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Identification of Gammaretroviruses Constitutively Released from Cell Lines Used for Human Immunodeficiency Virus Research[▽]

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Three human cell lines used in human immunodeficiency virus research were found to be contaminated with previously undetected retroviruses. On the bases of partial nucleotide sequence, capsid protein antigenicity, vector mobilization, and receptor usage studies, these contaminants were shown to be replication competent and to belong to the *Gammaretrovirus* genus. While the TZM-bl cells harbor ecotropic murine leukemia virus (MLV), Jurkat J6 cells were found to release xenotropic MLV and the A3.01/F7 cells to produce gibbon ape leukemia virus. These findings highlight the importance of routine testing of cell lines for retrovirus contamination to prevent potential experimental artifacts and allow correct biohazard assessment.

Cell lines capable of supporting human immunodeficiency virus (HIV) replication are essential tools for the phenotypic characterization of virus isolates, for the investigation of molecular mechanisms in the virus life cycle, and for testing the susceptibility of HIV to antiviral agents.

To favor virus replication and ease detection of viral infectious events, the cell lines used are often modified by ectopic expression of gene products supporting HIV type 1 (HIV-1) entry and allowing quantification of HIV infectivity. One example is the HeLa-derived TZM-bl indicator cell line (15, 32, 44), which expresses both HIV receptors and Tat-dependent detector genes.

Other useful cell sublines are selected according to their phenotypic properties. For example, the A3.01 (8, 17) subline was derived from the lymphoblastoid cell line CEM-CCRF (16) based on its abilities to support T-tropic HIV-1 replication and to display cytopathogenicity following infection. To allow efficient replication of M-tropic HIV, the A3.01 cell line was later modified (A3.01/F7) to express CCR5 constitutively (10). Several sublines have also been derived from Jurkat cells (37), including the interleukin-2-producing E6.1 clone (45) and the high CD3-expressing J6 clone (19).

During our study of HIV-1 production from different cell lines, we noticed that transfection of Jurkat J6 cells (obtained from Jenny Underwood, Imperial College London) with an *env*-defective molecular clone, NL4-3/*env*$^-$ (31), results in the production of HIV-1 particles infectious for both CD4-positive and CD4-negative cells (not shown). Conversely, virions produced in a different clone of Jurkat cells (Jurkat E6.1; American Type Culture Collection [ATCC]) are not infectious for either CD4-negative or CD4-positive cell types. This evidence could suggest that Jurkat J6 cells express an Env-like factor which can support a CD4-independent entry pathway. Phenotypic mixing of HIV-1 with another retrovirus (11, 21, 40) could explain this unexpected observation, and the possibility that Jurkat J6 cells are contaminated with a retrovirus was investigated. The presence of reverse transcriptase (RT) activity in cell culture supernatants was monitored with a real-time PCR-based RT assay, a variation of the previously described fluorescent product-enhanced reverse transcriptase assay (2), employing brome mosaic virus RNA (39) as the template and Sybr green I as the fluorescent dye on a LightCycler (Roche) real-time PCR platform. Cell-free culture supernatants from Jurkat J6 and control cell lines (Table 1 and Fig. 1) were subjected to ultracentrifugation, and RT activity in the pelleted samples was measured. Significant RT activity was detected in Jurkat J6 cells, indicating the presence of a retrovirus. Interestingly, RT activity was also detected in samples derived from A3.01/F7 (National Institute for Biological Standards and Control [NIBSC], United Kingdom) and TZM-bl cells, suggesting that a retrovirus also contaminates these two cell lines.

To investigate whether contamination had occurred in our laboratory, the three cell lines found to release RT activity were reobtained from external sources and tested within 5 days of their acquisition (not shown). RT activity was detected again in supernatants of Jurkat J6 cells obtained from the London Research Institute, of A3.01/F7 cells newly obtained from the National Institute for Biological Standards and Control (United Kingdom), and of TZM-bl cells newly obtained from the NIH AIDS Research and Reference Reagent Program, indicating that the cell lines had acquired a retrovirus infection outside our laboratory.

To characterize the contaminant retroviruses, genus-specific RT-PCR was used to detect beta- or gammaretrovirus RNA released in cell culture supernatants. Cell-free supernatants from RT-positive and -negative cell cultures were ultracentrifuged, RNA was extracted from the pellets, and RT-PCR was used to amplify a region in *pol* of beta- or gammaretroviruses with primer sets previously described (30). While no amplification could be observed with betaretrovirus-specific primers (not shown), a DNA fragment of the expected size was specifically obtained from the RT activity-releasing cells (Fig. 2A), indicating that Jurkat J6, A3.01/F7, and TZM-bl cells harbor a gammaretrovirus. This evidence was confirmed by detecting

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TABLE 1. Detection of RT activity in cell line supernatants

Cell line	RT[a]	Source
A3.01	−	NIH AIDS Research and Reference Reagent Program
A3.01/F7	+	NIBSC, United Kingdom
A549	−	Imperial College London
CEM-CCRF	−	ATCC
CEM-SS	−	NIH AIDS Research and Reference Reagent Program
HEK-293	−	ATCC
HEK-293T	−	ATCC
HeLa	−	ATCC
HT1080	−	University College London
Jurkat E6.1	−	ATCC
Jurkat J6	+	Imperial College and London Research Institute
Jurkat TAg	−	Dana-Farber Cancer Institute
Jurkat D1.1	−	ATCC
NIH-3T3	−	ATCC
TE671	−	University College London
TZM-bl	+	NIH AIDS Research and Reference Reagent Program

[a] +, RT activity present; −, RT activity absent.

gammaretrovirus Gag proteins (Fig. 2B) in cell lysates with an antibody raised against the capsid of a gammaretrovirus (the pig endogenous retrovirus) (4).

The contaminating retroviruses were further characterized by sequencing the RT-PCR products shown in Fig. 2A. BLAST analyses showed that the 211-bp sequences from the Jurkat J6 and TZM-bl cell retroviruses share the highest identity (100 and 99.5%, respectively) with several sequences derived from *pol* of murine leukemia virus (MLV), such as the retroviral genomes with accession numbers X94150 and AF033811, respectively. The 211-bp sequence from the A3.01/F7 cell retrovirus closely matched part of the *pol* gene of gibbon ape leukemia virus (GALV), sharing the highest identity with strains X (accession number U60065, 99.5% identity) and SEATO (accession number M26927, 93% identity).

To investigate the replication competency and confirm the receptor usage of these retroviruses, vector mobilization and cross-interference assays were carried out (Fig. 3).

Human TE671 and murine NIH 3T3 cells harboring an

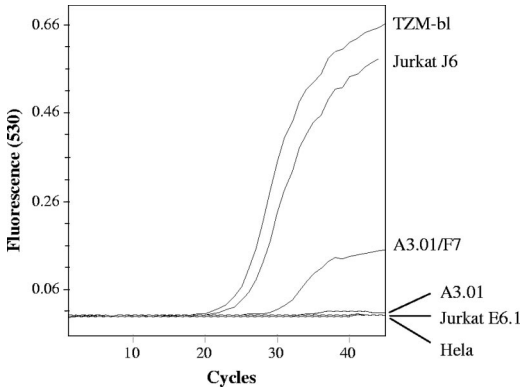

FIG. 1. Retrovirus contamination of three human cell lines. A real-time PCR-based RT assay was used. Amplification curves were obtained from samples derived from the indicated cell lines.

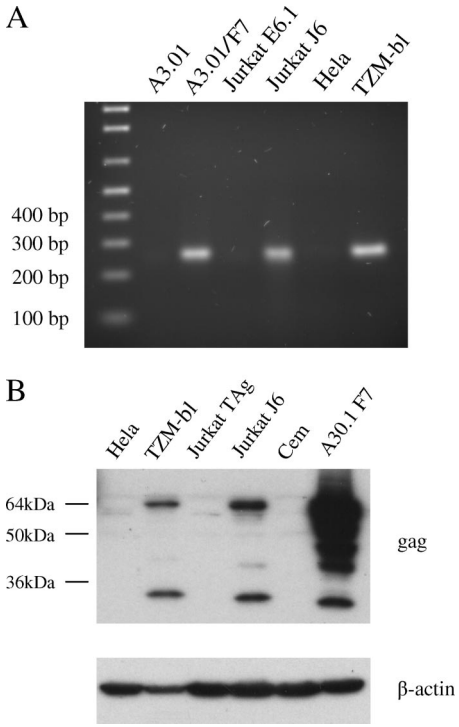

FIG. 2. Jurkat J6, A3.01/F7, and TZM-bl cells harbor a gammaretrovirus. (A) RT-PCR assay for gammaretrovirus *pol* RNA released by different cell lines. (B) Western blotting of cell lysates derived from the indicated cell lines and probed with a rabbit polyclonal antibody against the pig endogenous retrovirus capsid protein. Detection of β-actin is shown as a control.

MLV-based vector coding for β-galactosidase (TEL and 3T3L cells, respectively) (42) were inoculated with culture supernatants derived from A3.01/F7, Jurkat J6, and TZM-bl cells and grown for 10 days to allow any replication-competent retrovirus to propagate. Supernatants were then harvested and tested for the ability of retroviruses to transfer the MLV vector to human (TE671 or HEK 293) or murine (NIH 3T3) cells. To establish receptor usage, interference studies were carried out by inoculating the progeny LacZ retroviral pseudotypes onto target cells infected with MLV-X (xenotropic MLV, strain NZB), MLV-A (amphotropic MLV, strain 1504), GALV (42, 43), and MLV-E (Moloney MLV) (38).

As shown in Fig. 3, retroviruses harbored by A3.01/F7, Jurkat J6, and TZM-bl cells could all mobilize the MLV-based vector and produce high infectious titers in either human or murine cells, indicating their ability to replicate autonomously.

The retrovirus from TZM-bl cells could mobilize the LacZ vector from 3T3L cells but not TEL cells, suggesting the possibility that TZM-bl produces an ecotropic MLV for which human cells do not express a functional receptor (1). Accordingly, specific receptor interference of LacZ (TZM-bl) pseudotypes was observed on NIH 3T3 cells infected with MLV-E, further indicating that TZM-bl cells harbor an ecotropic MLV.

The Jurkat J6 cell retrovirus could mobilize the LacZ vector from human TEL cells (Fig. 3B), and the infectivity of the LacZ pseudotypes was specifically inhibited in cells

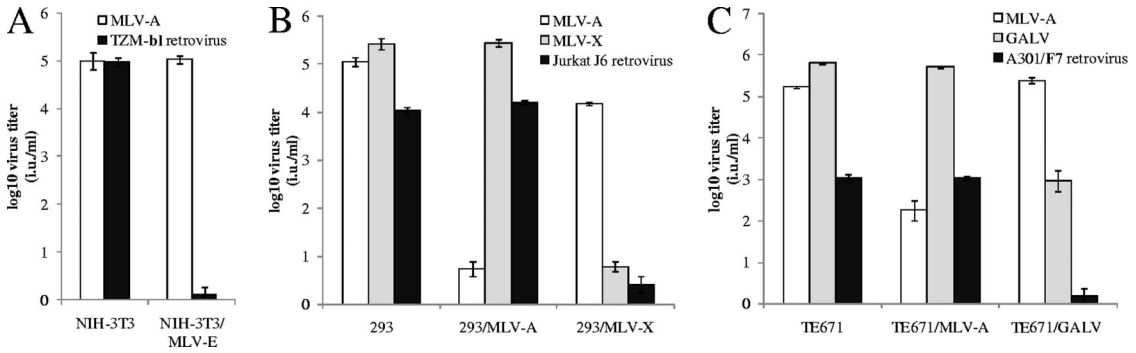

FIG. 3. Cross-interference analyses. LacZ pseudotypes were obtained by propagating TZM-bl (A), Jurkat J6 (B), and A3.01/F7 (C) cell retroviruses in 3T3L or TEL cells as described in the text. MLV-A (strain 1504), MLV-E (Moloney MLV), MLV-X (strain NZB), and GALV (strain X) were used as control retroviruses to test the specificity and efficiency of receptor interference. The retroviruses were inoculated onto uninfected or infected target cells as indicated. i.u., infectious units.

infected with MLV-X, suggesting that this retrovirus is a xenotropic MLV.

Finally, the retrovirus from A3.01/F7 cells could mobilize the MLV-based vector from human TEL cells and strong specific inhibition of LacZ pseudotype infectivity was observed on GALV-infected cells, further indicating that A3.01/F7 cells are contaminated with GALV.

Contamination of animal cell lines with retroviruses has been described several times in the literature (3, 7, 13, 14, 18, 24, 27, 34, 41, 46), and the most frequent contaminants are reported to be gammaretroviruses (7, 13, 14, 18, 27, 34, 35, 46). A likely cause of contamination with MLV is their passage through animals as xenoimplants (18, 23). Since xenotropic MLV frequently represents mouse endogenous retroviruses, the presence of MLV-X in Jurkat J6 cells suggests that the cells were passaged through a mouse. However, details describing the history of this cell line are not available to confirm this theory.

Since TZM-bl and A3.01/F7 cells were both established with retrovirus vectors expressing CCR5 (10, 32), their contaminant retroviruses could have originated from recombination events in the packaging cells used to generate such vectors. The retroviral vector used to express CCR5 in TZM-bl cells was obtained by a coculture of Ψ2 (ecotropic) and PA12 (amphotropic) murine packaging cell lines (32), which are known to generate helper retroviruses following a single recombination event between the vector and the packaging construct (12, 25, 26). The presence of ecotropic MLV in TZM-bl cells indicates that a replication-competent retrovirus arose in the ecotropic Ψ2 packaging cells and infected PA12 cells to acquire the transient amphotropic host range required to enter human cells.

Since the retrovirus vectors commonly used are based on MLV packaging constructs and no *gag-pol* GALV-based retrovirus packaging cell lines have been described, the presence of GALV in A3.01/F7 cells is more difficult to explain and laboratory contamination, rarely reported in the literature (9, 28, 29), remains the most plausible explanation.

The unknown presence of retroviruses in cell lines could confound experiments, as highlighted by our initial observation of unexpected rescue of *env*-negative HIV-1 by Jurkat J6 cells. HIV phenotypic mixing and contamination with gammaretrovirus particles could have occurred in those cases where one of these cell lines has been used for virus production (5, 10, 36) and might have affected the experimental outcomes. Such unnoticed retrovirus contamination seems to be frequent among widely used cell lines. In addition, when the contaminants have the potential to infect human cells, such as MLV-X and GALV, they could introduce a further biohazard, either by representing a direct risk of infection to the operator or by altering the virulence and tropism of infectious agents following phenotypic mixing and/or recombination. Because of their high sensitivity and their ability to detect divergent retroviruses, PCR-based RT assays are the ideal tools for monitoring retrovirus contaminations (2, 6, 20, 22, 33, 39). Given the simplicity and low cost of these assays, screenings should be performed routinely to identify accidental retrovirus laboratory contaminations or to detect replication-competent recombinants in retrovirus vector-transduced cells.

Nucleotide sequence accession numbers. Sequences obtained from Jurkat J6, TZM-bl, and A3.01/F7 cell retroviruses were deposited in GenBank with accession numbers FJ172350, FJ172351, and FJ172352, respectively.

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