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Endogenous retroviruses as potential hazards for vaccines

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ABSTRACT

Retroviruses are classified as exogenous or endogenous according to their mode of transmission. Generally, endogenous retroviruses (ERVs) are not pathogenic in their original hosts; however, some ERVs induce diseases. In humans, a novel gammaretrovirus was discovered in patients with prostate cancer or chronic fatigue syndrome. This virus was closely related to xenotropic murine leukemia virus (X-MLV) and designated as xenotropic murine leukemia virus-related virus (XMRV). The origin and transmission route of XMRV are still unknown at present; however, XMRV may be derived from ERVs of rodents because X-MLVs are ERVs of inbred and wild mice. Many live attenuated vaccines for animals are manufactured by using cell lines from animals, which are known to produce infectious ERVs; however, the risks of infection by ERVs from xenospecies through vaccination have been ignored. This brief review gives an overview of ERVs in cats, the potential risks of ERV infection by vaccination, the biological characteristics of RD-114 virus (a feline ERV), which possibly contaminates vaccines for companion animals, and the methods for detection of infectious RD-114 virus.

1. Exogenous and endogenous retroviruses (ERVs)

Retroviruses are classified as exogenous or endogenous according to their mode of transmission. Exogenous retroviruses are transmitted horizontally by infection, and they infect somatic cells but not germ line cells. On the other hand, endogenous retroviruses (ERVs) are retroviruses that have been integrated into germ line cells [5]. ERVs are inherited by offspring from parents in a classical Mendelian fashion. ERVs occupy about 10% of mammalian genomes and are mostly inactivated by deletions and mutations with stop codons [5]; however, some ERVs retain open reading frames (ORFs) which encode proteins. Certain ERVs express envelope proteins (Env) that block pathogenic exogenous retroviruses; for instance, cats express the Env of endogenous feline leukemia virus (FeLV) that block exogenous FeLV subgroup B [10].

Exogenous retroviruses are classified into seven genera, *i.e.*, alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus, spumaretrovirus, and lentivirus. ERVs are divided into at least three classes, I, II and III [5]. Type I ERV is closely related to exogenous counterparts of gammaretrovirus and epsilonretrovirus. Type II and III ERVs are similar to alpharetrovirus and betaretrovirus, and spumavirus, respectively.

2. Potential risk of infection by ERVs

Technical innovation of animal engineering enables us to develop genetically engineered pigs for the purpose of xenotransplanting pig organs or tissues to humans; however, pigs have replication-competent ERVs, termed porcine ERVs (PERVs) [17]. The discovery of PERVs able to infect human cells led to the halt of the clinical trials of xenotransplantation, and the risks of PERVs in xenotransplantation have been investigated extensively. Due to the presence of infectious ERVs in non-human species, the control subjects in xenotransplantation were expanded by the recent definition of xenotransplantation. Xenotransplantation (from animals to humans) is now defined as follows; any procedure that involves the transplantation, implantation, or infusion into a human recipient of either (a) live cells, tissues or organs from a non-human animal source, or (b) human body fluids, cells, tissues or organs that have had ex vivo contact with live non-human cells, tissues or organs.

Generally, ERVs are not pathogenic in their original hosts; however, some ERVs induce diseases; for example, ERVs from AKR mice induce lymphoma in their hosts [11]. Certain ERVs infect new hosts and induce diseases; there was an incident in which an ERV from Asian rodents infected Gibbon apes and induced lymphoma [19]. Moreover, a retrovirus emerged in koalas in Australia about two hundred years ago, and endogenized [19]. The virus, named koala retrovirus, induces neoplastic diseases and immune suppression in the new host. In humans, a novel gammaretrovirus was discovered recently in patients

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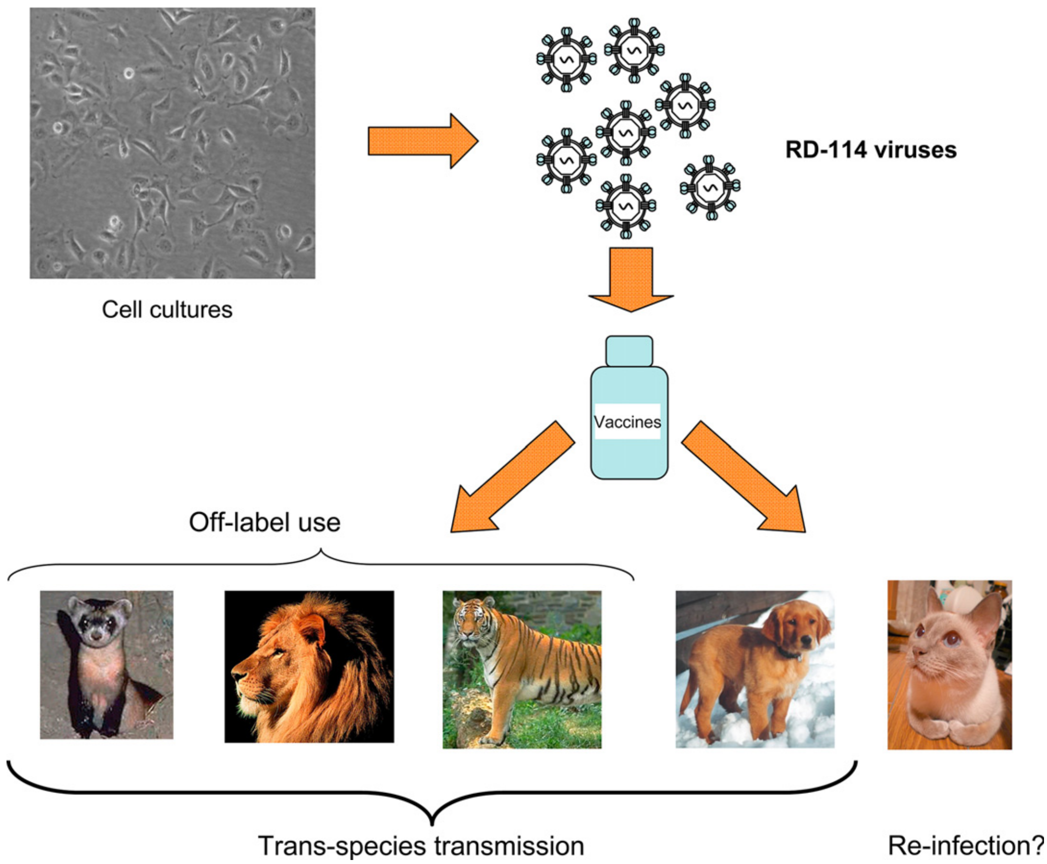

Fig. 1. Potential risks of contamination of RD-114 virus in live attenuated vaccines. Certain feline cell lines produce RD-114 viruses. If these cells are used for manufacturing vaccines for feline and canine infectious viral agents, RD-114 virus will contaminate the vaccines. RD-114 virus-contaminated vaccines may be used in companion animals and exotic animals as off-label use.

with prostate cancer [21]. This virus was closely related genetically to the xenotropic murine leukemia virus (X-MLV) and designated as xenotropic murine leukemia virus-related virus (XMRV). Currently, it is still controversial whether XMRV induces prostate cancer; however, several reports have been published, strengthening the link between infection with XMRV and prostate cancer in the USA. Quite recently, XMRV was found to be frequently isolated from patients with chronic fatigue syndrome in the USA [8] and a relationship between XMRV infection and the disease is suspected. The origin and transmission route of XMRV are still unknown at present; however, XMRV is considered to be derived from ERVs of rodents because X-MLVs are ERVs of inbred and wild mice.

In the veterinary science area, at least mice, pigs, cats and chickens have infectious ERVs. Many live attenuated vaccines for animals are manufactured by using cell lines from these animals. In addition, several live attenuated vaccines are manufactured by using cells which are known to produce infectious ERVs; however, the risks of infection by ERVs from xenospecies have been ignored. According to the definition, the use of vaccines manufactured using cells from xenospecies is not xenotransplantation. The discovery of XMRV prompted us to study the risks of ERVs in live attenuated vaccines. This brief review focuses on feline ERVs possibly contaminating vaccines for companion animals.

3. ERVs in cats

At least two ERVs, endogenous FeLV and RD-114 virus, are present in the cat genome. In addition to these ERVs, two additional ERVs have been reported. Bonner and Todaro reported that cats may contain a third group of ERV, distantly related to the primate virus MAC-1 [2]. Haapala et al. also reported a novel endogenous retrovirus which is related to RD-114 virus [6]; however, no further studies have been performed on these ERVs. There are about 20 copies of endogenous FeLV in the cat genome, and at least two loci have ORF encoding Env [7]. Extensive genetic analyses revealed that there is no infectious locus in the cat genome. On the other hand, all domestic cats (*Felis catus*) have an entire RD-114 genome and sometimes the virus is produced spontaneously or induced in vitro from feline cells by several chemical reagents [14].

4. Biological characteristics of RD-114 virus

Besides domestic cats, the provirus of RD-114 virus is also present in the genome of other feline species belonging to the genus *Felis*; however, there is no information on whether they also have infectious loci. RD-114 viral genomes have not been detected in large felids, such as lions and pumas; therefore, it is considered that RD-114 virus endogenized in the ancestral species of the genus *Felis* before branching into each species of the genus. In early studies, RD-114 virus was found to be closely related to baboon endogenous retrovirus (BaEV); therefore, RD-114 virus was considered to have originated in baboons. Van der Kyel et al. reported that RD-114 virus is a recombinant virus between a feline endogenous retrovirus termed FcEV and a type D simian retrovirus [22]. The *gag*–*pol* region of RD-114 virus is similar to gammaretroviruses, and the *env* region is closely related to betaretroviruses and is nearly identical to BaEV. Now, it is considered that BaEV infected an ancestor of the domestic cat lineage, but it was a *de novo* recombinant that made its way into the cat germ line [22].

RD-114 was first isolated from a human tumor cell line (RD cells) derived from a human rhabdomyosarcoma after passage through fetal cats, and is thought to be xenotropic, *i.e.*, RD-114 virus does not productively infect feline cells. However, RD-114 virus is known to infect several feline cell lines [1,4] in addition to cell lines from xenospecies such as humans and dogs; therefore, RD-114 virus is polytropic, but is not strictly xenotropic in vitro. In human cell lines, RD-114 virus interferes with BaEV, simian retroviruses 1, 2, 3, 4 and 5 (primate betaretroviruses), avian reticuloendotheriosis virus, and duck spleen necrosis virus; therefore, these retroviruses are considered to utilize the same receptor in human cells. In 1999, two groups independently identified the receptor for this large interference virus group [13,18]. The receptor for RD-114 virus is a sodium-dependent neutral amino acid transporter, termed ASCT2 [9]. ASCT2 is a multi-spanning (8 times) transmembrane protein with 10 hydrophobic regions (2 regions do not traverse the cell membrane) and five extracellular loops [9]. Both mice and humans have two types of ASCT molecules, termed ASCT1 and ASCT2. The homology between ASCT1 and ASCT2 is approximately 57% and amino acids transported by ASCT1 and ASCT2 are not identical. RD-114 virus utilizes both human ASCT1 and ASCT2, but the virus uses ASCT2 more efficiently than ASCT1. RD-114 virus does not infect murine NIH3T3 cells; however, the virus infected the cells when they were treated with tunicamycin [9]. BaEV infects NIH3T3 cells, but mouse ASCT2 does not function as BaEV receptor. BaEV utilizes ASCT1 instead of ASCT2 to infect murine cells [9]. RD-114 virus productively infects both canine and feline cell lines; however, there are no reports on the usage of ASCT molecules as the receptor for RD-114 virus in these species. Recently, it was reported that human endogenous retrovirus W (HERV-W) encodes an intact *env* ORF called syncytin-1, which is involved in placental morphogenesis. Interestingly, HERV-W Env also utilizes ASCT2 as a receptor. It is unknown whether the RD-114 viral sequence is involved in placental morphogenesis in cats.

RD-114 virus is also used as a retroviral vector for gene therapy. The pseudotype virus bearing RD-114 viral Env is physiologically stronger than that bearing MLV Env [12]. Interestingly, viruses bearing RD-114 Env produced from certain human cells are stable in fresh human serum. In contrast, viruses bearing amphotropic MLV Env are sensitive, even when produced from human cells [12]. This phenotype is advantageous to in vivo gene therapy in humans. In dogs, X-linked severe combined immunodeficiency was corrected by intravenous injection of concentrated RD-114-pseudotyped retrovirus vector encoding the interleukin-2 receptor γ chain [20]. These findings clearly indicate that dogs are susceptible to pseudotype virus bearing RD-114 virus Env in vivo.

5. Potential contamination of vaccines for companion animals by RD-114 virus

Infectious RD-114 virus and RD-114-like virus are known to be produced from several feline cell lines, such as Crandel-Rees feline kidney (CRFK) cells (ATCC CCL-94) [1], MCC cells derived from feline large granular lymphoma [3], and FER cells (ECACC catalogue number: 90031401) derived from feline fetal fibroblast cells. RD-114 virus grows efficiently in feline and non-feline cell lines except murine cells. RD-114 virus can contaminate viral vaccines for cats and dogs when RD-114 virus-producing cells are used for vaccine manufacturing. Indeed, seed stocks of some vaccines are currently prepared by using feline cells that may produce RD-114 virus (Fig. 2).

Since all feline cells have the potential to produce infectious RD-114 virus, exclusion of RD-114 virus requires the elimination of RD-114 viruses from seed stocks and the use of non-feline cells for vaccine production.

Contaminated vaccines may be used in cats and dogs, and exotic animals such as ferrets and large felids reared in zoos as off-label use. The susceptibility of vaccinated animals to RD-114 virus is

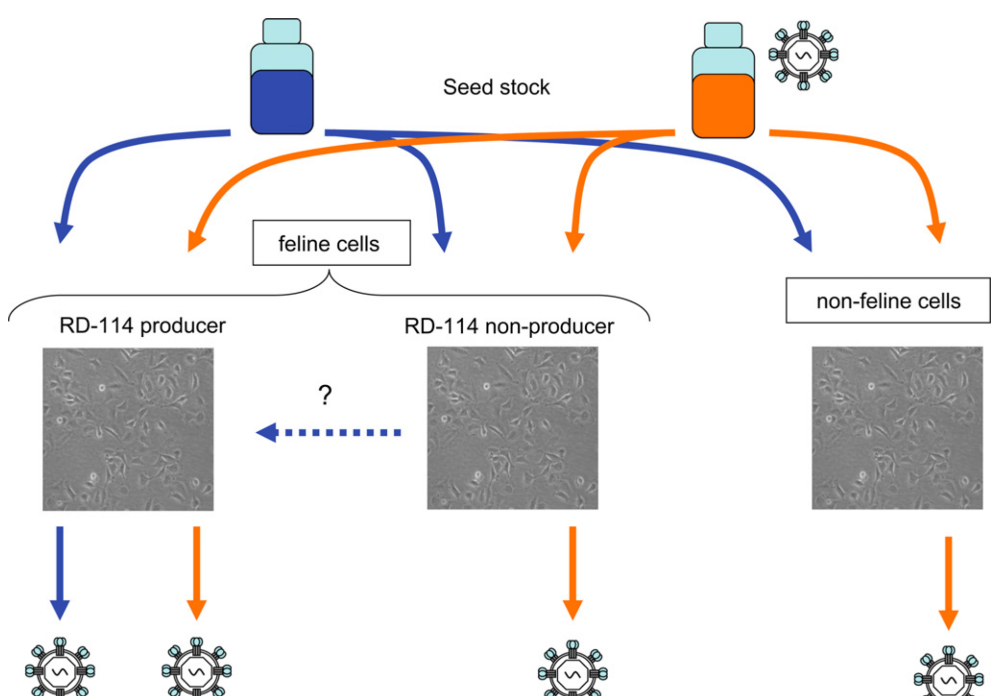

Fig. 2. Possible routes of contamination of RD-114 virus in vaccines. RD-114 virus grows efficiently in feline and canine cell lines and some viral seed stocks are prepared by using feline cells which produce RD-114 virus. When seed stocks are contaminated with RD-114 virus, vaccines may be contaminated with RD-114 virus. When vaccines are manufactured using RD-114 virus-producer cells, the vaccines are contaminated with RD-114 virus. RD-114 non-producer cells may change spontaneously to produce RD-114.

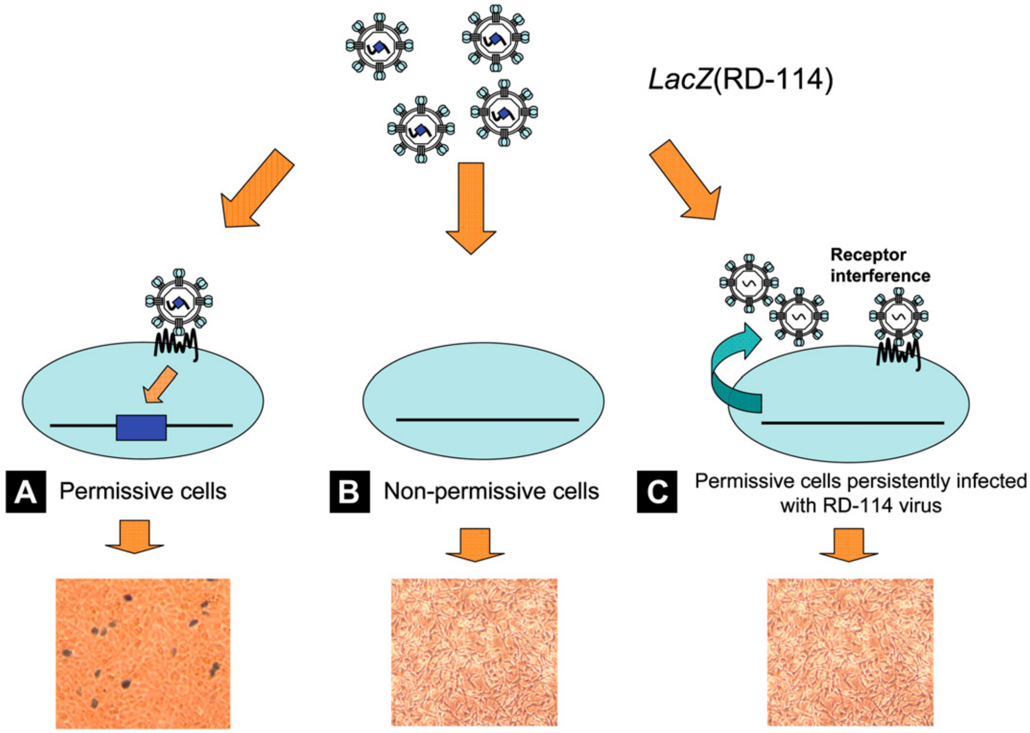

Fig. 3. Principles of LacZ assay and receptor interference. Details are described in the text.

unknown at present; however, RD-114 virus may be transmitted to these non-feline species because of the xenotropic features of the virus in vitro. In addition, RD-114 virus may infect cats when they are inoculated with infectious RD-114 virus because cats have functional receptors for RD-114 virus.

6. Detection systems of RD-114 virus

There are several methods to detect retroviruses, such as electron microscopy, reverse transcriptase (RT) activity assay, RT-polymerase chain reaction (RT-PCR), and real-time RT-PCR tests;

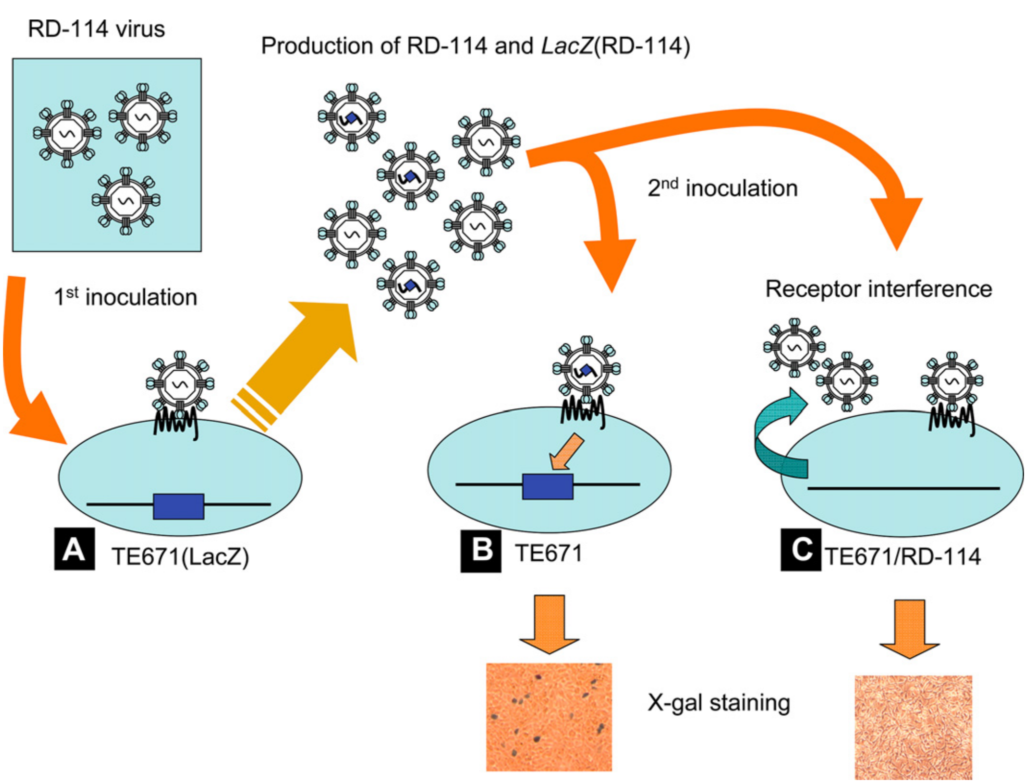

Fig. 4. Principle of LacZ marker rescue assay. Details are described in the text (modified from Sakaguchi et al. [15] with permission from the publisher).

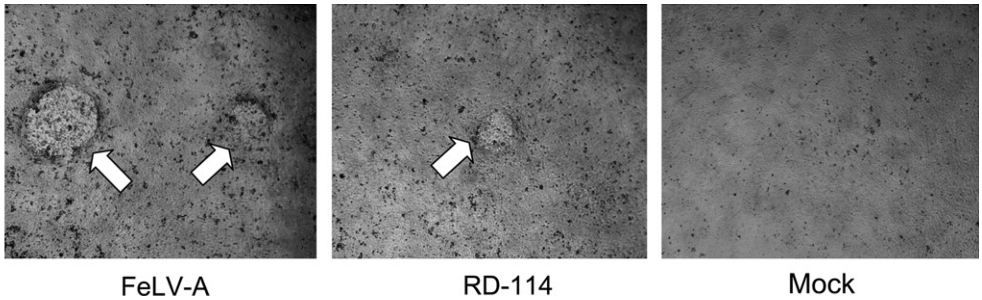

Fig. 5. Foci induced by RD-114 virus and FeLV subgroup A (FeLV-A) in QN10S cells. Mock-infected QN10S cells are also shown. Cells were observed with a phase-contrast microscope. White arrowheads indicate transformed foci (reproduced from Sakaguchi et al. [16] with permission from the publisher).

however, these assays do not measure the infectivity of the virus. Feline cell lines may produce non-infectious viral particles and cellular RNAs may be transcribed. To test infectious ERV particles, it is necessary to check the infectivity of cells susceptible to RD-114 virus. Because RD-114 virus does not induce cytopathic effects like other gammaretroviruses, and antibodies against RD-114 virus are not commercially available, we developed a LacZ marker rescue assay [15] and a focus assay using sarcoma (+) lymphoma (−) (S+L−) cells [16].

6.1. LacZ marker rescue assay

The principle of the LacZ marker rescue assay is illustrated in Figs. 3 and 4. *LacZ* pseudotype virus bearing RD-114 virus Env (*LacZ* (RD-114)) infects permissive cells expressing receptors for RD-114 virus (Fig. 3A). After inoculation of *LacZ* (RD-114), the cells are fixed and stained with β-gal. The nuclei of infected cells are stained blue, because the *LacZ* gene product contains a nuclear localization signal. If the cells do not express functional receptors, they are not infected with the virus (Fig. 3B). When permissive cells are persistently infected with RD-114 virus, the cells are resistant to *LacZ* (RD-114) due to receptor interference (Fig. 3C).

Prior to the LacZ marker rescue assay, human TE671 cells, which are derived from rhabdomyosarcoma cells and highly susceptible to RD-114 virus, are transduced with *nlsLacZ* gene to become TE671 (LacZ) cells (Fig. 4A). From the transgene, mRNA of *LacZ* gene with a packaging signal of MLV is constitutively expressed. Because RD-114 virus has viral core proteins belonging to gammaretroviruses, the mRNA with the packaging signal of MLV can be incorporated into the RD-114 viral core and rescued. To detect infectious RD-114 virus, samples are inoculated into TE671 (LacZ) cells, which are cultured for more than 12 days (Fig. 1A). When RD-114 virus is present in the sample, the virus grows efficiently in TE671 (LacZ) cells, and produces RD-114 virion. Most virions from the cells contain *LacZ* gene in the particles. The culture supernatant of the cells is then inoculated into either TE671 cells (Fig. 4B) or TE671 cells persistently infected with RD-114 virus (TE671 (RD-114)) (Fig. 4C). Two days later, cells are stained with β-gal and examined for the presence of *LacZ* (RD-114) in the culture supernatant of TE671 (*LacZ*) cells inoculated with the sample. Using this protocol, we could detect infectious RD-114 virus in end-point-diluted samples earlier than in single-step PCR using genomic DNA of RD-114 virus-inoculated TE671 cells [15].

6.2. Focus assay

The presence of replication-competent gammaretroviruses in biological materials, such as retroviral vectors for gene therapy, is assessed by the S+L− assay (focus assay). Exogenous FeLVs can be detected and titrated using feline S+L− cell lines, such as QN10S cells. Because QN10S cells are susceptible to a pseudotype virus bearing RD-114 Env [1], we applied QN10S cells for the S+L− assay to detect infectious RD-114 virus [16]. Consequently, QN10S cells formed foci by infection with RD-114 virus; however, the foci induced by RD-114 virus were generally smaller than those induced by FeLV (Fig. 5). In addition, the sensitivity of the S+L− assay was lower than that by the LacZ marker rescue assay for detection of infectious RD-114 virus [16]. Therefore, the S+L− assay using QN10S cells is not suitable to detect a small amount of RD-114 virus; however the assay will be useful for virological studies of RD-114 virus.

7. Concluding remarks

As long as feline cells are used to produce vaccines, there is a risk that infectious RD-114 virus contaminates live attenuated vaccines. Because RD-114 virus productively infects cells from cats and dogs, the virus can infect these animals in vivo. Since certain ERVs infect new host species and induce diseases, the potential risks of infection by ERVs in humans and animals should be reconsidered. Recently, we developed a sensitive assay system, LacZ marker rescue assay, to detect infectious RD-114 virus. We are currently investigating the presence of infectious RD-114 virus in commercial live attenuated vaccines for companion animals.

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