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A Novel Murine Retrovirus Identified during Testing for Helper Virus in Human Gene Transfer Trials

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An important requirement for the use of retroviral vectors in human gene transfer experiments is the avoidance of human exposure to replication-competent (helper) retroviruses. To meet this requirement, we used a sensitive marker rescue assay for helper virus to screen vector-transduced cells prior to reinfusion into patients. This assay utilized *Mus dunni* cells harboring a retroviral vector that can be rescued by helper retroviruses. The assay indicated the presence of helper virus in medium exposed to hematopoietic cells from all patients tested, including six patients with various cancers and one patient with Gaucher's disease, whether or not the patient cells had been exposed to retroviral vectors. All of the helper viruses were in a single interference group. We have now shown that treatment of the *M. dunni* marker rescue assay cells with 5-iodo-2′-deoxyuridine or hydrocortisone can activate production of an apparently identical helper virus, which we have named *M. dunni* endogenous virus (MDEV). Thus, production of virus in the assays of patient materials was likely due to exposure of the marker rescue assay cells to the hydrocortisone present in the hematopoietic cell growth medium. MDEV does not belong to any of the known murine leukemia virus groups by interference analysis, and we have called the new group multitropic because of the wide range of cells from different species that MDEV can infect.

Viral vectors are currently used in the majority of human gene transfer trials, in large part because of efficient mechanisms that viruses have evolved to transfer genetic material into cells. An important issue in experiments utilizing viral vectors is the possibility of generation of replication-competent (helper) virus due to recombination of viral components during vector production. While testing can provide a statistical measure of the likelihood of helper virus in a vector preparation, the possibility of human exposure to helper virus cannot be excluded. Because the health consequences of recombinant viruses in humans cannot be accurately predicted, there has been a constant effort by investigators and regulatory agencies to increase the stringency of helper virus testing to reduce the risk of human exposure to helper virus.

We have employed a sensitive marker rescue assay to screen for helper virus in retroviral vector preparations and in vector-transduced hematopoietic cells prior to reinfusion into patients as part of two clinical gene transfer trials. One trial is designed to study the contribution of granulocyte colony-stimulating factor-mobilized peripheral blood hematopoietic cells to long-term hematopoietic reconstitution by marking the cells with a retroviral vector containing the *neo* gene prior to reinfusion into patients. The other trial is designed to treat Gaucher's disease by transfer of the glucocerebrosidase gene into hematopoietic cells to correct the metabolic defect responsible for the disease. Both trials involve harvest of granulocyte colony-stimulating factor-mobilized peripheral blood hematopoietic cells followed by transduction of the cells by daily exposure to retroviral vectors during 5 days of culture. After transduction, the cells are cryopreserved for patient administration pending safety testing, including testing for helper retroviruses.

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Because screening of large amounts of the retroviral vector preparations did not reveal helper virus, we were surprised to see positive assay results from testing of medium from hematopoietic cell cultures for all of the patients tested to date. Initial experiments indicated that the viruses came not from the individual components of the hematopoietic culture medium but from the human hematopoietic cells. However, further characterization of the viruses has shown that they are all similar if not identical and that they likely derive from a previously uncharacterized endogenous virus in the *Mus dunni* cells used in the marker rescue assay. By interference analysis, the new virus represents a new mouse retrovirus group.

MATERIALS AND METHODS

Nomenclature. Cells that contain a virus or vector are indicated by the cell name followed by a slash and the name of the virus, e.g., NIH 3T3/10A1 or PA317/LXSN. A retroviral vector in its viral form is indicated by the vector name followed, in parentheses, by the name of the helper virus or packaging cells used to pseudotype the vector, e.g., LAPSN(10A1) or LAPSN(PA317). Throughout this paper, the pseudotype of a retroviral vector refers only to the viral envelope proteins present on the vector virions. For example, vectors produced by PA317 cells will be referred to as having an amphotropic pseudotype because of the presence of amphotropic Env proteins in the virions, even though the Gag and part of the Pol proteins in these virions are derived from Moloney murine leukemia virus (MoMLV) (14).

Cell culture. The following cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS): *M. dunni* tail fibroblasts (gift from Bruce Chesebro, originally described in references 4 and 13), NIH 3T3 (thymidine kinase-negative) cells (14), PA317 amphotropic retrovirus packaging cells (14), PM571 polytropic retrovirus packaging cells (20), PE501 ecotropic retrovirus packaging cells (17), HeLa human cervical carcinoma cells (11), NRK rat kidney cells (7), and CCC-81 cat cells transformed with Moloney murine sarcoma virus (10). G355 (G355-5) feline embryonic brain cells, G355 cells infected with RD114 endogenous cat virus, and PG-4 Moloney murine sarcoma virus-infected G355 cells (all gifts from Donald Blair [8, 12]) were grown in McCoy's medium with 15% FBS.

In helper virus assays, special care was taken to prevent cross-contamination of cultures. In particular, when aerosols might be generated during trypsinization and passaging of the cells, only one culture was manipulated in the laminar flow hood at a time. Culture medium was not exposed to aerosols, and once a sterile

pipette had been used to withdraw medium from the bottle for distribution to cultures, it was never inserted into the bottle again.

Marker rescue assay. *M. dunni* cells containing the N2 retroviral vector (1) (dunni/N2) or the LAPSN retroviral vector (19) (dunni/LAPSN) were made by transducing *M. dunni* cells with helper-free retroviral vectors generated by using PA317 retrovirus packaging cells (14) and then selecting the cells in G418 for 1 week to ensure the presence of the vectors in all cells in the populations. A similar procedure was used to generate G355 cat cells containing the LAPSN vector (G355/LAPSN). The marker rescue assay was performed by seeding dunni/N2, dunni/LAPSN, or G355/LAPSN cells at 10^6/10-cm dish on day 1, exposing the cells to the test sample (medium or hematopoietic cells) in the presence of 4 μg of Polybrene (Sigma) per ml on day 2, passaging the cells 1:10 or 1:20 every 2 or 3 days, feeding confluent dishes of the cells on day 15 or later, harvesting and filtering (pore size, 0.45 μm) the medium the day after feeding, and assaying the medium for the presence of the N2 or LAPSN vector by using *M. dunni* cells as targets for infection. During passage, the cells were kept at high density to facilitate virus spread. Cells were passaged for the first week (at least) in 10-cm dishes and later in 6-cm dishes. Polybrene was present only during the initial exposure of the marker rescue assay cells to test samples and was not added during passage of the cells.

Retroviral vector assay. The LAPSN vector was assayed by seeding target cells at 5×10^4 or 10^5/6-cm dish on day 1, feeding the cells with fresh medium containing 4 μg of Polybrene per ml and adding the LAPSN vector on day 2, and fixing and staining the cells for alkaline phosphatase (AP) expression encoded by the LAPSN vector as described previously (9) on day 4. Assay for the N2 virus was identical except that, instead of staining for AP, the medium was changed to medium containing 0.75 mg of G418 (active) per ml on day 3 and colonies were stained with Coomassie brilliant blue G (Sigma; 1 g/liter in 40% MeOH–10% acetic acid) on day 8.

Human hematopoietic cell transduction. Details of the human clinical trials have been published (25, 26). Briefly, patients received granulocyte colony-stimulating factor to mobilize hematopoietic stem cells into the circulation, peripheral blood cells were collected by leukapheresis, and $CD34^+$ hematopoietic cells were isolated. $CD34^+$ cells were transduced with the LN gene marking vector (17) produced by using PA317 cells (14) or with the LgGC retroviral vector that encodes glucocerebrosidase (unpublished results) produced by using PG13 cells (15), by cultivation of the cells in Iscove's modified Dulbecco's medium containing 50% retroviral vector stock, 12.5% heat-inactivated (56°C for 30 min) FBS, 12.5% heat-inactivated (56°C for 30 min) horse serum, 90 μM hydrocortisone sodium succinate, 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, 0.4 mg of L-glutamine per ml, 0.01 mM 2-mercaptoethanol, 4 μg of protamine sulfate per ml, and recombinant human interleukin-1β, -3, and -6 and stem cell factor (each at 50 ng/ml). Every day one-half of the culture medium was replaced with fresh vector-containing medium containing protamine sulfate but without fresh cytokines. After 5 days of cultivation, the hematopoietic cells and culture medium were harvested and the cells were cryopreserved for patient administration after safety testing.

Endogenous virus activation. In an initial experiment, dunni/LAPSN cells were plated at 2×10^5/35-mm-diameter well of 6-well dishes on day 1. On day 2, the cells were fed with medium containing 4 μg of Polybrene per ml and various concentrations of 5-iodo-2′-deoxyuridine (IdU) (30 μM to 3 mM), 5-bromo-2′-deoxyuridine (3 to 300 μM), or 5-azacytidine (300 nM to 30 μM) or no drug. Crystals of IdU were present at 3 mM IdU, so the medium was filtered with a 0.45-μm-pore-size cellulose acetate filter to remove the crystals. From day 3 through week 3, the cells were passaged in medium containing Polybrene but without continued addition of nucleosides. The cells were split in the dark for the first 2 weeks.

In the second experiment, dunni/LAPSN cells were plated at 4×10^5/6-cm dish in DMEM with 10% calf serum (heat inactivated at 56°C for 30 min) on day 1. On day 2, 10 dishes were fed with the same medium containing 4 μg of Polybrene per ml and saturated with IdU, while 5 dishes were fed with medium containing Polybrene but no drug. The medium saturated with IdU was first mixed at a concentration of 3 mM and vortexed periodically during incubation at 37°C for 30 min. After the remaining IdU crystals had settled to the bottom, the cells were fed with the saturated supernatant. From day 3 to week 4, the cells were split 1:3 to 1:10 every few days in medium containing Polybrene but no IdU. Exposure of three IdU-treated plates to fluorescent light or passage of the other seven dishes in the dark for 1 week prior to light exposure had no apparent effect on virus activation.

RESULTS

Detection of helper virus during testing of clinical samples. We used a marker rescue assay (Fig. 1) to screen for helper virus in medium from human hematopoietic cell cultures that had been exposed to retroviral vectors. Half of the medium in the hematopoietic cultures was replaced with vector-containing medium each day for 5 days, and the spent medium from days 1 and 5 was assayed for helper virus. Helper virus was detected in assays of culture medium from cells of all patients tested, while control assays were uniformly negative (Table 1). The majority of the patients had various cancers, including multiple myeloma, breast cancer, Hodgkin's lymphoma, and non-Hodgkin's lymphoma, while patient 7 had Gaucher's disease, a lysosomal storage disorder. In what follows, "patient 1 virus," "patient 2 virus," etc., refer to the helper virus produced following exposure of the marker rescue assay cells to culture medium from the respective patient's cells.

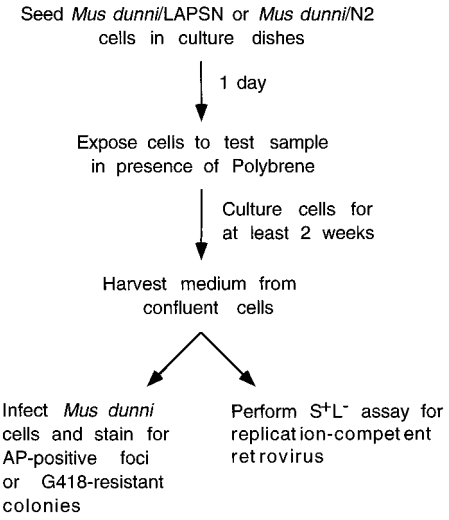

FIG. 1. *M. dunni* marker rescue assay for helper virus.

The medium from the patient cell cultures appeared to contain low levels of helper virus because occasional tests of 5-ml medium samples were negative (Table 1, patients 1 and 2). However, after exposure of dunni/LAPSN marker rescue assay cells to the culture medium from the patient cells and passage of the cells for 2 weeks, the LAPSN vector was produced at a high titer (up to 10^5 focus-forming units FFU/ml), suggesting production of the helper virus at a high titer. We could not detect the helper viruses by using standard focus induction assays, including S^+L^- assays utilizing PG-4 cat cells (12), CCC-81 cat cells overlaid with NRK cells (16), Mv1Lu mink cells (5), or SC-1 feral mouse embryo cells overlayed with

TABLE 1. *M. dunni* marker rescue assay for helper virus in medium samples from patient hematopoietic cell cultures

Patient[a]	No. of positive tests/total[b]			
	With vector[c]		Without vector, day 5	Control
	Day 1	Day 5		
1	ND	2/2,* 4/5,* 3/3	ND	0/1,* 0/5,* 0/2
2	ND	2/2,* 2/5*	ND	0/1,* 0/5*
3	5/5	5/5	1/1	0/2
4	5/5	5/5	1/1	0/3
5	ND	5/5	1/1	0/3
6	5/5	5/5	1/1	0/3
7	5/5	5/5	ND	0/3

[a] Patients 1 to 6 had various cancers; patient 7 had Gaucher's disease.

[b] Medium (5-ml samples) harvested from patient hematopoietic cells on the indicated day after initiation of culture or control medium (DMEM plus 10% FBS) was assayed for helper virus by the dunni/LAPSN or dunni/N2 (*) marker rescue assay. Results of independent experiments are listed for patients 1 and 2. Positive tests gave vector titers of 20 to 10^5 FFU/ml. When no LAPSN or N2 vector was detected (<1 FFU/ml), the test was judged negative. ND, not done.

[c] LN(PA317) for patients 1 to 6; LgGC(PG13) for patient 7.

TABLE 2. Interference of patient 1 and 2 viruses with each other[a]

Target cells	LAPSN titer (FFU/ml) for the following pseudotype:		
	Patient 1	Patient 2	Amphotropic
dunni/N2	6×10^4	5×10^4	8×10^5
dunni/N2 + patient 1 virus	20	25	9×10^5
dunni/N2 + patient 2 virus	80	50	7×10^5

[a] Target cells were plated at 5×10^4/6-cm dish and infected 1 day later with LAPSN vectors pseudotyped by the patient viruses or by LAPSN produced by using PA317 cells (amphotropic pseudotype). Cells were stained 2 days after infection for AP^+ foci, and a vector titer in FFU per milliliter was calculated. Further experiments showed that an additional four of four independent patient 1 virus isolates in dunni/N2 cells and an additional two of two patient 2 virus isolates in dunni/N2 cells interfered with patient 1-pseudotyped LAPSN virus, confirming results from the experiment shown.

Mv1Lu mink lung cells (5) (data not shown; mink S^+L^- assays were performed by Microbiologic Associates Inc., Rockville, Md.). Therefore, the helper virus titer was estimated by using limiting dilution analysis and the *M. dunni* rescue assay for virus detection. By this technique, medium from a dunni/LAPSN cell culture that had been exposed to medium from patient cells was estimated to contain between 10^6 and 5×10^6 infectious units of helper virus per ml.

Detection of helper virus in assays of hematopoietic cell culture medium was not related to exposure of the hematopoietic cells to retroviral vectors, because parallel cultures that had not been exposed to vectors also tested positive (Table 1, patients 3 to 6). This result is consistent with the fact that we have never found helper virus in the vector stocks by marker rescue or other helper virus assays.

In all experiments we assayed standard culture medium (DMEM with 10% FBS) in parallel with the patient samples as a negative control, and all of these assays were negative (Table 1). In one experiment, hematopoietic culture medium that had not been exposed to human cells was analyzed in the marker rescue assay and found to be negative in five replicate assays of 5-ml medium samples each (data not shown). We also analyzed the FBS, horse serum, and cytokines used in the medium in independent assays and never found helper virus in these components. In addition, the FBS and horse serum used were heat inactivated at 56°C for 30 min, and we have found that the titer of the patient virus pseudotype of the LAPSN vector is reduced $>5 \times 10^4$-fold by heating briefly to 56°C.

All recovered helper viruses are in the same interference group. We used interference analysis to compare the different patient viruses. Viruses from the first two patients were initially isolated by using dunni/N2 cells. To test for interference between these viruses, we transferred virus from the dunni/N2 cells to dunni/LAPSN cells and then passaged the cells for 2 weeks to allow helper virus spread. These cell populations produce the LAPSN vector with a patient 1 or patient 2 virus pseudotype and may also produce some N2 virus. We used virus from the patient virus-infected dunni/LAPSN cells to infect the original patient virus-infected dunni/N2 cells and measured AP^+ foci to determine the infection rate. Table 2 shows that LAPSN pseudotyped by either patient 1 or patient 2 virus can efficiently infect dunni/N2 cells but is inhibited from infecting dunni/N2 cells previously infected with either patient 1 virus or patient 2 virus. In contrast, amphotropic pseudotype LAPSN virus infects dunni/N2 cells and dunni/N2 cells infected with patient 1 virus or patient 2 virus equally well. This analysis shows that patient 1 and patient 2 viruses are in the same interference group and that amphotropic murine virus is in a different interference group.

Since dunni/LAPSN cells were used in the marker rescue assay for patients 3 to 7, we tested the ability of the isolated viruses to transfer AP to dunni/N2 cells or dunni/N2 cells infected with patient 1 virus. The transduction rate of all of the patient virus isolates was reduced >250 to 20,000-fold in dunni/N2 + patient 1 virus cells in comparison with uninfected dunni/N2 cells (data not shown). Some of the viruses had a low titer on uninfected dunni/N2 cells and did not detectably transduce dunni/N2 + patient 1 virus cells, so only a lower limit for inhibition (>250-fold) could be established. All viruses detected in medium from patient cells that had not been exposed to retroviral vectors were in the same interference group as those from patient cells that had been exposed to retroviral vectors. Thus, the interference data show that all of the viruses isolated during testing of medium samples from the human hematopoietic cells were in the same interference group and thus were closely related if not identical.

Activation of an *M. dunni* endogenous retrovirus (MDEV) that is in the same interference group as the patient viruses. Endogenous retroviruses present in the cellular genome can be activated by a variety of chemicals (6). We tested whether such viruses could be activated in dunni/LAPSN cells by various concentrations of IdU, 5-bromo-2′-deoxyuridine, or 5-azacytidine. At the highest concentration of IdU we detected LAPSN vector production from the dunni/LAPSN cells 3 weeks after exposure to the drug. This virus proved to be in the same interference group as the patient viruses (data not shown).

In a second experiment, 10 dishes of dunni/LAPSN cells treated with a high dose of IdU and 5 dishes of untreated dunni/LAPSN cells were passaged independently and assayed for virus production. Virus production was not detected in any of the cultures at 1 week (<1 AP^+ FFU/ml), but at time points ≥2 weeks virus production was detected in 10 of 10 IdU-treated cultures (6×10^3 to 5×10^4 AP^+ FFU/ml on *M. dunni* cells at week 4). The five untreated cultures of dunni/LAPSN cells were negative for virus production over the 4-week course of the experiment (<1 AP^+ FFU/ml). Infection by the IdU-activated virus was blocked in cells infected with patient virus 1 (500- to 3,000-fold inhibition), showing that all of the independently activated viruses were in the same interference group as the patient viruses and probably derive from one endogenous provirus, which we will call MDEV.

Given that the viruses detected during screening of medium from human hematopoietic cells were all identical by interference analysis and matched the MDEV activated by IdU, we reexamined the ability of the hematopoietic culture medium to activate MDEV from *M. dunni* cells. In two independent experiments, we detected virus after cultivation of dunni/LAPSN cells with the hematopoietic culture medium in four of four replicate assays in each experiment (data not shown). In prior screens of components of the medium, we had ignored chemically defined components as being unlikely sources of virus. However, the medium contained 90 μM hydrocortisone (to inhibit growth of contaminating lymphoid cells in the hematopoietic cell cultures), and steroids can increase expression of some retroviruses; thus, the hydrocortisone might activate expression of MDEV. Indeed, treatment of dunni/LAPSN cells with 90 μM hydrocortisone in regular culture medium activated virus in two of two assays (Table 3). Hydrocortisone at 1 μM could also induce virus production but less effectively. Infection by all viruses induced by hydrocortisone, as well as infection by LAPSN with a patient 1 virus pseudotype, was completely blocked by the presence of patient 1 virus in the target cells (Table 3). Amphotropic virus from PA317/LAPSN cells infected dunni and dunni/N2 + patient 1 virus cells equally well, showing that there was not a general block to

TABLE 3. Activation of the endogenous virus in *M. dunni* cells by hydrocortisone[a]

Cells assayed for LAPSN vector production	AP^+ FFU/ml[b] on the following target cells:	
	dunni	dunni/N2 + patient 1 virus
dunni/LAPSN		
No HC	<1, <1	<1, <1
+1 μM HC	<1, 10	<1, <1
+90 μM HC	2×10^4, 5×10^4	<1, <1
dunni/LAPSN + patient 1 virus	3×10^4	<1
PA317/LAPSN	4×10^6	3×10^6

[a] dunni/LAPSN cells were seeded at 10^6/10-cm dish on day 1, the medium was replaced with DMEM plus 10% FBS and the indicated concentrations of hydrocortisone (HC) on day 2, and the cells were passaged every 2 or 3 days for 2 weeks prior to measurement of LAPSN vector production by the cells.
[b] Pairs of results correspond to independent cultures passaged and assayed in parallel for LAPSN production.

TABLE 5. Lack of interference by patient viruses with infection by vectors having amphotropic, polytropic, or xenotropic murine virus pseudotypes[a]

Target cells	LAPSN titer (FFU/ml) for the following pseudotype:		
	Amphotropic	Polytropic	Xenotropic
dunni	2×10^6	9×10^4	1×10^6
dunni/N2	2×10^6	8×10^4	9×10^5
dunni/N2 + patient 1 virus	2×10^6	1×10^5	6×10^5
dunni/N2 + patient 2 virus	2×10^6	8×10^4	9×10^5
dunni/N2 + AM-MLV[b]	20	8×10^4	6×10^5

[a] Target cells were infected with the LAPSN vector produced by using PA317 cells (amphotropic pseudotype), PM571 cells (polytropic [mink cell focus-forming virus] pseudotype), or dunni/LAPSN cells infected with NZB xenotropic virus (22) (xenotropic pseudotype). Cells were stained 2 days after infection for AP^+ foci.
[b] AM-MLV, amphotropic MLV (16).

retrovirus infection in the dunni/N2 + patient 1 virus cells (Table 3). Thus, the presence of hydrocortisone in the hematopoietic culture medium can explain the frequent production of helper virus in marker rescue assays of medium from the human hematopoietic cultures.

A G355 cat cell-based marker rescue assay fails to detect helper virus in medium exposed to human hematopoietic cells. While we have shown that the hematopoietic culture medium can activate production of MDEV from *M. dunni* cells, we have not ruled out the possibility that the human hematopoietic cells also produce a related virus. To address this possibility, we used a marker rescue assay based on G355 cat cells to test samples of the culture medium from the human hematopoietic cells, in the hope that endogenous viruses might not be activated in G355 cells. Five 5-ml hematopoietic medium samples from day 5 of the patient 6 hematopoietic cell culture were all negative for helper virus in the G355 cat cell-based marker rescue assay (Table 4), while five 5-ml samples from the same culture were all positive in the *M. dunni*-based marker rescue assay (Table 1). In addition, the virus isolated in the *M. dunni* assay of culture medium from patient 6 cells scored positive in the G355 cat cell-based marker rescue assay (Table 4), showing that this assay was capable of detecting the virus if it was present in the culture medium from patient 6 cells. Thus, we have good evidence that the day 5 culture medium from the patient 6 hematopoietic cell culture was helper free and, therefore, that the virus produced in the *M. dunni* assay of the same medium must be MDEV.

MDEV represents a new murine retrovirus interference group. The LAPSN vector having either an amphotropic, a polytropic (mink cell focus-forming virus), or a xenotropic pseudotype was used to infect *M. dunni* cells that were previously infected with patient 1 virus, patient 2 virus, or amphotropic MLV or that were not infected with helper virus (Table 5). Amphotropic-, polytropic-, and xenotropic-pseudotype LAPSN vectors transduced dunni/N2 cells or dunni/N2 cells that were infected with virus from patient 1 or patient 2 equally well, showing that the patient viruses do not interfere with entry of these murine viruses. In all cases the presence or absence of the N2 retroviral vector in the *M. dunni* cells had no effect on the AP transduction rate, as expected, since the N2 vector encodes only Neo and makes no viral proteins. As a control for viral interference, dunni/N2 cells infected with amphotropic virus were efficiently infected by polytropic or xenotropic LAPSN vectors but not by the amphotropic LAPSN vector, as expected. Since MDEV and the patient virus isolates are in the same interference group, these results show that MDEV is not in the amphotropic, polytropic, or xenotropic interference group.

Since *M. dunni* cells are poorly infected by some ecotropic retroviruses, including MoMLV, we used NIH 3T3 mouse cells to conduct additional interference tests. The presence of eco-

TABLE 4. G355 cat cell-based marker rescue assay for helper virus in day 5 medium from the patient 6 hematopoietic cell culture

Sample	LAPSN vector titer (FFU/ml)[a]
No virus	<1, <1, <1
Patient 6 culture medium (5 ml)	<1, <1, <1, <1, <1
dunni/LAPSN + patient 6 virus	
1 ml	4×10^4
10 μl	4×10^4
100 nl	<1
AM-MLV[b] (1 μl)	5×10^7

[a] Sets of results correspond to independent cultures passaged and assayed in parallel.
[b] AM-MLV, amphotropic MLV (16).

TABLE 6. Lack of interference by MoMLV, AM-MLV, and 10A1 MLV with transduction by a vector having a patient 1 virus pseudotype[a]

Target cells	LAPSN titer (FFU/ml) for the following pseudotype:			
	Patient 1 virus	Ecotropic	Amphotropic	10A1
NIH 3T3	200	2×10^6	5×10^6	10^7
NIH 3T3/MoMLV	200	500	4×10^6	10^7
NIH 3T3/AM-MLV	400	10^6	40	9×10^6
NIH 3T3/10A1	200	10^6	3	200

[a] Target cells were seeded at 10^5/6-cm dish on day 1, infected with LAPSN virus with the indicated pseudotype on day 2, and stained for AP^+ foci on day 4. Helper virus-infected NIH 3T3 cells were generated by transfection of the cells with molecular clones of MoMLV (pMLV-K [18]), amphotropic MLV (AM-MLV) (pAM [16]), or 10A1. The permuted 10A1 virus DNA clone (pB6 [23], gift from David Ott and Alan Rein, National Cancer Institute Frederick Cancer Research and Development Center, Frederick, Md.) was cut with the *Sal*I restriction endonuclease and religated to generate intact 10A1 provirus circles prior to transfection into NIH 3T3 cells. Cells were passaged for several weeks to allow complete virus spread. Viruses used for infection were derived from dunni/LAPSN + patient 1 virus cells (patient 1 virus pseudotype), PE501/LAPSN cells (ecotropic pseudotype), PA317/LAPSN cells (amphotropic pseudotype), or NIH 3T3/LAPSN + 10A1 virus cells (10A1 pseudotype). The experiment shown was repeated two more times with similar results.

TABLE 7. Wide host range of the patient viruses

Target cells[a]	Species	Virus titer[b] for the following pseudotype:		
		Patient 1 virus	Patient 2 virus	AM-MLV[c]
HeLa	Human	2×10^4	1×10^4	1×10^6
NIH 3T3	Mouse	2×10^3	1×10^3	$>10^6$
M. dunni	Wild mouse	2×10^4	1×10^4	2×10^6
NRK	Rat	1×10^3	5×10^2	2×10^5
CCC-81	Cat	5	<5	1×10^6
G355	Cat	2×10^3	2×10^3	7×10^5
D17	Dog	1×10^3	ND	ND

[a] The LAPSN vector was used for infection of the G355 and D17 cells, while the N2 vector was used for all other cell types.
[b] In CFU per milliliter for the N2 vector and in FFU per milliliter for the LAPSN vector. ND, not determined.
[c] AM-MLV, amphotropic MLV.

tropic, amphotropic, or 10A1 virus in the mouse cells did not inhibit infection by the LAPSN vector with a patient 1 virus pseudotype (Table 6), indicating that MDEV and the patient viruses are not in these mouse virus classes. Control experiments showed that infection by an ecotropic LAPSN vector (produced by using PE501 cells) was blocked by ecotropic virus (MoMLV) but not the other viruses, that amphotropic LAPSN vector (produced by using PA317 cells) was blocked by amphotropic or 10A1 virus but not by ecotropic virus, and that the 10A1 pseudotype LAPSN vector was blocked by 10A1 but not the other viruses, all as expected. 10A1 virus can use both the amphotropic receptor Ram1 and the gibbon ape leukemia virus (GALV) receptor Glvr-1 in mouse cells (21), which is why 10A1 blocks infection by amphotropic virus but amphotropic virus does not block 10A1 virus infection. Together with the interference data obtained with *M. dunni* cells, these results show that MDEV is not in any of the five known MLV interference groups (24).

The host range of MDEV is broad and includes human, mouse, rat, cat, and dog cells (Table 7). Additional experiments showed that MDEV also infects Chinese hamster ovary (CHO) cells efficiently (data not shown). Interestingly, MDEV poorly infected CCC-81 cat cells, possibly because of expression of the RD114 endogenous cat virus. We therefore tested whether MDEV was in the RD114 virus interference group. We detected a low level of interference (10- to 20-fold) with MDEV infection by RD114 virus in G355 cat cells but no interference when the assays were performed with D17 dog cells (data not shown). Two other viruses shown to be in the RD114 class by interference assay in human cells (27), spleen necrosis virus and Mason-Pfizer monkey virus (SRV-3), did not interfere with patient virus infection (data not shown). Thus, MDEV shows a limited relationship to the RD114 endogenous cat retrovirus.

DISCUSSION

M. dunni cells were adopted by the U.S. Food and Drug Administration for helper virus testing of materials to be used in human gene transfer trials because they are susceptible to infection and allow replication of members of all previously characterized murine retrovirus classes (13). A notable exception to this generalization is that ecotropic MoMLV poorly infects these cells (13), but since ecotropic virus does not infect human cells, this is not a significant concern for human gene transfer trials. Since retrovirus packaging cells in common use are typically based on the NIH 3T3 mouse cell line and the retroviral vectors and defective helper virus components present in the packaging lines are typically derived from murine retroviruses, the choice of *M. dunni* cells allows detection of all anticipated helper viruses in these cases. However, we have found that an endogenous virus can be activated and spread in *M. dunni* cells following treatment with hydrocortisone or IdU; thus, steroids or other factors present in test samples can give false-positive results in this helper virus assay. We have also observed an apparent example of spontaneous activation of virus from dunni/LAPSN cells cultured under standard conditions (data not shown), and the virus was in the same interference group as the chemically activated viruses described in this report. In the context of a clinical gene transfer trial, such false positives can be very costly and time-consuming and may delay therapeutic treatment. Marker rescue assays that are less prone to such false-positive results would thus be desirable.

Another problem with the *M. dunni*-based marker rescue assay is that the assay is incapable of detecting some nonmurine retroviruses, including GALV. We have developed retrovirus packaging lines based on GALV (15) that promote more efficient infection of some myeloid and lymphoid cells (2, 3, 28) and are currently using a GALV-based packaging cell line in our attempt to treat Gaucher's disease by gene therapy (patient 7). In this case, we have employed a marker rescue assay based on a human cell line, in addition to the *M. dunni* marker rescue assay, to detect potential helper viruses having a GALV pseudotype.

Several factors complicated our progress in resolving the source of the helper virus detected in screening of patient materials. We initially focused on testing of components of the hematopoietic culture medium that were likely sources of virus, such as the horse serum, and could not detect virus in these materials. Chemical medium components, such as hydrocortisone, were judged unlikely virus sources. In addition, an initial test of five 5-ml aliquots of the complete hematopoietic culture medium was negative for helper virus, and since the clinical-grade components of the medium were expensive, we did not routinely test additional hematopoietic culture medium samples. We cannot explain this initial result in light of later experiments in which multiple samples of the hematopoietic culture medium all tested positive. Other misleading evidence included results from an experiment in which peripheral blood hematopoietic cells from one of the patients (prior to $CD34^+$ purification or cultivation in hematopoietic medium) scored positive for helper virus when cultivated with the *M. dunni* assay cells in standard culture medium (DMEM plus 10% FBS), and the recovered virus was in the same interference group as the other patient isolates (data not shown). This appeared to be good evidence that the source of the virus was the patient cells. We still cannot explain why these cells tested positive, but the result may be due to spontaneous activation of MDEV or activation of MDEV by factors secreted by the hematopoietic cells.

In summary, the virus isolates produced by IdU or hydrocortisone treatment of *M. dunni* cells, or produced by exposure of the *M. dunni* cells to human hematopoietic cells or medium from human hematopoietic cell cultures, are all in the same interference group and probably derive from a single endogenous virus in the *M. dunni* cells (MDEV). MDEV represents a new murine retrovirus interference group, for which we suggest the name "multitropic" because of the wide range of cells from different mammalian species that can be infected by MDEV. Efforts are under way to derive molecular clones of the virus that would help understand its relationship to other retroviruses.

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