Expression of Human Adenosine Deaminase in Mice Transplanted with Hemopoietic Stem Cells Infected with Amphotropic Retroviruses

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Summary

Amphotropic recombinant retroviruses were generated carrying sequences encoding human adenosine deaminase (ADA). Transcription of the human ADA gene was under control of a hybrid long terminal repeat in which the enhancer from the Molony murine leukemia virus was replaced by an enhancer from the F101 host-range mutant of polyoma virus. Hemopoietic stem cells in murine bone marrow were infected with this virus under defined culture conditions. As a result, 59% of day-12 colony forming unit spleen (CFU-S) stem cells became infected without any in vitro selection. Infected CFU-S were shown to express human ADA before transplantation and this expression sustained upon in vivo maturation. Mice transplanted with infected bone marrow exhibited human ADA expression in lymphoid, myeloid, and erythroid cell types. Moreover, human ADA expression persisted in secondary and tertiary transplanted recipients showing that human ADA-expressing cells were derived from pluripotent stem cells. These characteristics of our amphotropic viruses make them promising tools in gene therapy protocols for the treatment of severe combined immunodeficiency caused by ADA deficiency. In this respect it is also relevant that the viral vector that served as backbone for the ADA vector was previously shown to be nonleukemogenic.

Deficiency of adenosine deaminase (ADA;[1] adenosine aminohydrolase, EC 3.5.4.4) activity is associated with an autosomally inherited form of severe combined immunodeficiency (ADA$^-$SCID) disease (1, 2). It has been suggested that this form of SCID is caused by a defect in T and B cell differentiation due to the accumulation of adenine nucleosides as the result of the absence of functional ADA (2). The cloning of sequences encoding human ADA (hADA) (3–5) and the recessive nature of the disease allowed studies aimed at the development of gene therapy protocols for ADA$^-$SCID patients (6–9). The objective of such a gene therapy protocol would be to repopulate the lymphoid blood cell compartment of the patients by introducing a functional ADA gene into their hemopoietic stem cells (HSC).

To date, successful transfer of foreign genes into pluripotent hemopoietic stem cells (PHSC) has been performed using recombinant ecotropic retroviruses with a host range limited to mice (e.g., 10). Studies with amphotropic viruses, which can also infect human cells, are few (7) and did not provide evidence for gene transfer into PHSC. Another limitation of previous studies has been that several vectors appeared to be incapable of directing sustained expression in hemopoietic cells in vivo. It has been reported, for example, that a number of vectors that are active in mature hemopoietic cells are not expressed in blood cells of animals transplanted with infected hemopoietic stem cells (6, 7, 11). Likewise, a great number of vectors are transcriptionally inactive in undifferentiated embryonal carcinoma (EC) cells, and it has been suggested that an analogy exists in the mechanisms responsible for the expression block in EC cells and in hemopoietic cells in vivo (7, 12, 13). To overcome this repression phenomenon in the hemopoietic system, most investigators introduced additional promoters within the viral transcription unit (8, 9, 12, 14, 15). Although this has led to some progress in the expression patterns, the outcome of such alterations seems unpredictable, which is witnessed by the fact that a number of strong promoters that act constitutively in various cell types were inactivated when introduced into HSC as part of retrovirus vectors (6, 9, 11, 16).

[1] *Abbreviations used in this paper:* ADA, adenosine deaminase; BMC, bone marrow cells; CFU-S, CFU-spleen; dCF, deoxycoformycin; EC, embryonal carcinoma; hADA, human ADA; HSC, hemopoietic stem cells; LTR, long terminal repeat; Mo-MuLV, Moloney murine leukemia virus; PHSC, pluripotent HSC; Xyl-A, xylofuranosyl-adenine.

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Since the enhancer sequences in the long terminal repeat (LTR) of retroviruses are known to be responsible for expression specificity (17), our efforts to obtain expression in hemopoietic cells in vivo have been directed towards alterations of this element. We have recently described (13) the construction of a recombinant retrovirus in which a marker gene was placed under the transcriptional control of a hybrid LTR designated ΔMo+PyF101 (18). In this construct the enhancer from Moloney murine leukemia virus (Mo-MuLV) was replaced by the enhancer of a mutant polyoma virus (PyF101) that was selected to grow in EC cells. The vector exhibited useful expression patterns since it could direct gene expression in EC cells as well as in HSC. Moreover, upon transplantation of infected bone marrow cells (BMC) into lethally irradiated mice, virus expression was sustained in hemopoietic cells of the engrafted recipients (13). Its characteristics render the ΔMo+PyF101-LTR quite useful for the design of vectors to be employed in future gene therapy protocols. An additional advantage of such vectors would be that (in contrast to Mo-MuLV based vectors) they are derived from a nonpathogenic retrovirus, since Davis et al. (19) have shown that the leukemogenic potential of Mo-MuLV is abolished when its LTR is replaced by the ΔMo+PyF101-LTR. We have therefore set out to construct vectors in which sequences encoding human ADA are under the transcriptional control of the ΔMo+PyF101-LTR. This paper describes the construction of such a virus and its capacity to efficiently infect and express in cell types relevant for future gene therapy purposes. In this respect it is of importance that the virus used in these studies has an amphotropic host range, which permits infection of human cells as well.

Materials and Methods

Vector Construction. The pLgAL(ΔMo+PyF101) vector carries a 1.9-kb SstII (blunt)-ClaI ADA cDNA containing fragment (1.4-kb SstII fragment from pAMG1 [20] followed by 478 bp of Mo-MuLV sequences from a HpaI to a ClaI site encompassing nucleotides 7198 to 7676 [21]) located 3′ from Mo-MuLV sequences stretching from the 5′ LTR and including the first 420 nt of the *gag* gene (an NcoI (blunt)-EcoRI (blunt) fragment derived from pN2 [22], a kind gift from R. Hoeben, Leiden University). Downstream from the ADA sequences the vector carries the ΔMo+PyF101-LTR (a ClaI-KpnI fragment from ΔpMLV-C/R/B(+PyF101) [18]) followed by cellular DNA originally isolated as sequences flanking a provirus (a KpnI-EcoRI fragment from pZIPneoSV(X)1 [23]). For propagation in *Escherichia coli* the retrovirus sequences were cloned into pBR322 (EcoRI-SalI (blunt)).

Virus Production and Cell Culture. NIH/3T3, ψCRE and ψCRIP (24; kindly supplied by the authors) cell lines were routinely maintained in high glucose (4.5 g/l) α-modified essential medium, supplemented with 10% heat-inactivated FCS (Seromed, Berlin, FRG), 100 μg/ml streptomycin (Gist-Brocades, Delft, The Netherlands), and 100 U/ml penicillin (Gist-Brocades).

Recombinant virus-producing cell lines were generated by transfection of 20 μg of plasmid DNA onto ψCRE cells using the calcium-phosphate technique (25) as modified by Chen and Okayama (26). 48 h later the cells were selected for ADA overexpression by splitting them into medium containing 4 μM xylofuranosyl-adenine (Xyl-A) and 10 nM of the specific ADA-inhibitor deoxycoformycin (dCF) (27), kind gifts from Drs. N.R. Lomax (Drug Synthesis and Chemistry Branch, NCI, Bethesda, MD) and R.E. Kellems (Baylor College of Medicine, Houston, Texas), respectively. After 10 d of selection resistant clones were pooled and expanded. Filtered (0.45 μm pore size) supernatant culture medium from these cells was used to infect ψCRIP cells. Upon Xyl-A/dCF selection, individual virus-producing clones were isolated and expanded.

Amphotropic virus-producing cell lines were initially assayed for virus production by infecting NIH/3T3 cells that had been plated 10^5 cells/60-mm dish in medium containing 4 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO) 24 h before infection. After 2 h of infection with 2 ml of culture supernatant from the producer cells, fresh culture medium was added and the cells were allowed to grow for four more days. Zymogram analysis of these infected cells allowed semiquantitative evaluation of virus titers by comparing the intensity of the human and the endogenous murine ADA activities. Titers were determined quantitatively on the basis of Xyl-A/dCF resistance of infected NIH/3T3 cells. NIH/3T3 cells plated as before were infected for 2 h with serial diluted virus supernatant. After 24 h the cells were reseeded 1:40 in medium containing 4 μM Xyl-A and 1 nM dCF. Upon 10 d of selection resistant colonies were scored.

The presence of replication-competent virus was tested by marker rescue. NIH/3T3 cells infected with ZIPneoSV(X)1 virus (23) were seeded 10^5 cells/60-mm dish in medium containing 4 μg/ml polybrene. 24 h later these cells were infected with cell-culture supernatant or murine serum. Fresh culture medium was added after 2 h and the cells were passaged for 7 d without any selection. The culture medium was refreshed and supernatant was harvested 2 h later. The filtered supernatant was used to infect NIH/3T3 cells. 24 h later the medium was replaced by medium containing 1 mg/ml G418 (Geneticin; Gibco, Paisley, Scotland). After 10 d of selection resistant colonies were scored. The sensitivity of this assay was determined by mixing the virus supernatant with known concentrations of amphotropic replication-competent 4070A virus. From these experiments we deduced that the presence of one focus forming unit/ml, as determined by S^+L^- assay (28), could reproducibly be detected in this assay.

DNA and Enzyme Analyses. Hemopoietic cells were isolated from various tissues for the analysis of proviral integration and human ADA expression. Spleen colonies and total spleen or thymus samples were flash-frozen in liquid N_2 directly after dissection. Peripheral blood samples were taken by puncturing the orbital plexus. PBL were purified from these samples by erythrocyte lysis in 155 mM NH_4Cl, 12 mM $NaHCO_3$, 89 μM EDTA. B cells were obtained by dispersing splenic tissue through nylon mesh and subsequent stimulation with LPS for 4 d, upon which mononuclear cells were isolated by Ficoll (LSM; Organon Teknika, Durham, NC) density-gradient separation, resulting in a >99% pure B cell population (29). Splenic high density nucleated cells were obtained by Ficoll separation and subsequent erythrocyte lysis of pelleted cells. BMC were harvested from the femora and tibiae. Most samples were stored at −80°C until analysis.

High molecular weight DNA for Southern analysis was isolated as described previously (30). Genomic DNA (10 μg) was digested with the appropriate restriction enzymes and subjected to electrophoresis in a 0.6 or 0.7% agarose gel. The DNA was transferred to a membrane and hybridized with either the ^{32}P-labeled NcoI-EcoRI hADA-cDNA fragment from pAMG1 (20), or the XbaI Polyoma F101 enhancer-containing fragment from ΔpMLV-C/R/B(+PyF101) (18) according to standard procedures.

Isozyme-specific ADA activity was detected in lysates of in vitro cultured cells, murine hemopoietic tissues, dissected spleen colo-

nies, or blood samples by zymogram analysis as described by Meera Khan et al. (31).

Murine Bone Marrow Culture and Transplants. BMC were obtained by flushing the femora and tibiae of 7-wk-old BCBA (C57BL/KaLwRij × CBA/BrARij)F_1 mice. The bone marrow was enriched for hemopoietic stem cells on a metrizamide density gradient (sp.gr. <1.08 g/cm^3) (32). Using this procedure we routinely find 30–80% of day 12 CFU-S in the low density fraction. 10^5 low density BMC/ml were cocultivated for 72 h with a 70% confluent irradiated (20 Gy) monolayer of virus-producing cells in the standard medium described above under "Virus Production and Cell Culture," supplemented with human rIL-1α (Biogen, Geneve, Switzerland), murine rIL-3, and 0.4 μg/ml polybrene. When a preselection for ADA overexpression was to be performed, the cocultivation period was reduced to 48 h, during which the cells were pretreated with 20 nM dCF. Subsequently, the nonadherent cells were removed and cultured for an additional 24 h under serum-free conditions, either with or without the addition of 4 μM Xyl-A and various concentrations of dCF. The cells were intravenously injected into syngeneic 12–16-wk-old lethally irradiated (8.5 Gy) recipient mice. The equivalent of 2 × 10^4 and 10^5 unselected or 10^5 and 10^6 selected BMC was injected for the analysis of individual day 12 CFU-S–derived spleen colonies. For long-term expression studies mice were repopulated with the equivalent of 10^6 or 5 × 10^6 unselected BMC, including the adherent cell layer.

Results

Production of Recombinant hADA Viruses. In previous experiments we have successfully used the ΔMo+PyF101-LTR to overcome the expression block encountered by retroviruses in the hemopoietic system (13). Based on this experience we constructed the retroviral vector pLgAL(ΔMo+PyF101) (Fig. 1). This vector carries the human ADA cDNA downstream from a Mo-MuLV-LTR, whereas the ΔMo+PyF101-LTR was used as a 3′LTR. Due to the fact that U3 in the resulting retroviral genome will be derived from the 3′LTR, the alteration in the U3 region of the ΔMo+PyF101-LTR is expected to be present in both LTRs of the resulting recombinant retroviruses. In addition, a fragment from Mo-MuLV that stretches from the 5′LTR up to position 420 of the *gag* gene was included 5′ of the hADA cDNA gene.

ψCRE ecotropic packaging cells transfected with pLgAL(ΔMo+PyF101) were selected for ADA overexpression with Xyl-A and dCF (27). Zymogram analysis of the resistant cells revealed the presence of new isozymes with electrophoretic mobilities identical to those of the human ADA isozymes. Culture supernatant of these cells was used to infect ψCRIP amphotropic packaging cells. Upon selection with Xyl-A/dCF, individual resistant clones were expanded. Virus production from these clones was first tested by analyzing the presence of human ADA activity in NIH/3T3 cells infected with viral supernatant. We interpreted the relative intensity of human ADA activity as compared with the endogenous murine activity to be due to variations in the virus titers issuing from the ψCRIP clones. The clone showing the highest virus production by this criterium was designated POC-1 and further analyzed.

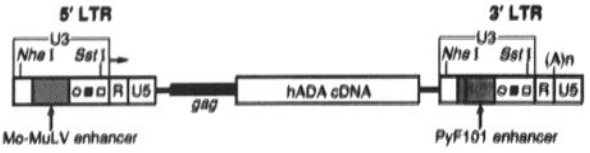

Figure 1. Structure of the retroviral vector pLgAL(ΔMo+PyF101). The open box represents the human ADA cDNA. The hatched and crossed boxes indicate the Mo-MuLV- and PyF101-enhancer sequences within the viral LTRs, respectively. The Mo-MuLV *gag*-sequences, as well as the positions of the TATA-box (*open circle*), the CAAT-box (*closed rectangle*), and G/C motif (*open rectangle*) in the viral promoter are indicated. The sites for transcription initiation (*arrow*), polyadenylation [(A)n], and relevant restriction enzymes are also shown.

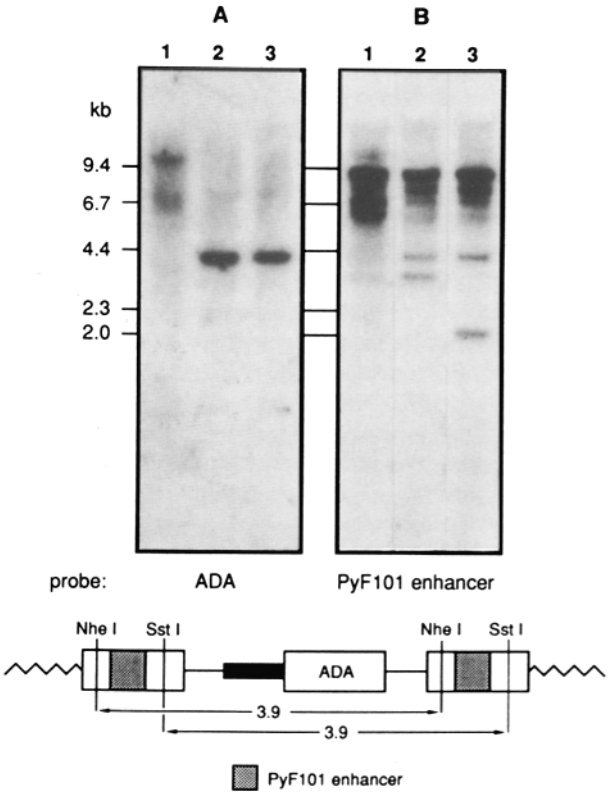

Figure 2. Southern analysis of NIH/3T3 clones infected with POC-1 virus. The structure of the LgAL(ΔMo+Py101) provirus in the genome of infected cells is shown in a schematic drawing. Genomic DNA (10 μg) from ψCRIP cells (lane *1*) and two independent NIH/3T3 clones infected with POC-1 virus (lanes *2* and *3*) was digested with SstI (*A*) or NheI (*B*) and fractionated on a 0.7% agarose gel. The Southern blots shown in *A* and *B* were hybridized with a hADA cDNA probe and a PyF101 enhancer probe, respectively. The positions of DNA molecular weight markers are indicated.

Since the provirus in the POC-1 cell line has undergone one round of replication we expected the U3 regions in both proviral LTRs to be derived from the 3′LTR of the shuttle vector. The provirus in POC-1 should therefore carry the PyF101 enhancer in both the 5′ and the 3′LTR. The same proviral structure is expected in cells that are infected with virus supernatant of POC-1. To test these predictions, DNA from two individual NIH/3T3 cell lines that were infected with POC-1 virus supernatant was subjected to Southern blot analysis. Genomic DNA was digested with restriction en-

zymes that cut once within the LTR at either side of the inserted PyF101-enhancer (NheI and SstI, respectively). When probed with the PyF101-enhancer (Fig. 2 *B*), the lanes containing NheI-digested DNA from both infected target lines revealed a fragment identical in size to the SstI-digested proviral fragment hybridizing to the ADA cDNA (Fig. 2 *A*). This proves that the PyF101-enhancer is indeed present in both LTRs. The additional fragments hybridizing to the PyF101-enhancer probe represent flanking genomic sequences.

A virus titer of the POC-1 supernatant was determined by selection of NIH/3T3 cells infected with it for Xyl-A/dCF resistance and was shown to be 4.4×10^3 CFU/ml. This rather low value is probably due to the fact that it relies on the functional overexpression of human over murine ADA. Such measurements usually result in titers lower than those obtained by means of dominant selectable markers (8). This notion is supported by our observation that the infection of 2×10^5 NIH/3T3 cells with 2 ml of virus supernatant resulted in a cell population exhibiting similar levels of human and murine ADA activity (not shown). Cell lines derived from individual infected cells always contained an amount of human ADA equal or less than that of the endogenous enzyme. This suggests that in our mass-infection experiments most NIH/3T3 cells became infected, which would require a multiplicity of infection that is ~20 times higher then the titer measured as Xyl-A/dCF^R CFU/ml. The absence of replication-competent viruses in the supernatant of the POC-1 cell line was demonstrated by a marker-rescue assay (see Materials and Methods). The amphotropic host range of our ADA virus was confirmed by successful restoration of the enzyme defect in a lymphoblastoid cell line derived from an ADA$^-$SCID patient upon cocultivation with POC-1 cells (not shown).

Integration and Expression of LgAL(ΔMo + PyF101) in Murine Hemopoietic Stem Cells and their Mature Progeny. We set out by studying integration and expression in the class of hemopoietic stem cells that can clonally grow out to form macroscopic colonies on the spleen 12 d after their injection into lethally irradiated recipients (day 12 CFU-S). To infect day 12 CFU-S with LgAL(ΔMo+PyF101) we cocultivated murine low-density bone marrow cells for 48 h with an irradiated monolayer of POC-1 cells. To study the relation between hADA expression in the infected CFU-S (before transplantation) and in their mature progeny present in day 12 spleen colonies, we adapted the Xyl-A/dCF selection procedure to select for ADA-overexpressing bone marrow cells. After cocultivation, the nonadherent cells were collected from the irradiated monolayer and cultured for another 24 h under serum-free conditions either with or without 4 μM Xyl-A and various concentrations of dCF. Lethally irradiated recipients were injected with these bone marrow cells and 12 d later the numbers of spleen colonies were scored. Spleen colonies were dissected and zymogram analysis revealed 7% of them to express the hADA gene when no preselection was performed.

As can be seen in Fig. 3, the preselection for ADA overexpression resulted in a [dCF]-dependent decrease in day 12 CFU-S survival, whereas the frequency of hADA-expressing

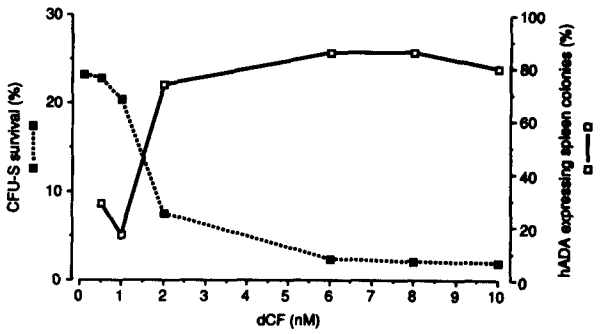

Figure 3. Day 12 CFU-S survival and human ADA expression in day 12 CFU-S–derived spleen colonies upon infection and preselection of murine bone marrow. The selection was performed for 24 h using a combination of 4 μM Xyl-A and 0.1, 0.5, 1.0, 2.0, 6.0, 8.0, or 10.0 nM dCF, respectively. The percentage day 12 CFU-S survival is given as compared with the CFU-S survival without preselection (i.e., bone marrow cultured under identical conditions without the addition of Xyl-A and dCF). The frequency of hADA-expressing spleen colonies was assessed by zymogram analysis on individually dissected spleen colonies. The percentage hADA-expressing day 12 spleen colonies at 4 μM Xyl-A + 0.1 nM dCF is not given since an insufficient number of individual spleen colonies could be analysed (at this stringency 0 of 8 colonies tested expressed hADA).

spleen colonies increased. At 2 nM dCF, the CFU-S survival (7.6%) closely resembled the infection efficiency (7%) and at this stringency the percentage of hADA-expressing spleen colonies rose to 73%. The procedure did not result in a 100% expression frequency at any of the stringencies used. We assume that this is brought about by a fraction of day 12 CFU-S that remained in G_0 during the period of infection and preselection, and consequently were resistent to retroviral infection and to the selection procedure.

We have also shown that this 24-h selection with Xyl-A/dCF could only be achieved in the absence of the virus-producing fibroblasts. This evidence came from an experiment in which bone marrow was cocultivated for 2 d with an irradiated NIH/3T3 cell line that did not produce any virus, but carried a copy of the LgAL(ΔMo+PyF101) virus. The Xyl-A/dCF selection was performed at 20 nM dCF, but this time in the presence of the fibroblast cells. Upon injection into irradiated recipients the number of day 12 spleen colonies was shown not to be reduced, and as expected, none of 25 spleen colonies tested expressed human ADA (data not shown). Apparently, the irradiated fibroblasts are able to detoxify the culture medium of Xyl-A.

The fact that ADA preselection resulted in a [dCF]-dependent increase in the number of hADA-expressing spleen colonies proves that the stem cells were overexpressing ADA before transplantation and subsequently sustained this expression upon in vivo differentiation into the more mature cell types present in day 12 spleen colonies.

Sustained Expression of LgAL(ΔMo + PyF101) in the Murine Hemopoietic System. To study the expression of human ADA in cells derived from infected hemopoietic cells with a more extensive self-renewing capacity than the day 12 CFU-S, mice were transplanted with 10^6 cocultured BMC to allow

a complete regeneration of the hemopoietic system. To study whether the Xyl-A/dCF selection procedure could also be applied to hemopoietic stem cells with regenerating capacity, a preselection at various stringencies was included in the procedure. 30 d after transplantation human ADA expression was analyzed in peripheral blood cells. All mice tested (23/23) expressed the human ADA gene. However, a clear [dCF]-dependent decrease in day 30 survival was observed. As can be seen in Table 1, all mice that received bone marrow that was preselected with 4 μM Xyl-A and 0.1 nM dCF survived the radiation. Strikingly, an increased selection stringency of 1 nM dCF resulted in the abolishment of radiation-protective capacity of the bone marrow, whereas the CFU-S-survival was not significantly affected. These data indicated to us that repopulating hemopoietic stem cells are extremely sensitive to the Xyl-A/dCF selection procedure. Complete radiation protection could not be obtained upon transplantation of bone marrow preselected at dCF concentrations above 0.1 nM and at this stringency no increase in the percentage of human ADA-expressing day 12 spleen colonies was observed (see Fig. 3). From this, we concluded that the applied preselection did not result in the survival of enough infected hemopoietic stem cells exhibiting radioprotective capacities.

In a further attempt to increase the efficiency of gene transfer we prolonged the cocultivation period from 48 to 72 h. Bone marrow was enriched for hemopoietic stem cells on a metrizamide density gradient (32) and low density cells representing 25% of the total marrow aspirate were cocultivated with POC-1 cells. The toxicity of the complete procedure was assessed by determining the recovery of day 12 CFU-S. Normal bone marrow contained 228 ± 12 SEM day 12 CFU-S per 10^6 cells. After stem cell enrichment and 72-h cocultivation, the recovery of day 12 CFU-S was 25% (56 ± 6 SEM/10^6 normal bone marrow equivalents). Zymogram analysis demonstrated that 59% of the day 12 spleen colonies expressed the human ADA gene (not shown), indicating that the 3 d of cocultivation resulted in a significantly higher infection efficiency than 2 d (7%). In vivo infection of CFU-S could be excluded by control experiments in which irradiated POC-1 virus-producing cells were mixed with normal bone marrow shortly before transplantation. The resulting spleen colonies always remained negative for hADA-expression (37 colonies tested).

10 mice transplanted with 10^6 cocultivated bone marrow cells were killed and analyzed for hADA expression 34 d after transplantation. In Fig. 4 the results are summarized. All mice expressed hADA in erythrocytes and spleen cells. In addition, five mice showed human ADA expression in PBL and/or thymus cells, proving that the infected hemopoietic stem cells gave rise to hADA-expressing cells of erythroid and lymphoid lineages. To study the self-renewing capacity of the stem cells responsible for the observed hADA expression pattern, bone

Table 1. *Stem Cell Parameters and hADA Expression after Cocultivation, ADA Preselection, and Transplantation of Murine Bone Marrow*

Selection stringency	Day 12 CFU-S*	30-d radiation protection‡	hADA expression in PBC§
No selection	123 ± 14	10/10	10/10
4 μM Xyl-A + 0.1 nM dCF	29 ± 6	8/8	8/8
4 μM Xyl-A + 0.5 nM dCF	28 ± 4	5/8	5/5
4 μM Xyl-A + 1.0 nM dCF	25 ± 8	0/7	

* Number of viable day 12 CFU-S present in 10^6 injected bone marrow cells, given as mean ± SEM.
‡ 30 d survival following 8.5 Gy irradiation and transplantation with the equivalent of 10^6 BMC is given as the number of surviving mice per total tested.
§ hADA expression was tested at day 30 in peripheral blood cells of all surviving animals and is given as the number of hADA-expressing mice per total tested.

mouse number	RBC	PBL	spleen	thymus
1	+	-	+	+
2	+	-	+	-
3	+	-	+	-
4	+	+	+	+
5	+	+	+	-
6	+	-	+	-
7	+	-	+	-
8	+	-	+	-
9	+	+	+	n.d.
10	+	+	+	n.d.

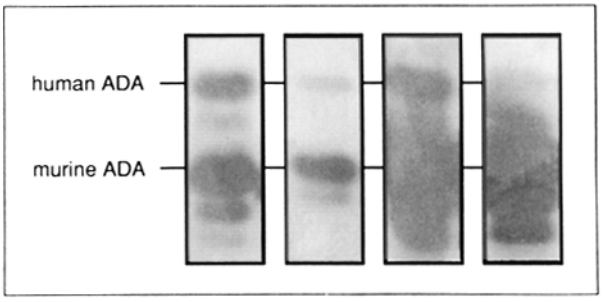

Figure 4. Human ADA expression in hemopoietic tissues of mice transplanted with LgAL(ΔMo+PyF101)-infected bone marrow. The top panel gives the results from zymogram analyses on RBC, PBL, spleen, and thymus from 10 mice killed 34 d after transplantation with 10^6 cocultured bone marrow cells. Examples of hADA$^+$ zymograms from each tissue are shown in the bottom panel. The positions of human and mouse ADA are indicated.

marrow was harvested from the four mice that showed hADA expression in PBL (mice 4, 5, 9, and 10) and transplanted into secondary irradiated recipients. These animals received either 10^5 or 5×10^6 BMC and were killed at day 15 or 34, respectively, for hADA expression analysis. At day 15, hADA expression was analyzed in peripheral RBC, bone marrow, and spleen. At day 34 PBL and thymus cells were included in the analyses, as well as splenic B cells, obtained by stimulation with LPS, and splenic high density nucleated cells of mainly myeloid origin. Some of the secondary recipients from mice 4, 5, and 9 exhibited hADA expression limited to the erythroid lineage. On the other hand, transplantation of bone marrow from mouse 10 into secondary recipients resulted in a persistence of hADA expression in lymphoid, myeloid, and erythroid lineages. Zymograms of organs from representative mice killed at day 15 and 34 are shown in Fig. 5. hADA expression was observed in all tissues examined, except in thymus cells. The latter could have been caused by the fact that thymus tissue has extremely high endogenous ADA levels, which hinders the detection of hADA in this organ. Not unexpectedly, the observed expression also persisted upon retransplantation into tertiary recipients (not shown).

Lineage-specific integration analysis was performed on DNA extracted from tissues from mouse 10 and its secondary recipients by digestion with EcoRI, which does not cut in the provirus, and Southern analysis. As can be concluded from Fig. 6, hADA-expressing hemopoietic tissues from all secondary recipient mice tested exhibit one major proviral integration site, indicating that the amphotropic hADA virus infected at least one pluripotent hemopoietic stem cell. In addition, our data provide evidence against in vivo reinfection due to the generation of replication-competent virus. The absence of replication-competent virus in the serum of all secondary transplanted animals was further confirmed via a marker-rescue assay, as described in Materials and Methods.

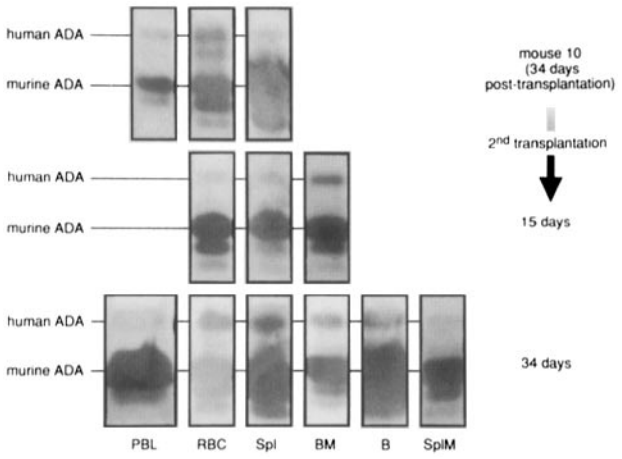

Figure 5. Zymograms of hemopoietic tissues from mouse 10 and two representative hADA-expressing animals killed at day 15 or 34. The positions of the human and murine isozymes are indicated. Spl, spleen; BM, bone marrow; B, splenic B cells; SplM, splenic high-density nucleated cells (myeloid).

Discussion

In our efforts to overcome the expression block encountered by retroviral vectors in the hemopoietic system, we have chosen to alter the enhancer sequences present in the retroviral LTR. Previously we have described sustained expression of the *neo*^{R} gene in murine hemopoietic cells. This was accomplished using a replication-defective retrovirus in which the Mo-MuLV enhancer sequences present in the LTR were replaced by the enhancer element from the polyoma host-range mutant F101 (13). An extra advantage of this alteration in the LTR is offered by the fact that it renders Mo-MuLV nonpathogenic. Here we describe the construction of a retroviral vector in which the human ADA cDNA is placed under transcriptional control of this ΔMo+PyF101-LTR. With this construct a cell line designated POC-1 was generated that produced amphotropic replication-defective retroviruses carrying the hADA gene. Subsequently, experiments were performed to test the efficacy of this virus in future gene therapy protocols for the treatment of ADA$^-$SCID disease.

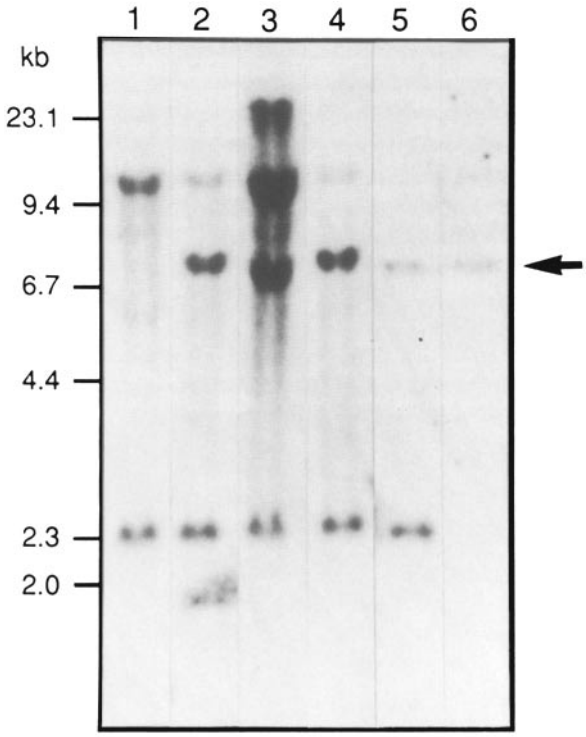

Figure 6. Southern analysis of DNA isolated from hemopoietic cells from primary recipient mouse 10 and secondary recipients transplanted with bone marrow from mouse 10. The DNA was digested with EcoRI, which does not cut in the provirus, separated on a 0.6% agarose gel, blotted to a membrane, and hybridized to the ADA cDNA probe. Lane *1*, spleen from mouse 10; lanes *2–3*, spleens from two individual secondary recipients killed 15 d after transplantation; lanes *4–6*, B cells from three individual secondary recipients killed at day 34 after transplantation. The positions of DNA molecular weight markers are indicated. The 11.5- and 2.3-kb bands hybridizing to the ADA probe represent crosshybridization to endogenous murine ADA sequences. In all secondary recipients a 7.5-kb provirus integration fragment, indicated by the arrow, is clearly present.

Using POC-1–derived viruses we achieved efficient modification of the murine hemopoietic system in vivo. We set out to measure infection and expression efficiency in CFU-S. It appeared that a 48-h cocultivation of murine bone marrow with the virus-producing cells resulted in only 7% of the day 12 CFU-S being infected. A considerable increase in infection efficiency (up to 59%) was achieved by a prolongation of the coculture period to 72 h. We conclude from these data that most CFU-S became susceptible to retrovirus infection at the third day of stimulation by IL-1 and IL-3, possibly due to the fact that more stem cells have left G_0 at that point. The POC-1 cell line did not exert a significant toxic effect on the day 12 CFU-S during this cocultivation period, and in contrast to previous reports by other investigators (11, 33), a pretreatment of bone marrow donor mice using 5-fluorouracil was not required for the high infection efficiency. The latter is of importance for future gene therapy procedures where such a taxing pretreatment is best avoided. Expression of hADA in infected day 12 CFU-S and their progeny was quantified by adapting the Xyl-A/dCF selection procedure (27). Whereas recently Lim et al. (33) reported that this selection procedure requires exposure of cells for 7–10 d, we show here that a selection for only 1 d is sufficient to kill uninfected day 12 CFU-S under serum-free conditions in the absence of fibroblast cells. A preselection of infected bone marrow using Xyl-A/dCF resulted in a clear [dCF]-dependent increase in the frequency of hADA-expressing spleen colonies. This proves that infected CFU-S were overexpressing ADA before transplantation and subsequently sustained this expression upon in vivo differentiation. We envisage that this quantitative selection method might find wider application, e.g., for studying infection of more primitive cells than the day 12 CFU-S such as those that can contribute to the long-term survival of lethally irradiated mice. However, we observed an exceptionally high sensitivity of cells with radioprotective capacities to the Xyl-A/dCF selection procedure. To select for ADA overexpression in such primitive cells a more careful titration of the employed dCF concentration is therefore required.

For long-term expression studies we reconstituted mice with cocultured bone marrow cells without applying any in vitro selection. Upon hemopoietic reconstitution for 34 d all mice (10/10) expressed hADA in their erythrocytes and spleen cells. 50% of these mice also expressed human ADA in PBL or thymic cells. To further assess the nature of the infected cells responsible for the observed expression patterns, proviral integration analysis and retransplantation experiments were performed. The secondary recipients from one of these mice showed a persisting hADA expression pattern in lymphoid, myeloid, and erythroid lineages. DNA analysis revealed that the chromosomal position of the provirus was identical in different hemopoietic cells from these secondary transplanted mice. We concluded from this that these cells were descendants from one PHSC that was infected with our virus. Recently, similar results were obtained using ecotropic viruses (33, 34). Our results prove that PHSC can also be infected with amphotropic retroviruses, a finding that has direct implication for the application of such viruses in protocols for human gene therapy.

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