Helper Virus Induced T Cell Lymphoma in Nonhuman Primates after Retroviral Mediated Gene Transfer

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Summary

Moloney Murine Leukemia Virus (MoMuLV) causes T cell neoplasms in rodents but is not known to be a pathogen in primates. The core protein and enzyme genes of the MoMuLV genome together with an amphotropic envelope gene are utilized to engineer the cell lines that generate retroviral vectors for use in current human gene therapy applications. We developed a producer clone that generates a very high concentration of retroviral vector particles to optimize conditions for gene insertion into pluripotent hematopoietic stem cells. This producer cell line also generates a much lower concentration of replication-competent virus that arose through recombination. Stem cells from rhesus monkeys were purified by immunoselection with an anti-CD34 antibody, incubated in vitro for 80–86 h in the presence of retroviral vector particles with accompanying replication-competent virus and used to reconstitute recipients whose bone marrow had been ablated by total body irradiation. The retroviral vector genome was detected in circulating cells of five of eight transplant recipients of $CD34^+$ cells and in the circulating cells of two recipients of infected, unfractionated bone marrow mononuclear cells. Three recipients of $CD34^+$ cells had a productive infection with replication-competent virus. Six or seven mo after transplantation, each of these animals developed a rapidly progressive T cell neoplasm involving the thymus, lymph nodes, liver, spleen, and bone marrow. Lymphoma cells contained 10–50 copies of the replication-competent virus, but lacked the retroviral vector genome. We conclude that replication-competent viruses arising from producer cells making retroviral vectors can be pathogenic in primates, which underscores the importance of carefully screening retroviral producer clones used in human trials to exclude contamination with replication-competent virus.

Retroviral vectors have the capacity to transfer genes with high efficiency into a broad spectrum of target cells (1–4). Such vectors have considerable flexibility with respect to the nature of the transferred genetic information. Generally, use of appropriate regulatory and/or RNA processing signals provides sufficient gene expression to achieve the desired experimental or therapeutic goal. Indeed, the first human gene therapy protocols are based on the use of retroviral vectors (5).

Most retroviral vector systems use some components of the Moloney murine leukemia virus (MoMuLV)[1] (1–4). This virus induces T cell leukemia/lymphomas in rodents with long latency primarily by the mechanism of insertional muta-

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[1] *Abbreviations used in this paper:* MCF, mink cell focus; MoMuLV, Moloney murine leukemia virus; rgIL-3, recombinant gibbon interleukin 3; rhG-CSF, recombinant human granulocyte colony-stimulating factor; rhGM-CSF, recombinant human granulocyte/macrophage colony-stimulating factor; rhSCF, recombinant human stem cell factor; STLV, simian T cell leukemia virus; WBC, white blood cell.

genesis (6–8). The transcriptional control elements of the integrated provirus activate adjacent cellular genes leading to dysregulated cell proliferation and the development of neoplasms. Viral replication in vivo is required for disease induction. In mice infected with MoMuLV there is consistent emergence of xenotropic viruses by recombination of the MoMuLV genome with cellular sequences (6, 7). These xenotropic viruses, detected by their capacity to induce foci on mink cells, are called mink cell focus (MCF) viruses. There is evidence that the MCF envelope protein activates the IL-2 receptor (9) and may play a role in oncogenesis when expressed in preneoplastic thymus cells.

The use of MoMuLV components for generation of therapeutic viral vectors, despite the known capacity of this virus to induce neoplasms in rodents, is based both on recombinant DNA engineering strategies and in vivo safety studies. Retroviral vectors are produced in packaging cells (murine fibroblasts) that contain two or more genetic components introduced by conventional gene transfer strategies (1, 10). The vector proviral genome contains both the coding sequences for the gene to be expressed in target cells and the LTRs and adjacent sequences that provide required packaging, replication, insertion, transcriptional control, and RNA processing signals (1–4). Packaging cell lines contain a second proviral genome designed to express the viral proteins necessary for infectious virus formation. In early packaging lines, this "helper" genome was mutated to remove the genetic element necessary for packaging of its own full-length RNA transcript into virus particles (10). Although useful for experimental purposes, such "first generation" packaging cell lines consistently gave rise to replication-competent viruses by recombination between the RNA transcript derived from the helper genome and that derived from the vector genome, and therefore could not be used for human gene therapy applications (1–4). Second generation packaging cell lines contain a helper proviral genome into which additional mutations had been introduced to reduce the probability of recombination and thus replication-competent virus production (11). In third generation packaging lines, genetic components encoding the ecotropic gag/pol and amphotropic envelope proteins were separated on different transcriptional units, thereby reducing the likelihood of generation of replication-competent virus by recombination (12–14). In addition to these changes in the helper genome, unnecessary sequences have been eliminated from the vector genome and mutations introduced into that portion of the gag region retained to achieve high viral titers (1, 2). These modifications in the vector genome reduced the likelihood that replication-competent virus would emerge from producer clones currently used to generate vectors for use in human studies (14).

Support for use of MoMuLV-based systems for generation of therapeutic gene transfer vectors was derived from safety studies in primates that suggested that murine amphotropic replication-competent virus was not pathogenic (4, 16, 17). Large amounts of amphotropic virus, introduced by intravenous injection or by implantation of virus-producing autologous fibroblasts, established only transient infection in normal or immunosuppressed animals but failed to induce disease. Sera from rhesus monkeys rapidly reduced the titer of amphotropic virus by two orders of magnitude, and replication-competent virus could not be detected in the sera of infected animals (16).

Our work has focused on the use of retroviral vectors for gene transfer into hematopoietic stem cells. Although strategies have been developed for reasonably efficient gene transfer into murine repopulating stem cells (18, 19), retroviral mediated gene transfer into primate stem cells has been more difficult to achieve (18–20). We developed a retroviral producer clone that generates a very high concentration of retroviral vector particles (10^9/ml) allowing a high multiplicity of infection for the target stem cell population (21). This producer clone was derived by use of a vector genome and packaging cell lines known to give rise to replication-competent virus by recombination, and indeed our clone produced a lower level of replication-competent virus (10^3–10^4/ml). Because murine amphotropic replication-competent viruses were thought to be nonpathogenic in primates (16, 17), we utilized this producer clone as an experimental tool for optimizing conditions for gene transfer into primate hematopoietic stem cells. In the course of these experiments, we observed that replication-competent virus is capable of inducing an aggressive T cell neoplasm in primates analogous to the disease observed in rodent species (6–8).

Materials and Methods

Bone Marrow Transplantation. Young adult rhesus macaques (*Macaca mulatta*) that were serologically negative for simian T cell leukemia virus (STLV), simian immunodeficiency virus, and simian retrovirus, and had blood type B, were used in these studies. Animals were quarantined and housed in accordance with the guidelines set by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Public. No. NIH 85-23, revised 1985) and the policies set by the Veterinary Resource Program of the National Institutes of Health. Before the transplantation procedure, indwelling central venous catheters were established by standard surgical techniques (22).

Day 0 was designated as the day of the bone marrow cell reinfusion. 100 ml of autologous peripheral blood were collected on days −11 and −10 and stored at 4°C. After warming to 37°C, this peripheral blood was used for transfusion on day −4 at the time of bone marrow harvest. On day −9, 70 mg/kg of 5-Fluorouracil was administered as a single intravenous bolus (23) to all except two animals. These two received recombinant human stem cell factor (rhSCF) (24) intravenously (200 μg/kg/day) for days −14 to −4. On day −4, ~150 ml of bone marrow containing 1–4 × 10^9 total cells was harvested surgically from the femurs, iliac crests, and ischial tuberosities into heparinized syringes under general anesthesia. On day −2 and/or −1, animals received total body γ-irradiation to ablate the endogenous bone marrow (Table 1). Two animals were transplanted with the total mononuclear cell fraction after 4 d of direct coculture with the retroviral producer cells (21). These animals received no 5-FU or SCF before bone marrow harvest. Standard supportive care for bone marrow transplant recipients was initiated after the irradiation (22). Autologous or crossmatched whole blood transfusions were administered when the hematocrit was less

than 20% or when the platelet count fell below 20,000. Recombinant human granulocyte colony-stimulating factor (rhG-CSF) (25) at a dose of 5 μg/kg/day or recombinant human granulocyte/macrophage colony-stimulating factor (rhGM-CSF) (22, 26) was administered as a continuous intravenous infusion until the white blood cell (WBC) count reached 10,000/mm^3 (Table 1).

Immunoselection of CD34$^+$ Cells. Upon harvesting, the bone marrow was diluted with HBSS and passed through wire mesh (Baxter Healthcare Corp., Deerfield, IL) to remove clumps. Mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), and washed. Cells expressing CD34 were isolated by immunoselection as previously described (27, 28).

To estimate the progenitor concentration, bone marrow mononuclear cells and CD34$^+$ immunoselected cells were plated in methylcellulose in IMDM containing 30% FCS, 0.9% deionized BSA, and 10^{-4} M 2-ME (Terry Fox Laboratories, Vancouver, Canada). Unfractionated cells were plated at 5×10^4/ml, whereas the CD34$^+$ cells were plated at 100 and 1,000 cells/ml. rhSCF (100 ng/ml) (24), rhGM-CSF (26) (10 ng/ml), and recombinant erythropoietin (29) (2 U/ml) were added to the culture medium. Colonies were scored 12–14 d after initiation of triplicate cultures.

Retroviral Producer Cells. The cell clone, N263A2 (21), was maintained in DMEM (Biofluids Inc., Rockville, MD) with 15% FCS. Medium conditioned by amphotropic producer cells during the 12–18 h of proliferation from semiconfluency to confluency contained $\sim 10^9$ infectious neo^R vector particles and 10^3–10^4 replication-competent virus particles per ml. Fresh conditioned medium was used without freezing for all bone marrow cell infections.

Culture of Bone Marrow Cells. Immunoselected CD34$^+$ cells were resuspended at 2×10^5/ml in DMEM plus 15% FCS supplemented with glutamine, penicillin, and streptomycin. One half of the total volume in which the cells were suspended was culture medium derived from the amphotropic producer cell line as described above. The amphotropic producer line had been engineered to secrete recombinant gibbon IL-3 (rgIL-3) and recombinant human IL-6 (rhIL-6) in low concentration (21). Polybrene (6 μg/ml), rhSCF (24) (100 ng/ml), and rhIL-6 (30) (50 ng/ml) were added. For certain animals, rhIL-3 (31) was also added at 10 ng/ml. One half of the medium was changed every 24 h and replenished with medium containing fresh virus, growth factors, and polybrene. Coculture of unfractionated mononuclear cells of two animals (Table 1) with retroviral producer cells was as previously described (21).

In some cases (Table 1), CD34-selected cells were cocultured with a previously established autologous stromal layer. The stromal cells were obtained by aspirating bone marrow from the iliac crest into preservative-free heparin 1–2 wk before initiation of the bone marrow transplantation protocol. This marrow was suspended in HBSS and mononuclear cells were recovered by density gradient centrifugation. These cells were washed and suspended at 2×10^6/ml in DMEM containing 15% FCS supplemented with antibiotics. Nonadherent cells were removed 48–72 h later. The adherent cells were washed 2–3 times with HBSS and fresh tissue culture medium was added. The adherent cells were passaged after trypsinization as necessary to allow continuous growth. 12–24 h before the main bone marrow harvest, when the adherent cells were 30–50% confluent, nonadherent cells were removed and fresh culture medium added. For coculture, CD34$^+$ selected cells were added (2×10^5/ml) in culture medium containing fresh virus, polybrene, and growth factors and maintained as specified above.

Immunophenotyping of Tumor Cells. Tumor samples were analyzed both by flow cytometry and by immunohistology. For flow cytometric analysis, suspensions of mononuclear cells from peripheral blood or tissues infiltrated with tumor were prepared by standard techniques including density gradient centrifugation. The following antibodies were used to characterize these cells. FITC-conjugated anti-CD2, PE-conjugated anti-CD20, and their isotype-matched controls were obtained from Coulter Immunology (Hialeah, FL); PE-conjugated anti-CD4 along with its isotype matched control were obtained from Becton Dickinson & Co. (Mountain View, CA); and FITC-conjugated anti-CD8 along with its isotype-matched control were obtained from Gen Trak, Inc. (Plymouth Meeting, PA). The rat mAb, 83A25 (32, 33), was used in these studies to recognize an epitope of the envelope gp70 molecule common to the different classes of murine leukemia retroviruses. The rat IgG2a isotype control was obtained from Zymed (San Francisco, CA). A PE-conjugated goat F(ab')$_2$ anti-rat Ig (Tago Inc., Burlingame, CA) was used as the secondary antibody. Antibodies were used at the concentrations specified by the manufacturers or based on titration to achieve maximal staining intensity on cells known to be antigen positive.

For immunohistological studies, portions of involved tissues were embedded in OCT (Miles Laboratories, Naperville, IL) and snap frozen in 2-methyl butane mixed with dry ice. Air-dried acetone–fixed frozen sections were stained using the avidin–biotin complex immunoperoxidase procedure as previously described (34). The panel of mAbs used included Leu2a (CD8), Leu3a, (CD4), Leu5b (CD2), Leu12 (CD19), Leu16 (CD20), MY4 (CD14), (Becton Dickinson & Co.), LCA (CD45) (Dako Corp., Carpinteria, CA) and Bf1 (β chain of the TCR) (T Cell Sciences, Inc., Cambridge, MA). All of the above antibodies, developed against human hematopoietic cell surface antigens, crossreact with the corresponding cell surface antigens in the rhesus monkey.

DNA Analysis. DNA was recovered from peripheral blood cells and tumor tissues by standard techniques (35). The vector and replication-competent amphotropic proviral genomes were detected by PCR amplification using 400 ng of genomic DNA as a template with specific primers as previously described (21). The sequence of the primers used to amplify sequences in the vector genome or in the amphotropic or ecotropic envelope gene have been previously published (21) whereas those for the rhesus globin genes are given in the legend to Fig. 4. Conditions used for amplification PCR were as follows: amphotropic or ecotropic envelope gene or neo^R proviral sequences: denaturation, 94°C for 1.5′; annealing, 52°C for 2.5′; elongation, 72°C for 3.0′ (25 cycles); and rhesus γ globin primers: denaturation, 94°C for 1′; annealing, 55°C for 1′; and elongation, 72°C for 1′ (25 cycles). Southern blot analysis was performed after restriction enzyme digestion using standard techniques (35).

Detection of Replication-Competent Virus. The S$^+$/L$^-$ assay was performed on serum samples and media conditioned by suspended tumor cells using PG4 cells (36). This assay detects replication-competent xenotropic or amphotropic retrovirus by mobilization of a focus forming proviral genome within the PG4 cells. DNA samples were assayed for STLV-I using a sensitive PCR-based assay (37). Samples of sera were analyzed for antibodies to the retroviral core protein, p30 (16), using a kit obtained from Victor Laboratories, (Burlingame, CA).

Results

Bone Marrow Transplantation. 14 animals were the subjects of these studies; eight animals were transplanted with

$CD34^+$ cells incubated in vitro for 80–86 h under conditions designed to introduce the retroviral vector; two animals received $CD34^+$ cells without in vitro culture; two irradiated animals received no cells; and two animals were transplanted with total bone marrow mononuclear cells cocultured with retroviral producer cells (Table 1).

The immunoselection procedure was highly efficient resulting in an average recovery of 4.0% of the starting mononuclear cells with an average 27-fold enrichment of clonogenic progenitors; 1.8–5.2% (average 3.5%) of the $CD34^+$ cells gave rise to colonies in vitro. The $CD34^+$ fraction contained 90–100% of the clonogenic progenitors. There was an average fourfold (range 2.0–7.6) increase in the number of cells during in vitro incubation of the CD34 selected cells.

The eight animals that received CD34 selected cultured cells recovered to a WBC count of >1,000/mm^3 in 20 ±

Table 1. *Characteristics and Outcome of Bone Marrow Transplantation*

Animal	Pretreat	Radiation dose (Rads)	Culture conditions‡	No. of cells infused	Days to WBC count of 1,000§	Clinical status
CD34 Selected cells						
88053	5-FU	500 × 2	SCF, IL-6	8.4×10^7	17	Lymphoma, died (Day 200)
88049*	SCF	500 × 2	Stroma SCF, IL-6	2.2×10^8	20	Lymphoma, died (Day 182)
15445	5-FU	500 × 2	Stroma SCF, IL-6, IL-3	4.3×10^7	19	Lymphoma, died (Day 206)
15186	5-FU	500 × 2	SCF, IL-6	1.0×10^8	20	Alive (Day 458)
15326*	SCF	500 × 2	Stroma SCF, IL-6	2.8×10^8	23	Alive (Day 385)
15222	5-FU	500 × 2	Stroma SCF, IL-6	1.8×10^8	24	Alive (Day 349)
15365	5-FU	500 × 2	Stroma SCF, IL-6	4.3×10^7	16	Alive Day (329)
15160	5-FU	500 × 2	Stroma SCF, IL-6, IL-3	3.6×10^7	19	Alive (Day 262)
Unfractionated Mononuclear Cells						
95A	None	600 × 2	Viral producer IL-3, IL-6	5.0×10^8	25	Alive (Day 790)
1474	None	600 × 2	Viral producer IL-3, IL-6	7.1×10^8	23	Alive (Day 798)
Controls						
88054	5-FU	850	–	1.2×10^7	16	Alive (Day 282)
88075	5-FU	1,000	–	3.0×10^7	14	Alive (Day 226)
15520	5-FU	500 × 2	–	–	24	Sepsis, died (Day 67)
16A	5-FU	500 × 2	–	–	22	Sepsis, died (Day 58)

* 5-FU, 5-Fluorouracil (70 mg/kg) given 5 d before the marrow harvest (Day 9). SCF, Stem cell factor (200 μg/kg) infused for the 10 d before marrow harvest (Days −14 to −4).

‡ SCF = Stem cell factor (100 ng/ml); IL-6 = (50 ng/ml); IL-3 = (10 ng/ml).

§ Animals 16A, 95A, and 1474 received GM-CSF starting on day 2 whereas the others received G-CSF starting on day 14. The growth factor infusion was continued until the WBC count exceeded 10,000/mm^3.

2.0 d and achieved full hematopoietic reconstitution (Table 1). The two animals that received $CD34^+$ cells that had not been cultured in vitro reconstituted to a WBC count of >1,000/mm^3 on day 14 or 16. Although the nontransplanted controls reached a WBC count of 1,000 on day 22 or 24 with growth factor support, engraftment was incomplete and survival brief (Table 1). The two animals (95A and 1474) that received unfractionated bone marrow mononuclear cells after coculture with retroviral producer cells also reconstituted fully although 22 or 24 d were required to reach a WBC of >1,000/mm^3.

Transfer of the Vector Genome into Hematopoietic Cells. After reconstitution, five of the eight experimental animals that received $CD34^+$ cells showed evidence of retroviral vector transfer into reconstituting hematopoietic cells. In four animals, the PCR signal with primers specific to the neo^R gene gave a signal intensity indicating that 0.1–1.0% of the circulating cells contained the N2 proviral genome (Fig. 1 and data not shown). The N2 genome was detected in these animals' circulating cells immediately postreconstitution and persisted during the period of follow-up in all animals in which the viral genome was initially detected. The PCR signal derived from the N2 proviral genome in the circulating cells of one animal (15445) was of an intensity, compared to standards, that suggested that 10% of the cells contained the viral genome (Fig. 1). DNA from T lymphocytes recovered by immunoselection and from granulocytes recovered by density gradient centrifugation gave PCR signals of intensities suggesting that 10 and 5% of the circulating cells of these lineages, respectively, contained the vector provirus (data not shown). These cell fractions were >95% pure as determined by cytological examination or FACS® analysis. The two animals transplanted with unfractionated mononuclear cells had 0.2–1.0% retrovirally marked cells in both T lymphocyte and granulocyte fractions for more than 1 yr posttranscription (data not shown).

Detection of Amphotropic Envelope Coding Sequences in Circulating Blood Cells. Over a period of several weeks, three of the animals in this study developed a rapidly progressive lymphoid neoplasm (see below), alerting us to the possibility that replication-competent virus may have been present in vivo. These animals had been clinically stable with full hematopoietic reconstitution before developing this syndrome 6–7 mo after transplantation (Table 1). Primers specific for the amphotropic envelope gene (21) were used in a PCR analysis of peripheral blood cell DNA obtained at various intervals after transplantation (Fig. 1). In the first samples obtained (days 16–22), a weak signal was detected in all three animals suggesting that 1.0–10% of the circulating cells contained the amphotropic envelope gene. However, by days 58–69 in all three animals, the signal detected was of an intensity equivalent to that observed with DNA from the amphotropic packaging cell line which suggests that virtually all of the circulating cells contained one or more copies of the amphotropic envelope gene. At this time, the WBC differential was unremarkable in the three animals. The amphotropic envelope gene was present in DNA prepared from purified T lymphocytes and granulocytes of animal no. 15445 (data not shown).

7 of the 10 animals that reconstituted with bone marrow cells exposed to the amphotropic virus have remained free of neoplastic disease for periods of observation ranging from 6–24 mo (Table 1). The wild-type amphotropic envelope gene was detected in DNA from peripheral blood leukocytes of six of these animals with a signal intensity suggesting that 0.1–0.5% of the circulating cells contained the coding sequences. In one animal (15365), a subsequent DNA sample from peripheral blood cells was negative for the amphotropic envelope gene sequences.

Detection of Replication-Competent Virus In Vivo. Plasma from two of the three tumor-bearing animals (88049 and 15445) was obtained at the time of death for assay for replication-competent virus. Infectious viral particles in titers of 10^4 or 10^5, respectively, was detected using serial dilutions of sera in the S^+L^- assay (Table 2). In contrast, sera obtained 5–24 mo after transplantation from the seven animals that failed to develop a tumor did not contain detectable replication-competent virus as determined in this assay. Serum was assayed for antibodies reactive with the MoMuLV core protein, p30,

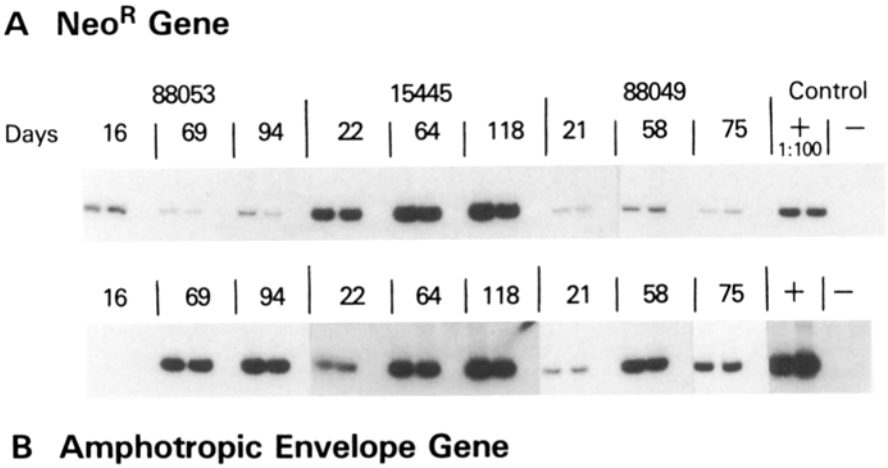


Figure 1. Detection of the vector and amphotropic envelope sequences in circulating blood cells from three transplant recipients. (*A*) Primers specific for the vector genome (19) were used on template DNA prepared from circulating blood cells at various intervals after transplantation. The positive control was a 1 to 100 dilution ($\mu g/\mu g$) of DNA from a producer line known to contain one copy of the vector genome with DNA from circulating cells of a control monkey. The DNA from this monkey also served as the negative control. (*B*) Primers specific for the amphotropic envelope sequences (21) were used with the same set of DNA samples as template. In this case, the positive DNA, used undiluted, was derived from the pA317 packaging cell line that contains 3–5 copies of the helper genome. The negative control DNA was derived from circulating cells of a nontransplanted recipient monkey.

Table 2. *Replication-Competent Virus in Transplant Recipients*

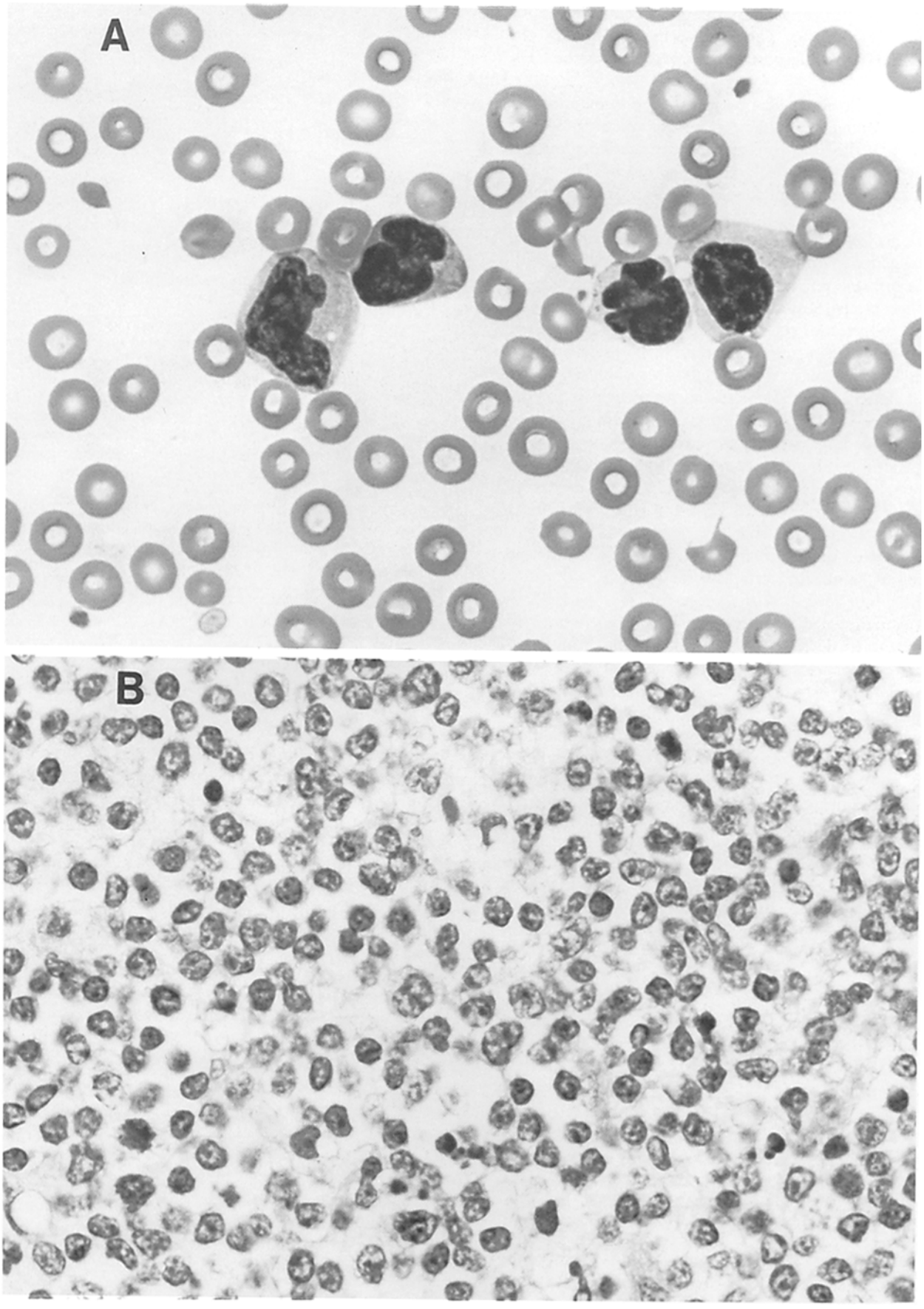
Animal	Estimated maximal percent of PBLs containing coding sequences*		Serum Abs to viral p30	Replication-competent virus in serum‡	Clinical status
	Vector	Amphotropic envelope			
		%			
CD34 Selected cells					
88053	1	100	ND	ND	Lymphoma, died (Day 200)
99049	1	100	–	10^4/ml	Lymphoma, died (Day 182)
15445	10	100	–	10^5	Lymphoma, died (Day 206)
15186	0	0.5	+	$<10^1$	Alive (Day 458)
15326	0	0.2	+	$<10^1$	Alive (Day 385)
15222	1.0	0.2	+	$<10^1$	Alive (Day 349)
15365	1.0	0	+	$<10^1$	Alive (Day 329)
15160	0	0.1	+	$<10^1$	Alive (Day 262)
Unfractionated Mononuclear Cells					
95A	2	<1	+	ND	Alive (Day 790)
1474	0.5	ND	+	ND	Alive (Day 790)

* These estimates are based on signal intensity of the PCR product compared to appropriately diluted controls.
‡ Detected in serial dilutions of serum by S^+L^- assay.

by Western blot methodology. Antibody was detected in the sera of the seven animals that remained free of disease, but was not detectable in the two animals with lymphoma (Table 2).

Characteristics of the Lymphoid Neoplasm. Two animals died 182 and 200 d, posttransplantation. At autopsy, each was found to have diffuse lymphadenopathy, a large thymic mass and hepatosplenomegaly. Pleural and pericardial effusions were present. One animal (88049) had severe pulmonary atelectasis due to pleural effusions, whereas the other (88053) died of a spontaneous splenic rupture. A third animal (15445) exhibited a progressive increase in WBC count over a period of 6 wk to more than 60,000/mm^3, most of which were lymphoblasts (Fig. 2 *A*) accompanied by progressive anemia (Hb = 5.9 gm/dl) and thrombocytopenia (28,000/mm^3). A chest x ray revealed an enlarged thymic shadow. The animal was euthanized on day 206 posttransplantation. Autopsy revealed pleural effusions and ascites, diffuse lymphadenopathy, thymic enlargement, hepatosplenomegaly, and tumor nodules in the kidney and liver. Histopathological study of all involved organs in each animal revealed a diffuse, high grade lymphoid neoplasm consistent with lymphoblastic lymphoma (Fig. 2 *B*). Immunophenotypic analysis of frozen sections prepared from both an enlarged lymph node and the thymic mass revealed a T cell phenotype (data not shown) with positive staining for the T cell–associated antigens Bf1, CD2, and CD8, but not for CD4. There was no staining for the B cell–associated antigens CD19 and CD20, nor was there staining for the myeloid associated antigen CD14. In addition, flow cytometric analysis of cell suspensions prepared from the lymph

Figure 2. Morphology of the neoplastic cells. (*A*) Peripheral blood from animal 15445, obtained 198 days posttransplantation and stained with the Wright-Giesma technique. (*B*) A section of thymus from animal 15445 stained with hematoxylin and eosin. ×400.


A
B

node and thymus of the same animal produced identical immunophenotypic results. The tumor cells were found to be CD2- (Fig. 3) and CD8-positive, but negative for the CD4 antigen.

Cytogenetic analysis was performed on spontaneously dividing cells from pleural fluid. An isochromosome 8 resulting from fusion of the long arms at the centromeric region was present in 3 of 12 spreads analyzed, one spread was tetraploid, and four other spreads had abnormalities on chromosome 3 or 4.

Lymphoma Cells Contain and Express an Amphotropic Proviral Genome and Produce Replication-Competent Virus. A PCR analysis using primers specific for the amphotropic envelope gene, verified the presence of its coding sequences in all tissues infiltrated with tumor cells from the three animals (Fig. 4). Ecotropic envelope sequences, known to be present in the producer line (21), were not detected in tumor cell DNA (data not shown). These same tissues were negative for the vector genome as reflected by analysis using primers specific for the neo^R coding and adjacent envelope sequences (Fig. 4). DNA samples from the tumor-infiltrated tissues of the three animals were also negative for STLV-type I sequences as shown by PCR analysis with STLV-I specific primers (data not shown). Primers specific for globin gene sequences provided a control to verify that each DNA sample was capable of serving as an effective template for PCR.

Southern blot analysis with a probe for envelope gene sequences demonstrated a proviral genome in DNA from all tissues examined with a copy number ranging from 10 to more than 50 as estimated by densitometric analysis (Fig. 5). Insertion site analysis using an enzyme that yields a junction fragment demarcated by a site in the proviral genome and a second in adjacent DNA gave a complex pattern with differences among the DNA samples from the three animals (Fig. 5). In contrast, the proviral genome copy number was similar among DNA samples obtained from three different diffusely infiltrated tissues of the same animal, and insertion site analysis gave a common pattern of bands for the three samples.

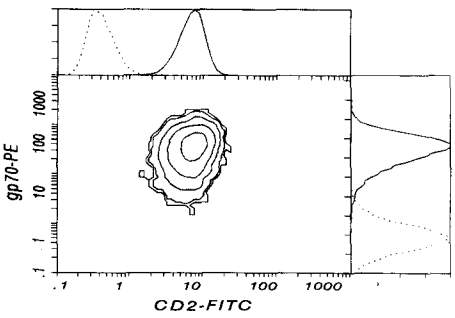


Figure 3. Expression of the retroviral envelope protein on the surface of neoplastic CD2 positive cells. A single cell suspension was prepared from the thymus of animal 15445 at the time of killing. The antibodies and isotope controls used are described in Materials and Methods. Isotope control (- - - - -). Antienvelope or CD2 antibody (———). The two-dimensional bitmap gives the data obtained with the two specific antibodies and indicates that more than 99% of cells were positive with both antibodies.

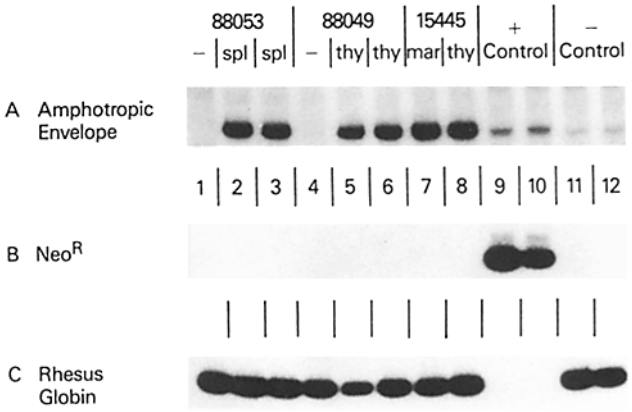


Figure 4. Detection of the amphotropic envelope gene sequences in tumor cell DNA and the absence of the vector genome in tumor cells. (Lane *1*) DNA extracted concurrently from circulating cells of a control monkey; (lanes *2* and *3*) duplicate DNA samples extracted from spleen cells of animal 88053; (lane *4*) DNA extracted concurrently from circulating cells of a control monkey; lanes *4* and *5* duplicate DNA samples extracted from the thymus of animal 88049; and (lanes *7* and *8*) DNA from marrow and thymus, respectively, from animal 15445. (*A*) Analysis with primers specific for the amphotropic envelope gene sequences (21). The positive control DNA (lanes *9* and *10*) was extracted from pA317 cells whereas the negative control DNA (lanes *11* and *12*) was recovered from mouse 3T3 cells. (*B*) Analysis with primers specific for the vector genome (21). The positive control was DNA extracted from the cell line N763 known to contain one copy of the proviral genome, whereas the negative control DNA (lanes *11* and *12*) was extracted from circulating cells of a nontransplanted recipient. (*C*) Analysis with primers specific for the rhesus γ-globin gene sequences: 5′ primer = 5′ GTTGGGAGTGAAGAAACTGC 3′ and 3′ primer = 5′ TAGCCTCAGACTCTGTTTGG 3′. The set of DNA samples analyzed were identical to those utilized in *B*.

A suspension of thymus cells from animal 15445 stained brightly and uniformly with an antibody specific for murine leukemia retroviral gp70 envelope protein (Fig. 3). These cells and the pleural fluid cells were cultured in vitro in RPMI containing 15% FCS and 5 U/ml of IL-1β (Cetus Corp., Berkeley, CA), 10 Units/ml of IL-2 (Genzyme Corp., Boston, MA) or a combination of both growth factors. Cells from each source continued to divide for ~2 wk. Conditioned medium harvested from each cell population after 48 h in vitro was found to contain replication-competent virus in the S^+L^- assay on PG4 cells at a titer of $\sim 10^4$/ml.

Discussion

The apparent pathogenesis of the neoplastic process in these primates resembled that resulting from MoMuLV infection of rodent species (6–8). Replication-competent virus infection was apparently established during the in vitro infection of immunoselected CD34$^+$ cells with subsequent amplification of virus within hematopoietic tissues. Expression of the amphotropic viral envelope protein was documented in neoplastic cells and seems likely to have been present in preneoplastic thymus cells as well. The genome of the monoclonal tumor cells was riddled with exogenous provirus providing multiple opportunities for insertional activation or inactiva-

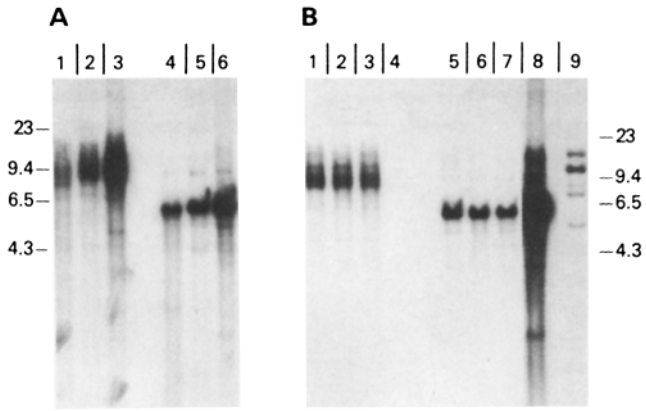


Figure 5. Southern blot analysis of DNA extracted from tumor tissues of the three animals having lymphoma. (*A*) (Lanes *1* and *4*) DNA from spleen of animal 88053; (lanes *2* and *5*) DNA from the thymus of animal 88049; and (lanes *3* and *6*) DNA from the thymus of animal 15445. Digestion was with PstI for the DNA in lanes *1–3* and with SstI in lanes *4–6*. PstI cuts toward the 5′ end of the amphotropic 4070 genome. This is the replication-competent virus likely to arise by recombination in the producer cell line (11, 14). The probe fragment containing envelope sequences detects a junction fragment containing proviral and genomic DNA sequences. SstI cuts in the LTRs of the predicted replication-competent viral genome and once internally yielding a 5.7-kb proviral fragment. (*B*) Analysis of DNA from three tissues of animal 15445. (Lanes *1* and *5*) bone marrow; (lanes *2* and *6*) thymus; and (lanes *3* and 7) lymph node. Lane *4* contains DNA from circulating cells of a control rhesus monkey. Lanes *1–7* were derived from a radioautograph exposed for 8 h whereas lanes *8* and *9* were derived from a radioautograph exposed for 48 h. Lane *8* contains the same DNA present in lane 7 whereas the DNA in lane *9* is derived from the pA317 packaging cell line. The DNA in lanes *1–4* was digested with PstI, and that in lanes *5–9* with SstI. The probe used was a ClaI fragment (4987–7645) from the envelope region of the helper genome (11).

tion of genes involved in the regulation of cell growth and regulation.

Establishment of a chronic replication-competent virus infection and the evolution of an aggressive neoplastic process was not predicted based on previous work in rhesus monkeys (4, 16, 17). However, there are at least two important differences between our protocol and those employed in the prior safety testing of the amphotropic helper virus. Total body irradiation in the doses we used to ablate bone marrow causes much more severe immunosuppression than the combination of prednisone and cyclosporin (38). Furthermore, the monkeys used in the prior safety studies had intact bone marrow and lymphoid function whereas the animals in our studies were recipients of autologous grafts from which the T cells had been removed. T cell depletion from marrow grafts is known to delay restoration of T cell–dependent immune functions in transplant recipients (39). We surmise that severe immunosuppression was important in disease pathogenesis.

Early amplification of a replication-competent viral infection after engraftment distinguished the three animals that developed lymphoma from the seven that did not, but no single experimental parameter can be implicated in the establishment and evolution of this infection. Neither the agent used for pretreatment (5-FU or SCF) nor the exact conditions of in vitro culture of the CD34 selected cells seemed relevant since these variables were different among the three animals in which neoplasms developed (Table 1). Rather, each of the seven animals that controlled the viral infection had detectable serum antibodies to a viral protein, whereas two animals with lymphoma that were studied apparently failed to mount a humoral immune response to this antigen.

In mice infected with MoMuLV, recombination between an endogenous retroviral genome and the MoMuLV leads to the emergence of a MCF virus that induces transformed foci of mink cells in vitro (6–8). The mixtures of replication-competent virus present in mice with lymphoma have a characteristic dual-tropic specificity. The xenotropic envelope protein is thought to have a role in the early pathogenesis of the neoplastic process, perhaps by heightening sensitivity of T cells to IL-2 (9). In contrast, MoMuLV-induced disease in rats is not accompanied by the emergence of a virus having unique host cell specificity. MoMuLV appears to be sufficient to cause disease in this species (6). The situation in the rhesus monkeys with lymphoma has not yet been resolved. There are at least two potential sources of a unique virus distinct from the replication-competent amphotropic virus that was demonstrable in medium derived from the producer clone (21). Recombination between this replication-competent virus and another proviral genome in the murine fibroblasts in vitro could have resulted in the emergence of a mixed population of virus from the producer clone. Conversely, productive infection in vivo by the original amphotropic replication-competent virus from the producer cell line could conceivably have mobilized a primate endogenous proviral genome (40) by recombination. Either mechanism could have led to emergence of complement-resistant virus with enhanced survival in rhesus plasma. Future studies will evaluate these possibilities by characterization of the replication-competent virus recovered from these animals.

In neoplasms induced by MoMuLV in rodents, several genes may be activated by proviral insertion. These include the *myc*, N-*myc*, *myb*, Ha-*ras*, LcK, and Ets-1 genes and several other less well characterized loci, designated M1vi 1–4 (4, 6, 8). Various combinations of two or more mutations are thought to be required for the emergence of a neoplastic process. In any specific tumor, such a combination of mutations is thought to arise sequentially leading to stepwise evolution of the neoplastic phenotype. The primate loci homologous to those characteristically disrupted in rodents by MoMuLV will be examined in future studies for evidence of proviral insertion.

Our work was undertaken to optimize conditions for retroviral mediated gene insertion into repopulating hematopoietic stem cells. The retroviral vector genome was identified in circulating blood and/or bone marrow cells in seven animals from the time of reconstitution and continued to be present in 0.1–10% of circulating cells throughout the period of observation (Table 2). In contrast, the replication-competent virus infection was progressive with a gradual increase in the proviral genome copy number in circulating cells (Fig. 1). We infer that insertion of the vector and replication-competent viral genomes were independent events. Supporting

this interpretation is the fact that the cells of one animal (15365), contained the vector but not the amphotropic envelope gene sequences (Table 2), and that the tumors contained the amphotropic envelope gene but not the vector genome (Fig. 4). Nonetheless, the ability of replication-competent helper virus to establish infection in these animals created the possibility for mobilization of the vector genome and its spread throughout hematopoietic tissues after bone marrow reconstitution. This concern is particularly relevant to the one animal in which about 10% of cells contained the vector genome (15445) since replication-competent virus appeared to have amplified very early after transplantation in this animal (Fig. 1). Therefore, we cannot unequivocally interpret our studies with respect to the use of these conditions for facilitating gene insertion into repopulating stem cells. Recent work in the canine model has yielded data documenting retroviral mediated gene insertion into cells capable of giving rise to multiple lineages (41, 42). Indeed, recent studies support coculture with autologous stroma to facilitate retroviral mediated gene transfer into primitive hematopoietic cells (43).

Our observations underscore the importance of avoiding replication-competent virus contamination in all retroviral vectors used for human gene transfer studies. Undoubtedly, the normal immune system provides a potent barrier to murine-type replication-competent viral infection, but many patients who are candidates for gene transfer protocols have various degrees of immune dysfunction due to intrinsic disease or because of having received chemoradiotherapy. An intrinsic barrier within the host to replication-competent virus–induced disease is not likely to be reliable in such patients. Fortunately, the modifications introduced into the helper and vector genomes in second and third generation packaging lines have rendered generation of replication-competent virus by recombination statistically improbable and practically undetectable (1–4, 11–14). Assiduous monitoring of clinical grade vector preparations for replication-competent virus adds another margin of safety. We conclude that our observations should not detract from future use of retroviral vectors for human gene therapy.

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References

1. Miller, A.D. 1990. Retrovirus packaging cells. *Hum. Gene. Ther.* 1:4.
2. Miller, A.D., and G.J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. *Biotechniques.* 7:980.
3. Linial, M.L., and A.D. Miller. 1990. Retroviral RNA packaging: sequence requirements and implications. *Curr. Top. Microbiol. Immunol.* 157:125.
4. Cornetta, K., R.A. Morgan, and W.F. Anderson. 1991. Safety issues related to retroviral-mediated gene transfer in humans. *Hum. Gene Ther.* 2:5.
5. Culver, K.W., W.F. Anderson, and R.M. Blaese. 1991. Lymphocyte gene therapy. *Hum. Gene Ther.* 2:107.
6. Tsichlis, P.N., and P.A. Lazo. 1991. Virus-host interactions and the pathogenesis of murine and human oncogene retroviruses. *Curr. Top. Microbiol. Immunol.* 171:95.
7. Famulari, N.G. 1983. Murine leukemia viruses with recombinant env genes: a discussion of their role in leukemogenesis. *Curr. Top. Microbiol. Immunol.* 103:75.
8. Tsichlis, P.N. 1987. Oncogenesis by moloney murine leukemia virus. *Anticancer Res.* 7:171.
9. Tsichlis, P.N., and S.E. Bear. 1991. Infection by mink cell focus-forming viruses confers interleukin 2 (IL-2) independence to an IL-2-dependent rat T-cell lymphoma line. *Proc. Natl. Acad. Sci. USA.* 88:4611.
10. Mann, R., R.C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell.* 33:153.
11. Miller, A.D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell Biol.* 6:2895.
12. Danos, O., and R.C. Mulligan. 1988. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc. Natl. Acad. Sci. USA.* 85:6460.
13. Markowitz, D., S. Goff, and A. Bank. 1988. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.* 62:1120.
14. Muenchau, D.D., S.M. Freeman, K. Cornetta, J.A. Zweibel, and W.F. Anderson. 1990. Analysis of retroviral packaging lines for generation of replication-competent virus. *Virology.* 176:262.
15. Markowitz, D., S. Goff, and A. Bank. 1988. Construction and use of a safe and efficient amphotropic packaging cell line. *Virology.* 167:400.
16. Cornetta, K., R.C. Moen, K. Culver, R.A. Morgan, J.R. McLachlin, S. Sturm, J. Selegue, W. London, R.M. Blaese,

and W.F. Anderson. 1990. Amphotropic murine leukemia retrovirus is not an acute pathogen for primates. *Hum. Gene Ther.* 1:15.
17. Cornetta, K., R.A. Morgan, A. Gillio, S. Sturm, L. Baltrucki, R. O'Reilly, and W.F. Anderson. 1991. No retroviremia or pathology in long-term follow-up monkeys exposed to a murine amphotropic retrovirus. *Hum. Gene Ther.* 2:215.
18. Nienhuis, A.W., K.T. McDonagh, and D.M. Bodine. 1991. Gene transfer into hematopoietic stem cells. *Cancer.* 67:2700.
19. Karlsson, S. 1991. Treatment of genetic defects in hematopoietic cell function by gene transfer. *Blood.* 78:2481.
20. Kantoff, P.W., A.P. Gillo, J.R. McLachlin, C. Bordignon, M.A. Eglitis, N.A. Kernan, R.C. Moen, D.B. Kohn, S.F. Yu, E. Karson et al. 1987. Expression of human adenosine deaminase in nonhuman primates after retrovirus-mediated gene transfer. *J. Exp. Med.* 166:219.
21. Bodine, D.M., K.T. McDonagh, S.J. Brandt, P.A. Ney, B. Agricola, E. Byrne, and A.W. Nienhuis. 1990. Development of high-titer retrovirus producer cell line capable of gene transfer into rhesus monkey hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA.* 87:3738.
22. Nienhuis, A.W., R.E. Donahue, S. Karlsson, S.C. Clark, B. Agricola, N. Antinoff, J.E. Pierce, P. Turner, and W.F. Anderson. 1987. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) shortens the period of neutropenia after autologous bone marrow transplantation in a primate model. *J. Clin. Invest.* 80:573.
23. Weider, R., K. Cornetta, S.W. Kessler, and W.F. Anderson. 1991. Increased efficiency of retroviral-mediated gene transfer and expression in primate bone marrow progenitors after 5-fluorocil-induced hematopoietic suppression and recovery. *Blood.* 77:448.
24. Martin, F.H., S.V. Suggs, K.E. Langley, H.S. Lu, J. Ting, K.H. Okino, C.F. Morris, I.K. McNiece, F.W. Jacobsen, E.A. Mendiaz et al. 1990. Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell.* 63:203.
25. Demetri, G.D., and J.D. Griffin. 1991. Granulocyte colony-stimulating factor and its receptor. *Blood.* 78:2791.
26. Gasson, J.C. 1991. Molecular physiology of granulocyte-macrophage colony-stimulating factor. *Blood.* 77:1131.
27. Kirshenbaum, A.S., J.P. Goff, S.W. Kessler, J.M. Mican, K.M. Zsebo, and D.D. Metcalfe. 1992. Effect of IL-3 and stem cell factor on the appearance of human basophils and mast cells from $CD34^+$ pluripotent progenitor cells. *J. Immunol.* 148:772.
28. Kirshenbaum, A.S., S.W. Kessler, J.P. Goff, and D.D. Metcalfe. 1991. Demonstration of the origin of human mast cells from $CD34^+$ bone marrow progenitor cells. *J. Immunol.* 146:1410.
29. Krantz, S.B. 1991. Erythropoietin. *Blood.* 77:419.
30. Kishimoto, T. 1989. The biology of interleukin-6. *Blood.* 74:1.
31. Yang, Y.C., A.B. Ciarletta, P.A. Temple, M.P. Chung, S. Kovacic, J.S. Witek-Giannotti, A.C. Leary, R. Driz, R.E. Donahue, G.G. Wong, and S.C. Clark. 1986. Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell.* 47:3.
32. Evans, L.H., R.P. Morrison, F.G. Malik, J. Portis, and W.J. Britt. 1990. A neutralizable epitope common to the envelope glycoproteins of ecotropic, polytropic, xenotropic, and amphotropic murine leukemia viruses. *J. Virol.* 64:6176.
33. Kadan, M.J., S. Sturm, W.F. Anderson, and M.A. Eglitis. 1992. Detection of receptor-specific murine leukemia virus binding to cells by immunofluorescence analysis. *J. Virol.* 66:2281.
34. Colligan, J.E., A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. Current Protocols in Immunology. 1991. John Wiley & Sons, Inc. New York.
35. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1971. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
36. Bassin, R.H., N. Tuttle, and P.J. Fischinger. 1971. Rapid cell culture assay technic for murine leukaemia viruses. *Nature (Lond.).* 229:564.
37. Dezzutti, C.S., A. Lazo, J.Y. Yee, J.R. Blakeslee, L.E. Mathes, B.G. Brown, and M.D. Lairmore. 1992. Detection of simian T-lymphotropic virus type I using the polymerase chain reaction. *Int. J. Cancer.* 50:805.
38. Job, G., M. Pfreundschuh, M. Bauer, K. zum Winkel, and W. Hunstein. 1984. The influence of radiation therapy on T-lymphocyte subpopulations defined by monoclonal antibodies. *Int. J. Radiat. Oncol. Biol. Phys.* 10:2077.
39. Daley, J.P., M.K. Rozans, B.R. Smith, S.J. Burkoff, J.M. Rappeport, and R.A. Miller. 1987. Retarded recovery of functional T cell frequencies in T cell-depleted bone marrow transplant recipients. *Blood.* 70:960.
40. Shih, A., E.E. Coutavas, and M.G. Rush. 1991. Evolutionary implications of primate endogenous retroviruses. *Virology.* 182:495.
41. Schuening, F.G., K. Kawahara, A.D. Miller, R. To, S. Goehle, D. Stewart, K. Mullally, L. Fisher, T.C. Graham, F.R. Appelbaum, et al. 1991. Retrovirus-mediated gene transduction into long-term repopulating marrow cells of dogs. *Blood.* 7:2568.
42. Carter, R.F., A.C.G. Abrams-Ogg, J.E. Dick, S.A. Kruth, V.E. Valli, S. Kamel-Reid, and I.D. Dube. 1992. Autologous transplantation of canine long-term marrow culture cells genetically marked by retroviral vectors. *Blood.* 79:356.
43. Moore, K.A., A.B. Deisseroth, C.L. Reading, D.E. Williams, and J.W. Belmont. 1992. Stromal support enhances cell-free retroviral transduction of human bone marrow long-term culture-initiating cells. *Blood.* 79:1393.