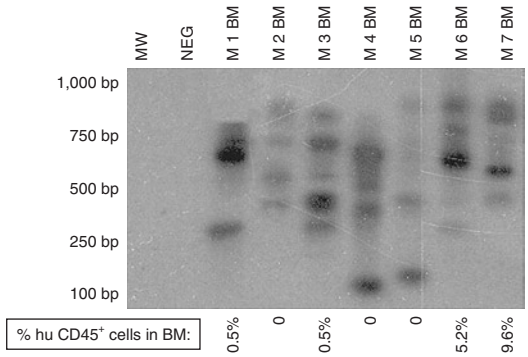


**Figure 4 Inverse PCR analysis of bone marrow (BM) from mice that had received human N2-transduced CD34$^+$ cells.** DNA was harvested from the marrow of bnx mice that were killed because they developed lymphoma. The extent of vector integration in each sample was assessed by clonal analysis using inverse PCR. The levels of engraftment by human CD45$^+$ cells, as assessed by fluorescence-activated cell sorting, are shown at the bottom of the blot. Polyclonal patterns were observed in each sample, even when the mouse had lost the human cell graft, thereby indicating that the N2 vector had passed to murine cells in the marrow.

of the 11 sick mice in this experiment. Polyclonal patterns were observed in each sample, even when the mouse had lost the human cell graft, thereby indicating that the N2 vector had passed to murine cells in the marrow. It must be noted that only the sensitivity of the assay was demonstrated by transplantation of N2 vector–transduced cells. These early retroviral vectors would, of course, never be used for human clinical trials. Better and more highly safety-modified MoMuLV-based vectors have been available for human gene therapy applications for over a decade, and these vectors were tested next.

## Biosafety testing for safety-modified MoMuLV-based vectors

We evaluated the potential for generation of RCR and assessed the risk of occurrence of adverse events in human HSCs and MSCs carrying two different safety-modified MoMuLV-based vectors: the LN vector in HSCs[9] and the LN-based L-IL-3-SN vector in MSCs (**Figure 1**). The two transduced cell types were cotransplanted into bnx mice.[10] A total of 481 bnx mice were analyzed for biosafety factors between 7 and 18 months after transplantation.

Packaging of the MoMuLV-based vector LN and its derivatives was done in the PA317 and PG13 cell lines. Half a million to one million human CD34$^+$ cells or 2,000 purified CD34$^+$/CD38– cells that had been exposed to the vectors were transplanted into each mouse. The average transduction efficiency of the engrafted human HSCs was 18%, as measured on recovery from the murine bone marrow.

In addition to receiving transplants of human CD34$^+$ and CD34$^+$/CD38$^-$ cells, each bnx mouse received a cotransplant of 1 million MSCs that had been engineered by the L-IL-3-SN vector to secrete human interleukin-3 (IL-3), so as to promote sustained human hematopoiesis. A total of 481 mice with transplants were monitored up to 18 months after cotransplantation of the HSC/LN and the MSC/L-IL-3-SN inoculum. The mice were observed at least once daily for signs of illness, which were defined as any one or more of the following: weight loss, hunching, lethargy, rapid breathing, skin discoloration or irregularities, bloating, hemiparesis, visibly enlarged lymph nodes, and visible solid tumors under the skin. Any signs of illness were logged as "adverse events" in the experiment, the mouse was immediately killed, and an autopsy was performed to establish the cause of illness. The presence of tumors and organ or lymph node abnormalities was specifically looked for. Organs, marrow, blood, and serum were banked. Tissue blocks for sectioning were prepared from all the organs (and from the visible tumor if present). DNA was prepared, as described,[11] from marrow, organs, blood, (and tumor if present), so as to determine whether vector elements were present in them, as described later.

Sera from mice that had experienced adverse events (117 of 481 mice followed, **Table 1**) were tested for the presence of RCR. RCR that could potentially be formed from recombination of the LN vector in HSCs, or from the L-IL-3-SN vector in MSCs, should potentially be detectable in the murine serum. The marker rescue assay was carried out as depicted in **Figure 2**. No RCR was detected in the serum of any mouse assayed. Tissue or tumor samples from mice that had suffered adverse events were also assayed (**Figure 2**). By testing for RCR in incubated tissue, we ruled out the possibility that RCR might have been present but inactivated by complement, which is present at normal levels in the sera of bnx mice. No RCR was rescued from any incubated tissue. More recent experiments, including the majority of those relating to biosafety testing of lentivirus *in vivo* (described later) were carried out in nude/NOD/SCID[11] or nonobese diabetic/severe combined immune deficiency (NOD/SCID) mice that lack complement activity.

Marrow, spleen, and blood cells were analyzed using FACS in order to determine whether mice that had experienced adverse events were engrafted with human hematopoietic cells. When human cells were present, an immunophenotyping FACS panel was performed to identify possible human leukemia (**Table 2**). Also, single-cell suspensions from each organ were analyzed using FACS in order to determine whether they were infiltrated with

**Table 1 Summary of adverse events (AEs) in long-term engrafted immunodeficient mice**

| # Mice followed | # Mice with AEs mice[a] | # AE mice with RCR in plasma | # AE mice with RCL in Plasma | # AE mice with human leukemia or solid tumor cells | # AE mice with vector in leukemia or tumor cells[b] |
|---|---|---|---|---|---|
| LN-based retroviral vectors | | | | | |
| 481 | 117 | 0 | — | 4 | 0 |
| Lentiviral vectors | | | | | |
| 149 | 7 | — | 0 | 0 | 0 |

[a]In the current study, an AE was defined as any instance of ill health in the mouse, as defined by any of the following: weight loss, hunching, lethargy, rapid breathing, skin discoloration or irregularities, bloating, hemi-paresis, visibly enlarged lymph nodes, or visible solid tumors under the skin. [b]Defined as either murine or human leukemic cells or solid tumors that carried vector integrants. None of the aberrant cells recovered from the mice in the current studies had evidence of vector integration.

**Table 2 Immunophenotyping panel used in the current studies to determine whether human hematopoietic cells were skewed toward one lineage, which might indicate the development of leukemia**

| FITC-conjugated antibody | PE-conjugated antibody |
|---|---|
| ASC | ASC |
| Human CD45 | ASC |
| Hu CD4 | Hu CD8 |
| Hu CD45 | Hu CD33 |
| Hu CD45 | Hu CD19 |
| Hu CD34 | Hu CD38 |
| ASC | Hu CD13 |
| Hu CD7 | Hu CD3 |
| Hu CD45 | Hu CD3 |
| Hu CD56 | Hu CD3 |
| Hu CD45 | Hu CD2 |
| Hu CD69 | Hu CD3 |

*Abbreviations*: FITC, fluorescein isothiocyanate; PE, phycoerythrin.
Definition of antibody pairs: ASC is the mouse ascites control, and is used to set the level of background, nonspecific labeling. CD4 versus CD8 identifies human T lymphocytes. CD45 is a panleukocyte marker, used against CD33 to identify human myeloid progenitors, and against CD19 to identify human B lymphocytes. Anti-CD34 and CD38 are used together to identify human stem/progenitor cells. A primitive phenotype for human cells is $CD34^+/CD38^-$. Anti-CD13, used alone against ascites, identifies human granulocytes. Anti-CD7 used against anti-CD3 identifies human T cells ($CD7^+/CD3^+$) versus immature T progenitors or other lineages ($C7^+/CD3^-$). CD3 is used against CD45 to provide an additional verification that mature human T cells exist in the murine bone marrow. Human natural killer cells will express CD56, and only a small subset coexpress CD3. CD2 is found on T cells and their progenitors. CD69 is the receptor for L-selectin on human T cells, and is used in conjunction with CD3 to determine the T cell activation state.

the leukemic cells. Human leukemic cells were found in only 4 of the 117 mice that had experienced adverse events. All the four human leukemias had infiltrated the murine liver, spleen, blood, and lymph nodes. Two of the leukemias were observed in two arms of the same experiment, 2 months after the transplantation, in mice #312 and #313. The leukemic cells that had developed in both mice were derived from the same marrow donor, and had a myelomonocytic phenotype ($CD33^+/CD13^+$). Two other human leukemias, positive for human $CD45^+$ and human $CD19^+$ but negative for all other panel markers (**Table 2**) spontaneously arose in mice #94 and #205, at 4 and 6 months after the transplantation. These two mice had received transplants of human cells from different donors.

Leukemic cells were isolated from the mice by FACS, and tested for the presence of vector DNA. All the samples were negative for the MoMuLV long- terminal repeat sequences and

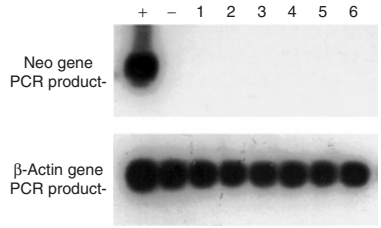


**Figure 5 Neo PCR for mice that carried human leukemic samples.** DNA samples obtained from the mice that carried human leukemic cells were subjected to PCR for the *neo* gene so as to determine whether proviral integration might have contributed to the leukemic transformation. PCR for the *β-actin* gene was used for confirming that adequate DNA was present in each sample. Samples, from left to right: Positive control = the PA317/ SV-neo (clone B) cell line, containing one copy of the neo gene per cell; negative control = PA317 cells; sample 1 = mouse #312, bone marrow; sample 2 = mouse #312, spleen; sample 3 = mouse #312, blood; sample #4 = mouse #205, bone marrow; sample 5 = mouse #205, spleen; sample 6 = mouse #205, blood.

for the neo gene (**Figure 5**). These data demonstrate that the leukemic events had arisen randomly and were not the result of insertional mutagenesis. Neither human nor murine leukemic cells can be recognized and rejected in immune-deficient mice that lack natural killer activity. Human leukemic cells that had likely arisen through random translocations or mutagenic events had quickly infiltrated the marrow, blood, and organs of the recipient mice. Given that leukemic cells with the same phenotype had arisen in two mice that had received transplants from the same donor (mice #312 and #313), there may have been cells that were already transformed or that were predisposed to leukemic transformation in the human marrow sample before the transplantation. The human B cell lymphoproliferation that arose in mice #94 and #205 may have been caused by the presence of Epstein–Barr virus-transformed cells in the initial sample used for the transplant.

The other 113 mice that experienced adverse events were tested for the presence of human cells, for vector DNA, and for RCR by marker rescue assay, as shown in **Figure 2**. Of these mice, 37 had developed solid tumors which were all determined to be of murine origin, and none had vector DNA present. None of the 37 mice had detectable RCR in their serum or any RCR produced from tumor tissue. The engineered human MSCs (a total of 459 million transduced, selected cells injected in all the experiments) never gave rise to solid tumors.

Seventy six other mice had adverse events that ranged from development of murine leukemia (51 mice) to scarred skin or discolored organs detected at autopsy. None of the serum samples

or affected tissues from these mice had vector integrants or produced RCR in the marker rescue assay.

### Biosafety testing for HIV-based vectors

We also analyzed the biosafety of HIV-1-based lentiviral vectors. A total of 149 immunodeficient bnx and NOD/SCID mice were given transplants of human $CD34^+$ and $CD34^+/CD38^-$ cells that had been transduced by lentiviral vectors as described.[12,13] The mice were monitored daily for the development of adverse events as described earlier. Although recombinant HIV-1 (if it were to be generated) would not infect murine tissues, replication-competent lentivirus (RCL) could infect $CD4^+$ human monocytes in NOD/SCID mice or T cells engrafted in the bnx mice; consequently, HIV-1 p24 antigen (the HIV core protein) should be detectable in the sera of such mice.

We monitored the mice daily for any irregularities as described earlier. The average duration of monitoring was 4 months in this series of experiments, because of the shorter life span of the NOD/SCID strain.[5] Of the 149 mice that received transplants only 7 experienced adverse events. One bnx mouse that received a transplant of human $CD34^+/CD38^-$ cells developed a subcutaneous tumor 3 months after the transplantation, and 6 other NOD/SCID mice developed lymphoma. Tissues, marrow, and blood cells were subjected to FACS analysis. The lymphoma cells in the NOD/SCID mice were determined to be murine pre-T cells, as previously documented in this strain.[14–16] Normal percentages of human $CD4^+$ T lymphocytes and $CD33^+$ monocytes[17] were found in the bnx mouse bone marrow, demonstrating that there had been no depletion by RCL. Again, the tumor in the bnx mouse was entirely composed of murine cells, reflecting the poor antitumor response of immunodeficient mice. Single-cell suspensions from the tumor and the organs (marrow, liver, spleen, and lung) were plated into tissue culture flasks for 24 hours to allow generation of recombinant virus, if present. The murine serum and the supernatants collected from the tissues were tested for the presence of HIV-1 p24 antigen using enzyme-linked immunosorbent assay. All samples tested negative for p24.

The other 142 mice (that did not experience adverse events) were allowed to proceed to the planned termination point of the experiment. After the animals were killed, normal distributions of human $CD4^+$ cells present in the murine bone marrow were found. Sera from an additional 16 mice were tested for p24 using enzyme-linked immunosorbent assay, and found to be negative for HIV-1 p24.

These studies demonstrate that there is very low risk from replication-competent vectors or the occurrence of adverse events when the Moloney murine leukemia–based retroviral vectors and HIV-1-based lentiviral vectors are used for delivering a marker gene such as neo. In view of the fact that some of the hurdles involved in the use of these vectors have been recently overcome and clinical efficacy can now be achieved in some settings with the use of the Moloney-based vectors, the documentation of the safety of these vectors is reassuring.

## DISCUSSION

In this report we describe an *in vivo* system designed to serve as an assay for potential risk assessment when retroviral or lentiviral vectors are considered for use in human clinical gene therapy applications. We used both HSCs and MSCs, cotransplanted into immune-deficient mice (that have no immune system and therefore cannot reject a transformed cell) for conducting these studies. A total of 630 immune-deficient mice were intravenously given transplants of transduced human stem cells, and followed for time points ranging from 2 to 18 months to assess the risk, if any, posed by the transduced cells. No vector-related adverse events were found during the detailed autopsies of each mouse.

In addition to vector testing, this xenotransplantation system also provides testing of the tumorigenic potential of the stem cells themselves. Bone marrow stroma is initially a heterogeneous cell mixture consisting of fibroblasts, endothelial cells, adipocytes, and macrophages. In these studies, we expanded the multipotent, myofibroblastic component known as MSCs. These cells have been shown to have the capacity to generate fat, smooth muscle, cartilage, and bone.[18–21] The primary human MSCs used in the current studies were derived from 84 different marrow donors. The MoMuLV-based vector, L-IL-3-SN, packaged in the PA317 cell line and transferring the neomycin resistance gene, was used for MSC transduction in all the animals. In order to achieve 100% transduction efficiency at the time of transplantation, the MSCs were rigorously selected for expression of the neo gene, using the toxic neomycin analog G418. Only MSCs that carried the neo transgene survived at the dosages of drug that were used (0.75 mg/ml active concentration), as documented by the death of 100% of the sham-transduced MSCs that were selected as controls in each experiment. The immune-deficient mice were continuously monitored for solid tumor formation after receiving transplants of MSCs. It has been reported in the literature that MSCs that are expanded over a period of 4–5 months can become malignant.[22] Malignancy from transplanted human MSCs was never observed in our *in vivo* xenotransplant model, perhaps because the MSC cultures were not carried longer than ~4 weeks, which has been suggested in the literature to be a "safe" culture period. We also did not allow the MSCs to become confluent at any point,[22] in order to avoid increasing the potential risk of cell fusion and other *in vitro* transformations. No human MSC-derived solid tumors were ever detected in any of the 630 mice tested. It is also important to note that in the large numbers of mice that received cotransplants of human MSCs, no ectopic bone, cartilage, or other unusual growths were observed. This finding is of importance not only to the stem cell field but also to the gene therapy field.

Of the 117 total adverse events observed in this study, 4 were caused by the spontaneous development of human leukemia, without vector involvement, and the remainder of the events were caused by the development of murine cancer in the murine tissues, again not caused by the retroviral vectors. No RCR was found in any of the mice that had murine leukemias or murine solid tumors (113 tested). Resected tumors lacked staining for human HLA, thereby demonstrating that all the solid tumors that had arisen were of murine origin. DNA analysis confirmed that no vector elements had been transferred. An equivalent incidence of development of murine solid tumors and leukemia was found in our immunodeficient bnx mouse breeding colony, where the animals are never exposed to recombinant DNA. The

spontaneous transformation of cells that cannot be rejected is observed frequently in mice that lack an immune system, and specifically in animals that lack natural killer cells. The incidence of development of adverse events, ranging from skin discoloration to the development of tumors or leukemia, in our unmanipulated bnx breeder colony was 268 in 954 mice. This rate is higher than in normal mice, perhaps in part because the rearrangement of immunoglobulin genes cannot occur properly in bnx mice, given their deficiency of Bruton's tyrosine kinase. The failure of successful gene rearrangement usually leads to the death of the cells, but may also lead to leukemogenesis in some cases. Also, the animals have no innate natural killer cell function to eliminate leukemic cells as they arise. The incidence of adverse events in the transduced stem cell-injected mice (117/481 = 24.3%) was not significantly higher than in the unmanipulated breeding colony (268/954 = 28%), $P > 0.5$. However, in spite of this background, the findings in our study are significant. It is this tendency toward tumor formation in our mouse model that allows us to say with confidence that a transformed cell could actually develop into a detectable malignancy rather than be eradicated by a residual immune system. This statement is further underscored by the fact that four human leukemias developed spontaneously from the transplanted donor cells. We also believe that because of the very large numbers of mice used in this transplant study we could actually detect the spontaneous transformation of human cells, an event that cannot be easily observed when smaller sample sizes are used.

In spite of reports in recent papers that transduced murine cells can reproducibly form malignancies in mice,[23,24] we could not observe tumor formation using transduced human hematopoietic cells despite the large number of animals studied. We as well as others believe that there is a difference between human cells and murine cells as regards the ease with which they get transformed into malignant cells. It has been established that human cells require multiple "hits" in order to become transformed and fully malignant, and usually the immune system would clear them from the system once the initial abnormalities occur.[25–29] Human tumors that arose in our model spontaneously from nonvector-related causes could be detected, and those events demonstrate the sensitivity of the system. However, leukemic events arising in human cells have been observed to arise several years after transduction, so a longer period than a murine model allows could be warranted.

During the duration of the study, no evidence could be observed of leukemia caused by insertional mutagenesis in the transduced human stem/progenitor cells. All the mice tested were free of human leukemic cells carrying intact or recombinant retroviral vectors, up to 18 months after the transplantation. No RCR was observed, in the sera or tissues of the mice tested (117 samples total), that had received human cells transduced by the current generation of safety-modified vectors. However, RCR was easily and sensitively detected when human cells that had been transduced with an older vector, N2, lacking the current safety modifications, was used. Because we wanted to set up a long-term study stretching over >10 years, and keeping in mind the requirement for consistency, we decided to continue to use the identical set of laboratory tests throughout the study so that no additional and uncontrollable variable would be introduced. We do understand that some amplified RCR assays developed over time may have higher sensitivity, but we were confident that we could detect emergent RCR in our experiments to a high degree of efficacy with the marker rescue assay described here, which has been used ever since the studies were initiated in the early 1990s. For transduction of hematopoietic cells we also used supernatant and packaging cell lines that had been certified by third-party companies to be free of RCR or helper virus, given that these supernatants were candidates for use in human clinical gene therapy trials. Also, our inverse PCR was the assay of choice when the study was conceived. It may not be as sensitive as the more modern linear amplification-mediated PCR assay,[30] but it did allow us to quantify integration events reliably. In future studies, particularly for testing lentiviral vectors, the use of linear amplification-mediated PCR and insertion site analysis[30,31] should be considered.

At a juncture when much more scrutiny is being directed toward clinical stem cell and gene therapy trials, it is reassuring that our study did not find increased tumor risk to be associated with the transplantation of either MoMuLV or lentivector-transduced human HSCs or MSCs.

## MATERIALS AND METHODS

***Isolation and transduction of MSCs.*** MSCs were engineered to secrete human IL-3 and cotransplanted into mice along with retrovector or lentivector-transduced cells in each experiment, so as to sustain viability of the human hematopoietic progenitors in the mice, as we have described earlier.[10] MSCs were isolated from the screens used for filtering bone marrow at the time of harvesting of bone marrow from normal donors. The screens were flushed in the Operating Room at Children's Hospital of Los Angeles, under an institutional review board–approved protocol. Bone spicules were collected by gravity sedimentation in tissue culture flasks in Dexter's original medium [350 ml Iscove's modified Dulbecco's medium, 75 ml screened heat-inactivated horse serum, 75 ml screened heat-inactivated fetal calf serum (Omega Scientific, Calabasas, CA), 5 ml L-glutamine (200 mmol/l stock; Gibco/BRL, Grand Island, NY), 2.5 ml Pen/Strep (stock = 10,000 U/ml penicillin and 10,000 μg/ml streptomycin; Gibco/BRL, Grand Island, NY), 500 μl 2-mercaptoethanol ($10^{-1}$ mol/l stock; Sigma, St. Louis, MO), and 500 μl hydrocortisone ($10^{-3}$ mol/l stock; Sigma, St. Louis, MO)]. MSCs were allowed to adhere to the flasks overnight, the nonadherent cells were removed, and the MSC layer was allowed to expand. Sub-confluent layers of primary MSCs were split by trypsinization (trypsin–EDTA; Sigma, St. Louis, MO). MSCs were not used for transduction until passages 3 or 4. At this point all $CD45^+$ hematopoietic cells were eliminated, except for occasional mature $CD14^+$ macrophages which comprised <1% of the culture.

Primary human MSCs derived from 84 different marrow donors were used in this study. Supernatant from the MoMuLV-based retroviral vector L-IL-3-SN[10] was added four times to <50% confluent MSC culture flasks over a 48-hour period. Protamine sulfate (final concentration = 4 μg/ml) was added once every 24 hours. MSCs were visually verified to be subconfluent before each aliquot of supernatant was added, because confluent cells are contact-inhibited and will not divide; division is needed in order to allow for retro-vector integration. The transduction efficiency was 20–40%, as measured by *neo*-PCR (data not shown). The transduced cells were selected in G418 (Geneticin; Gibco/BRL, Grand Island, NY) at a concentration of 0.75 mg/ml active drug for 5 days or until sham-transduced controls were completely killed. The G418-selected MSCs were then re-expanded for one or two passages and used for cotransplantation with sorted, transduced human stem and progenitor cells. One million transduced MSCs were transplanted into each bnx mouse, as described earlier.[10,32]

***Transduction of human hematopoietic progenitors.*** The MoMuLV-based vector LN and its derivatives[9] were used for progenitor cell transductions and assessment of biosafety factors relating to MoMuLV vectors. MoMuLV was packaged in PA317 and PG13 packaging cell lines, and vector supernatant was collected as described previously.[32–35] The MoMuLV vectors and packaging cells (having been candidates for human gene therapy clinical trials) were precertified by third party testing companies as being RCR-free. In order to determine the sensitivity of the system, N2 virus was transfected into the Psi2 packaging cell line and then transferred to the PA317 cell line. Hematopoietic $CD34^+$ stem and progenitor cells isolated from normal human bone marrow were exposed to retroviral supernatant three times at 24-hour intervals in the presence of appropriate stimulatory cytokines, as described earlier.[34,36–38] Production of second generation lentivector packaged with the vesicular stomatitis virus G-protein envelope and transduction of human $CD34^+$ and $CD34^+/CD38^-$ cells were carried out as described earlier.[12,13] $CD34^+/CD38^-$ cells comprised ~20% of the target cell population.

***Immune-deficient mice and cotransplantation.*** Six- to eight-week-old homozygous bg.bg/nu.nu/xid.xid (bnx) mice, bred at Childrens Hospital of Los Angeles (Los Angeles, CA), were used in all the studies. Cotransplantation of human hematopoietic progenitors [500,000–1,000,000 $CD34^+$ cells, isolated by immunomagnetic selection using Dynabeads (Dynal, Oslo, Norway), or 2,000 $CD34^+/CD38^-$ cells, purified by FACS], and 1,000,000 human MSCs engineered to secrete human interleukin 3, was carried out as described earlier.[10,32,34,39]

***Health monitoring.*** After cotransplantation of the engineered bone marrow progenitor/MSC inoculum, the mice were assessed twice a day on weekdays and once a day on weekends and holidays for signs of illness, as defined by one or more of the following: weight loss, hunching, lethargy, rapid breathing, skin discoloration or irregularities, bloating, hemi-paresis, visibly enlarged lymph nodes, and visible solid tumors under the skin. If any type of irregularity was observed, the mouse was immediately killed with 75% $CO_2$/25% $O_2$ narcosis and subjected to the full range of tissue and plasma banking and biosafety analyses as described later. If no signs of illness were observed, engraftment with transduced cells was allowed to continue until 7–18 months after the transplantation.

Immediately after an animal was killed, blood was collected from the renal artery into a heparinized microfuge tube. Plasma was separated by centrifugation in a microcentrifuge at 2,000 rpm. for 2 minutes, then collected and rapidly frozen. The remaining blood cells were kept at room temperature for the duration of the autopsy. The mouse was carefully examined for any external or internal abnormalities, irregularities in organ color or shape, tumors, and enlarged lymph nodes. If any abnormalities were noted, the tissue in question was excised for further testing. A portion was banked in tissue blocks, a portion was used for DNA preparation to determine whether vector elements were present, and a portion was dispersed into a single-cell suspension to test for the presence of human cells, using FACS. Another portion was plated in tissue culture flasks for 24 hours in minimal culture medium to allow for potential production of RCR, to be analyzed later using marker rescue assay (**Figure 2**).

***Determination of the presence of human hematopoietic cells in tissues recovered from the mice.*** Bone marrow was flushed from the four long bones of the hindlegs, and either used immediately for FACS analysis and cell sorting, or cryopreserved for later analysis. Spleen, liver, lung, and kidney, plus any abnormal areas of the mouse that might have been discovered upon autopsy (tumors, lymph nodes, discolored areas of organs, patches of skin, etc.), were dispersed into single-cell suspensions by manual dissociation and teasing out cells with fine scissors and tweezers, or into sieve cups if the portion of tissue was large enough. Debris was removed by allowing it to settle out, and single-cell suspensions were retained for viable cryopreservation, plating for marker rescue assay, DNA preparation, and antibody labeling for FACS.

In order to determine the total human hematopoietic cell content in the bone marrow and other organs, the single-cell suspensions were labeled with HLE-1 (antihuman CD45), a fluorescein isothiocyanate–conjugated antihuman panleukocyte antibody from Becton Dickinson (BD), or antihuman HLA (Sigma), and then subjected to FACS analysis. Analysis of 10,000–100,000 cells acquired from each tissue was performed using the CellQuest program (BD). In order to determine whether human leukemic cells were present in mice that had abnormal lymph nodes, or showed paleness of organs or bone marrow, or had displayed rapid breathing, pallor, or hunching before being killed, a panel of lineage-specific antibodies was used to test single-cell suspensions from marrow and organs (effective antibody combinations are shown in **Table 2**). Single-cell suspensions from the bone marrow, blood, or tissues of the mice were preincubated for 15 minutes on ice with unconjugated mouse immunoglobulin (Coulter, Hialeah, FL). Directly conjugated antibodies used for identifying human-specific cell surface antigens were HLE-1 [anti-CD45; Becton Dickinson (BD)], My9-RD1 (anti-CD33; Coulter), Leu-12 (anti-CD19; BD), Leu-3a (anti-CD4; BD), Leu-2a (anti-CD8; BD), Leu 5b (anti-CD2; BD), Leu 4 (anti-CD3; BD), Leu 9 (anti-CD7; BD), HPCA-1 and HPCA-2 (anti-CD34; BD), Leu 17 (anti-CD38; BD), My7 (anti-CD13; Coulter), Leu 19 (anti-CD56; BD), and Leu 23 (anti-hu CD69; BD). Samples were acquired on a FACScan (BD) flow cytometer and analyzed using the CellQuest software package (BD). Ten thousand events were acquired for each sample. The appropriate isotype controls were used in all cases, and parallel staining and FACS analyses were performed on normal human bone marrow and on transplant-free *bnx* mouse bone marrow controls, so as to confirm that none of the human-specific antibodies cross-reacted with murine cells.

***DNA preparation and testing for vector elements using PCR.*** Genomic DNA was extracted from murine bone marrow and organs, and from tumor tissue, if present. In the four instances where human leukemic cells were found in the mice, they were identified phenotypically and then isolated from the murine marrow by FACS. Genomic DNA was analyzed for the presence of the LN provirus using PCR for the *neo* gene, PCR for *β-actin* to confirm that DNA was present in all the samples, and for the MoMuLV long-terminal repeat in the case of isolated human cells, as described.[10,32]

***RCR and RCL analysis.*** Testing for the generation of RCR and RCL in the mice during the engraftment period was carried out using enzyme-linked immunosorbent assay for HIV-1 p24 (for RCL) (p24ELISA kit; Coulter, FL), and marker rescue assay (for RCR) on plasma and tissues from the mice that had experienced adverse events (**Figure 2**). Plasma was collected and immediately frozen, and single-cell suspensions were made from tissues as described earlier in the text. One million cells from each of the single-cell suspension samples were incubated in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum for a 24-hour period at 5% CO2 and 37 °C, so as to allow generation and release of recombinant virus, if present. The culture supernatant was collected and filtered through a 0.45-μm syringe filter after the 24-hour period. The supernatant and serum samples were then added to subconfluent plates of 3T3 and Hep-G2 cells (American Type Culture Collection) with 4 μg/ml protamine sulfate, and allowed to incubate for 24 hours. Each sample plate was tested in duplicate and, for each sample plate, an additional two control (3T3 and Hep-G2) plates were prepared and treated with medium and protamine sulfate only, as shown in **Figure 2**. The first set of test and control plates was selected in G418 to determine whether colony formation could occur consequent to transfer of the *neo* gene by recombinant viruses. The second set of plates was lysed for DNA preparation and tested for the neo gene (3T3 and Hep-G2) and MoMuLV long-terminal repeat sequences (Hep-G2) using PCR as described.[10]

***Statistical analyses.*** All analyses were performed using Excel 5.0 (Microsoft, WA). Average values are listed with SDs. The significance of each set of values was assessed using the two-tailed *t*-test, assuming equal variance.

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