Establishment of a LacZ Marker Rescue Assay to Detect Infectious RD114 Virus

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ABSTRACT. Cats have an infectious endogenous retrovirus, named RD114 virus, and there is a possibility that RD114 virus has contaminated live attenuated vaccines, for which feline cells are used as a substrate. To monitor infectious RD114 virus in vaccines for cats, we developed a LacZ marker rescue assay to detect infectious RD114 virus. Among four human cell lines examined, TE671 cells (human rhabdomyosarcoma) were most susceptible to RD114 virus and supported RD114 replication efficiently. Infection was enhanced approximately 5 times by the addition of polybrene at concentrations of 2 to 8 $\mu\text{g}/\text{ml}$ in the medium during viral adsorption. A 4-hr viral adsorption period was sufficient to obtain the maximum titer. By inoculating samples into TE671 cells transduced with the *lacZ* marker gene, the limiting diluted sample (*i.e.*, less than 10 infectious units) was detected at 12 days post-inoculation by the LacZ marker rescue assay. Based on the results obtained in this study, we propose a standard protocol of the LacZ marker rescue assay to detect infectious RD114 virus.

KEY WORDS: detection, endogenous retrovirus, RD114 virus.

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Endogenous retroviruses (ERVs) originate from exogenous retroviruses that have infected germline cells in the past [5]. ERVs are transmitted from parents to offspring by Mendelian's law. Most ERVs are defective and not infectious; however, cats have an infectious ERV in their genome, named RD114 virus [4, 10]. The RD114 virus is a recombinant comprising a *gag-pol* gene from a gammaretrovirus and an *env* gene from a betaretrovirus [19]. Generally, ERVs are not pathogenic in their original hosts; however, some ERVs induce diseases; for example, ERVs from AKR mice induce lymphoma in the hosts [13]. Certain ERVs infect new hosts and induce diseases; there was an incident in which an ERV from Asian wild mice (*Mus caroli*) infected Gibbon apes and induced lymphoma [9]. Moreover, a retrovirus emerged in koalas in Australia about one hundred years ago, and endogenized in koalas. This virus, called koala retrovirus, induces neoplastic diseases and immune suppression in the new host [6, 17, 18].

In humans, vaccines against mumps and yellow fever have been contaminated with ERVs from chickens [7, 8], as they were manufactured using chicken cells. There is a possibility that RD114 virus is contaminated in live attenuated vaccines, for which feline cells are used as a substrate. Actually, RD114-like virus is produced from a feline cell line, Crandell feline kidney cells [1]. The cells are used for several live attenuated vaccines for cats and dogs. To date, there is no definitive assay system to detect infectious RD114 virus in live attenuated vaccines. In this study, we established a LacZ marker rescue assay for infectious

RD114 virus.

MATERIALS AND METHODS

Cells: Human embryonic kidney (HEK) 293T cells (ATCC, CRL-11268), HeLa cells (human epitheloid carcinoma) (ATCC, CCL-2), HT1080 cells (human fibrosarcoma) (ATCC, CRL-12012), TE671 cells (human rhabdomyosarcoma) [14], TELCeB6/RDF cells [2], TELCeB6/FBFeLV-B cells [11] and TE671 cells persistently infected with RD114 virus (TE671/RD114 cells) [16] were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) (Invitrogen, Carlsbad, CA, U.S.A.).

Viruses: RD114 virus was prepared from the culture supernatant of TE671/RD114 cells. A pseudotype virus, termed *lacZ*(RDF) virus, was prepared from TELCeB6/RDF cells [2]. The *lacZ*(RDF) virus harbors an envelope of RD114 virus, the core of murine leukemia virus (MLV), and contains the *nlsLacZ* gene with a packaging signal derived from MLV as a genome. The *nlsLacZ* gene has a nuclear localization signal upstream from the open reading frame of the *lacZ* gene [2]. *lacZ*(FeLV-B) virus, having an envelope of FeLV-B, core of MLV and the *nlsLacZ* gene, was prepared from TELCeB6/FBFeLV-B cells [11]. To prepare another pseudotype virus, termed *lacZ*(RD114) virus, which has an envelope and core of RD114 virus and the *nlsLacZ* gene, TE671/RD114 cells were transduced with the *nlsLacZ* gene by inoculation with *lacZ*(FeLV-B), and designated as TE671(LacZ)/RD114 cells. The stock virus from the cells was used as *lacZ*(RD114) virus; however, it should be noted that both *lacZ*(RD114) pseudotype virus and RD114 virus

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(wild-type) are produced from the cells. All stock viruses were filtrated through a 0.45 μm filter unit (Acrodisc; PALL Co., Ann Arbor, MI, U.S.A.) and kept at -80°C until used.

Titration of pseudotype viruses: Target cells except HEK293T cells were seeded in 24-multiwell plates at a concentration of 8×10^4 cells/well (500 μl) and incubated one day before infection. HEK293T cells were seeded at a concentration of 4×10^5 cells/well (500 μl). Infectivities of gammaretroviruses and lentiviruses are enhanced by cationic polymers such as hexadimethrine bromide (polybrene) in viral adsorption [3]; therefore, infections were performed at 37°C by plating 500 μl of each dilution of viral supernatant into target cells with 8 μg of polybrene (Sigma-Aldrich, St. Louis, MO, U.S.A.) per milliliter, except for the experiment shown in Fig. 2A, in which we changed the concentration of polybrene in viral adsorption. Four hr post-infection, supernatants were replaced with fresh medium, and infected cells were incubated for 2 days before being subjected to X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) staining, as described previously [2], except for the experiment shown in Fig. 2B, in which we changed the viral adsorption period. Viral titers were determined by counting *lacZ*-positive foci, and expressed as focus-forming units (f.f.u.) per ml, as described previously [2].

LacZ marker rescue assay: TE671 cells were transduced with the *nlsLacZ* gene by inoculation of *lacZ*(FeLV-B) virus. Transduced cells, termed TE671(LacZ) cells, were stained with X-gal, and it was confirmed that almost all cells were transduced with the gene. To determine the growth kinetics of RD114 virus in TE671 cells, TE671(LacZ) cells (1.2×10^6 cells) grown in a 12.5 cm^2 culture flask (BD FALCON, Franklin, NJ, U.S.A.) were inoculated with 1,000-fold diluted stock RD114 virus (1 ml) (at a multiplicity of infection of 0.0005), and the viruses produced in the culture supernatants were titrated on TE671 cells as described above.

Sensitivity of the LacZ marker rescue assay: To evaluate the sensitivity of the LacZ marker rescue assay, serially diluted (10-fold) stock RD114 viruses (1 ml each) were inoculated into TE671(LacZ) cells (9×10^5 cells) grown in a well of a 6-multiwell plate. The inoculated cells were passaged at a split ratio of 1:6 at 1 day after inoculation and transferred to 25 cm^2 culture flasks. At 4, 8 and 12 days after inoculation, the cells were passaged at a split ratio of 1:10. The viruses produced in the culture supernatant were titrated on TE671 cells by the LacZ marker rescue assay. Simultaneously, virus-inoculated cells were subjected to DNA isolation at the indicated times, and proviral DNA was detected by polymerase chain reaction (PCR) tests described in the next section.

PCR tests: Total genomic DNA was isolated from cells using a QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA, U.S.A.). To detect RD114 proviruses, partial *pol* and *env* RD114 sequences were amplified using primers corresponding to part of the *pol* gene (forward primer, 5'-gagacccttactaattgac-3'; reverse primer, 5'-agtctctggtc-caggggtt-3') and *env* gene (forward primer, 5'-ttgccaagat-

tgttggtc-3'; reverse primer, 5'-gtaccggataagacttgac-3'). PCR conditions were as follows. The reaction mixture (total 50 μl) consisted of 2 μl of the DNA template (200 ng), 5 μl of $10 \times$ buffer containing 20 mM MgCl_2 (Ex Taq buffer; Takara, Tokyo, Japan), 0.25 μl of *Taq* polymerase (Ex Taq; Takara), 4 μl of 2.5 mM deoxynucleotide triphosphates, 1 μl of each primer (100 pmol/ μl), and 36.75 μl of distilled water. The amplification conditions were 94°C for 10 min followed by 30 cycles of amplification, consisting of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 90 sec, and then final extension at 72°C for 10 min. All PCR reactions were carried out in 200 μl thin-walled tubes using a thermal cycler (Robocycler gradient 96; Stratagene, La Jolla, CA, U.S.A.).

RESULTS

Sensitivity of human cell lines to RD114 virus: RD114 virus is a xenotropic ERV that infects human cells using an amino acid transporter, ASCT1, as a receptor [12, 15]. First, we examined the sensitivity of human cell lines to RD114 virus. The *lacZ*(RDF) virus was inoculated into four human cell lines, TE671, HEK293T, HeLa, and HT1080 cells, and the titers of the *lacZ*(RDF) virus were determined. The *lacZ*(RDF) virus infected all cell lines examined; however, HeLa cells were less sensitive to the virus (Fig. 1A).

Next, another pseudotype virus, *lacZ*(RD114) virus, was inoculated into human cell lines. The titers of the

Fig. 1. Sensitivity of various human cell lines to RD114 pseudotype viruses. *lacZ*(RDF) (A) and *lacZ*(RD114) viruses (B) were inoculated into TE671, HEK293T, HeLa and HT1080 cells. Three independent experiments were performed, and averages with standard deviations are shown.

lacZ(RD114) virus were similar to those of the *lacZ*(RDF) virus in the cell lines examined (Fig. 1B). The reason for the lower sensitivity of HeLa cells to both *lacZ*(RDF) and *lacZ*(RD114) viruses is unknown at present; however, it is possible that the promoter activity of the MLV long terminal repeat for expression of the *nlsLacZ* gene is low in the cells. The ratio of the titers of *lacZ*(RD114) and *lacZ*(RDF) viruses in HT1080 cells (0.50) was slightly lower than those in other cell lines (0.78 to 1.09), suggesting that RD114 virus infections were weakly restricted in HT1080 cells. From these data, we concluded that both HEK293T and TE671 cells are suitable target cells for detecting RD114 virus; however, HEK293T cells need collagen-coated plates for the assay, and we therefore selected TE671 cells for further studies.

Effect of a cationic polymer (polybrene) on viral adsorption: By the addition of polybrene from 2 to 8 $\mu\text{g/ml}$, the titer of *lacZ*(RD114) virus was enhanced about 5 times (Fig. 2A); however, the effect was slightly diminished at a concentration of 16 $\mu\text{g/ml}$ polybrene, possibly due to the cytotoxicity of the reagent.

Determination of optimal adsorption period: We determined the adsorption period to have the maximum titer of

the RD114 virus. The titer of *lacZ*(RD114) virus reached a plateau with a 4-hr adsorption period (Fig. 2B). The titer obtained by adsorption for 24 hr was not significantly different from that obtained by adsorption for 4 hr. These data indicate that a 4-hr adsorption period is sufficient to obtain the maximum titer.

Growth kinetics of RD114 virus in TE671 cells: TE671(LacZ) cells were inoculated with 1,000-fold-diluted stock RD114 virus, and viral production was monitored by the LacZ marker rescue assay (Fig. 3). The titer reached a plateau (5.6×10^5 f.f.u./ml) at 12 days after infection. These data indicate that TE671 cells support RD114 replication efficiently.

Sensitivity of the LacZ marker rescue assay: The sensitivity of the LacZ marker rescue assay was compared with that of PCR tests. By the LacZ marker rescue assay, *lacZ*-positive signals were detected in a 10^4 -fold-diluted sample at 4 days after inoculation (Fig. 4), and the endpoint dilution (10^5 -fold dilution) could be determined at 12 days after infection. By the PCR test using *env* primers, proviral DNA was detected to be as sensitive as the LacZ marker rescue assay; however, the sensitivity was lower when the PCR test was conducted using *pol* primers (Fig. 4).

Fig. 2. Determination of optimal concentration of polybrene (A) and viral adsorption period (B). (A) *lacZ*(RD114) virus was inoculated into TE671 cells with various concentrations of polybrene during viral adsorption. (B) *lacZ*(RD114) virus was inoculated into TE671 cells with different adsorption periods. Three independent experiments were performed, and averages with standard deviations are shown.

Fig. 3. Growth kinetics of RD114 virus on TE671(LacZ) cells. One thousand-fold-diluted stock RD114 virus was inoculated into TE671(LacZ) cells, and the viruses produced in the culture supernatants were titrated on TE671 cells by the LacZ marker rescue assay. Three independent experiments were performed, and averages with standard deviations are shown.

Receptor interference of RD114 virus: Finally, we examined whether *lacZ*(RD114) virus can interfere with homologous RD114 virus on TE671 cells. As a control, we used *lacZ*(FeLV-B) virus. *lacZ*(FeLV-B) virus infected both TE671 and TE671/RD114 cells; however, *lacZ*(RD114) virus infected only TE671 (Fig. 5). These data indicate that RD114 virus completely interferes with homologous RD114 virus on TE671 cells.

DISCUSSION

Many animal cell lines produce defective (*i.e.*, replication-incompetent) retroviral particles; however, some cells produce replication-competent (*i.e.*, infectious) as well as defective ERVs. In vaccines, contamination of replication-competent ERVs should be excluded; therefore, there is a need to monitor infectious ERVs. To detect retroviruses, reverse transcription (RT)-PCR, reverse transcriptase (RT) activity assay and enzyme-linked immunosorbent assay (ELISA) have been developed; however these assays also

Fig. 4. Comparison of sensitivity of LacZ marker rescue assay with PCR test. Serially diluted (10^{-1} to 10^{-6}) stock RD114 virus was inoculated into TE671(LacZ) cells (lanes 1 to 6). At the indicated times, culture supernatants and genomic DNA were subjected to the LacZ marker rescue assay and the PCR test, respectively. Genomic DNAs from TE671 (N) and TE671(RD114) cells (P) were used for the templates as negative and positive controls in the PCR tests. The numbers of foci counted in 2 wells of a 24 multi-well plate are shown in parentheses.

Fig. 5. Receptor interference of RD114 virus on TE671 cells. *lacZ*(RD114) and *lacZ*(FeLV-B) viruses were titrated on both TE671 and TE671/RD114 cells. Three independent experiments were performed, and averages with standard deviations are shown.

detect defective viral particles. To detect infectious ERVs exclusively, samples are inoculated into susceptible cells, and then the viruses replicated in the cells are detected. In this study, we showed that human TE671 cells are most susceptible to RD114 virus, and the cells support replication of the RD114 virus efficiently.

To detect replicated RD114 viruses in TE671 cells, several methods may be applicable: RT activity assay, PCR, RT-PCR, indirect immunofluorescent assay, immunostain-

ing, and a marker rescue assay; however, an anti-RD114 virus antibody which can be used for indirect immunofluorescent assay and immunostaining is not available to us at the moment. There is a risk of cross-contamination in the PCR and RT-PCR assays during the steps of DNA or RNA preparation and amplification. RT activity assay requires radioisotopes (³²P or ³H) with a facility for the radioisotopes or a commercial ELISA kit, which is rather expensive. A LacZ marker rescue assay can be carried out easily and does not require special equipment such as a thermal cycler, a UV microscope and an ELISA reader; therefore, we decided to establish the LacZ marker rescue assay and compare the sensitivity of the assay with the PCR test.

We could detect RD114 virus produced in TE671 cells inoculated with limiting diluted RD114 virus (*i.e.*, less than 10 infectious units) from 12 days after inoculation (passaged three times on day 1, 4 and 8) (Fig. 4). The LacZ marker rescue assay was as sensitive as the PCR test using *env* primers. We have not compared the sensitivity of the marker rescue assay with nested PCR to detect proviral DNA or RT-PCR to detect viral transcripts in infected cells. It is possible that these assays are more sensitive than single-step PCR and can determine the end-point dilution earlier than the LacZ marker rescue assay.

Taken together, in this study, we developed a method to detect infectious RD114 virus by the LacZ marker rescue assay. Based on the results, we propose a standard protocol of the assay (Fig. 6). Samples are inoculated into TE671(LacZ) cells and cultured for 12 days (passaged three times), and then the presence of the RD114 virus in the culture supernatant is detected by the LacZ marker rescue assay

Fig. 6. Standard protocol for detection of infectious RD114 virus. (A) Samples are inoculated into TE671(LacZ) cells, which are cultured for 12 days. If RD114 virus is present in the sample, the virus grows efficiently in TE671(LacZ) cells. The culture supernatant of the cells is then inoculated into both TE671 and TE671/RD114 cells. Two days later, the cells are stained with X-gal and examined for the presence of replicated RD114 virus. (B) Time schedule of the LacZ marker rescue assay.

on TE671 cells. The viral adsorption period should be more than 4 hr in the presence of 2 to 8 $\mu\text{g/ml}$ polybrene. The specificity of the RD114 virus can be checked by the receptor interference assay or the PCR test, as shown in Figs. 4 and 5. The assay is applicable for monitoring the contamination of infectious RD114 viruses in multivalent live attenuated vaccines for cats, because human TE671 cells are not susceptible to feline herpesvirus type 1, feline calicivirus and feline panleukopenia virus, which are the components of the vaccines.

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