RESULTS

Serologic and genetic analysis of the Hit family

Figure 1 shows the Hit family tree. As seen, we detected the Hit antigen in three generations of the family based on the serologic and genetic studies. These studies indicate that Hit^a was inherited from the paternal grandfather.

PLT genotyping of the Hit family members was performed using PCR with sequence-specific primers or PCR-RFLP for HPA-1, -2, -3, -4, -5, -6, -7, -8, and -15. The genotyping results are presented in Table 1. Although the genotypes of the mother's and infant's PLTs exhibited two incompatibilities (HPA-2 and HPA-3 types), the Hit serum did not react with any of the typed panel PLTs, including HPA-1a, -1b, -2a, -2b, -3a, -3b, -4a, -4b, -5a, -5b, -6a, and -6b antigens. Crossmatching with Hit maternal serum and the paternal and infant PLTs gave strongly positive results, both with and without chloroquine treatment, which removes HLA antigens from PLTs, suggesting the presence of alloantibodies against a private or a low-frequency antigen.

Furthermore, no HLA antibodies were detected in the mother's serum using the LABScreen PRA I and II kits. However, the results of the crossmatch test were consistent with the existence of an antibody against PLT antigens, which suggested the presence of a previously unidentified PLT-specific antigen.
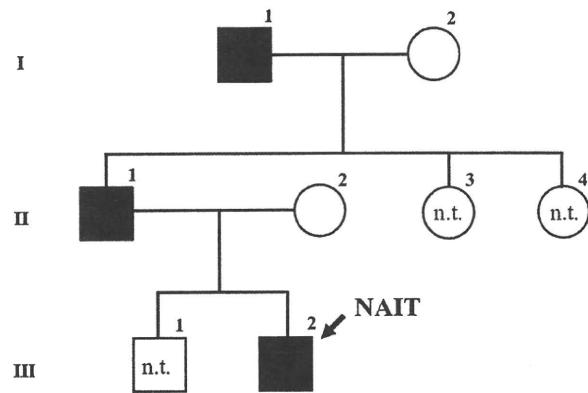
When the Hit mother's serum was analyzed using five-cell-lineage FCM analysis, only the father's PLTs were positive (data not shown). The results of five-cell-lineage FCM analysis also supported the existence of a PLT-specific antigen.

MAIPA and inhibition test using MoAbs to GP proteins

To localize the detected antigen molecule, Hit serum was employed using a modified MAIPA with anti-CD41a (GPIIb), anti-CD61 (GPIIIa), anti-CD49b (GPIa/IIa), or anti-CD42b (GPIb). The results of the MAIPA show that anti-CD41a and -CD61 were positive, whereas anti-CD42b and -CD49b were negative (data not shown). The MAIPA data indicated that the Hit antigen was located on the GPIIb/IIIa complex molecule. As the next step, we determined whether the antigen was located on GPIIb or GPIIIa by conducting an inhibition test using flow cytometry. We also performed inhibition tests using MoAbs specific for CD36 (GPIV), CD41a (GPIIb), CD42a (GPIX), and CD61 (GPIIIa). The data of the inhibition test indicated that the antibody in the Hit serum has specificity for an epitope on the GPIIIa molecule but not on GPIIb (Fig. 2).

Genetic analysis of the Hit antigen of the GPIIIa gene

On the basis of the serologic results, we anticipated that the epitope of the Hit antigen might exist on the GPIIIa molecule. We therefore analyzed the whole exon-specific sequences of the GPIIIa gene. The results showed that the ninth exon of the GPIIIa gene had a C to T substitution base at np 1297 (Fig. 3). This C>T mutation occurs at the same nucleotide position as the single-nucleotide polymorphism of HPA-7b, the latter of which involves a C-to-G substitution in the GPIIIa gene. The C-to-T substitution changes a CCC codon for proline into a TCC that codes for

Fig. 1. Pedigree of the Hit family of the infant with NAIT attributed to Hit^a. The thrombocytopenic infant is indicated by an arrow. (□) Hit^a (−); (■) Hit^a (+). n.t. = not tested.

TABLE 1. PLT alloantigens of the Hit family*

Family member	HPA genotype
Infant	1a/a, 2a/b, 3a/b, 4a/a, 5a/a, 6a/a, 7a/a,† 8a/a, 15a/b
Father	1a/a, 2a/a, 3a/b, 4a/a, 5a/a, 6a/a, 7a/a, 8a/a, 15a/b
Mother	1a/a, 2b/b, 3a/a, 4a/a, 5a/a, 6a/a, 7a/a, 8a/a, 15a/b

* Underlines indicate the incompatibilities between the mother and infant.
† HPA-7 type (7a/a) in this table does not indicate HPA-7b.

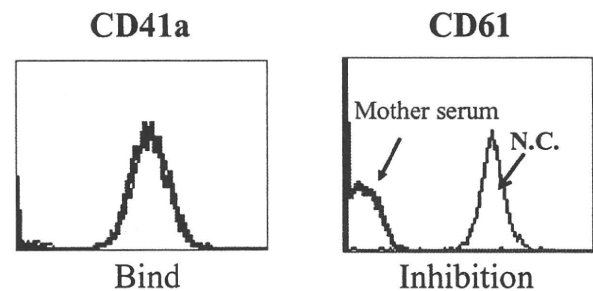### CD41a   CD61

Bind   Inhibition

Fig. 2. Flow cytometry inhibition analysis with the mother's serum or negative control serum and the father PLTs using various MoAbs against GP molecules. Among the antibodies tested, only CD61 (GPIIIa) reacted differently between the mother's serum and the negative control serum (N.C.).
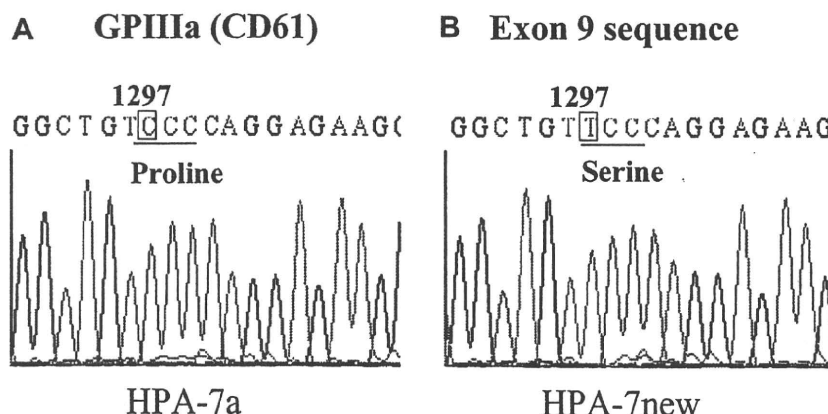
A GPIIIa (CD61)   **B Exon 9 sequence**

Fig. 3. Nucleotide sequence analysis of the GPIIIa gene from the Hit[a] (+) father (B) and a normal donor Hit[a] (–) (A). Exon 9 of the β3 gene was amplified by PCR, cloned using a TA cloning kit, and sequenced. A heterozygous C>T transition at np 1297 was identified in the father. This mutation results in the replacement of a proline with a serine at Position 407 in the wild-type (HPA-7a).
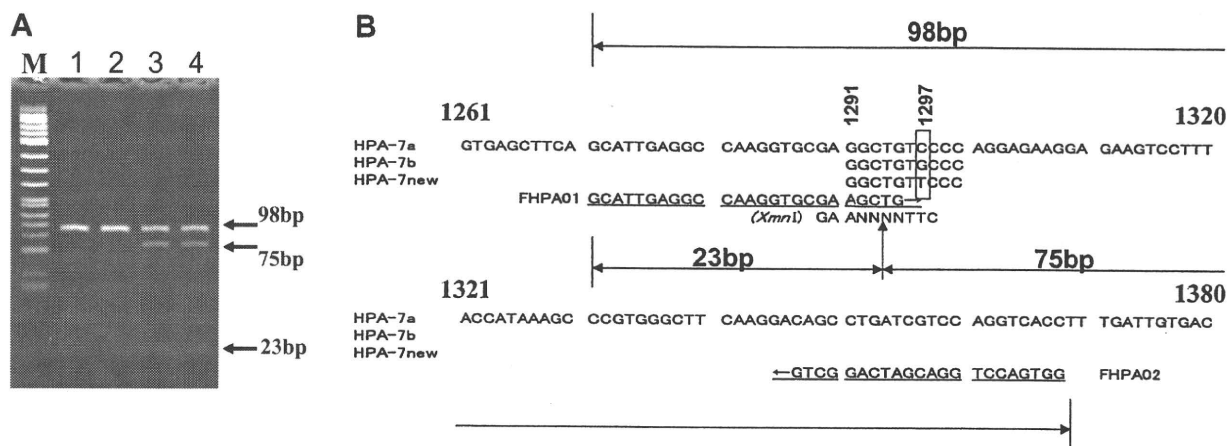

Fig. 4. (A) Amplified 98-bp GPIIIa fragments were digested with *Xmn*I endonuclease and subsequently analyzed electrophoretically using 10% polyacrylamide gel electrophoresis gels. Lane M = molecular weight marker, ΠX174/*Hinf*I digest; Lane 1 = undigested 98-bp PCR products from the mother; Lane 2 = *Xmn*I-digested PCR products from the mother Hit[a] (–); Lane 3 = *Xmn*I-digested PCR products from the father, Hit[a] (+) heterozygote; Lane 4 = *Xmn*I-digested PCR products from the infant, Hit[a] (+) heterozygote. (B) Restriction site map of the PCR-amplified 98-bp fragment of GPIIIa. The PCR strategy is shown, along with the fragments after RFLP analysis using *Xmn*I. A sequence of a part of Exon 9 of the wild-type GPIIIa gene (HPA-7a) from np 1261 to np 1380, and the sequences around the *Xmn*I restriction site in HPA-7b and the new HPA-7 allele are shown. One mismatch primer (FHPA01) sequence is also shown; this primer was used to create a restriction site in the new HPA-7 allele. The vertical arrow indicates the position of the restriction site for *Xmn*I. "HPA-7new" in this figure indicates the new third allele of HPA-7.

serine at Amino Acid 407 of the GPIIIa protein. The amino acid at the same position in HPA-7b is alanine. Furthermore, we confirmed the C>T mutation by PCR-RFLP analysis with a mismatch primer (Fig. 4) using the *Xmn*I, which digested the new third allele, but neither HPA-7a nor HPA-7b. Additionally, the PCR products treated with *Bsp1286*I, which digests only the HPA-7b allele, gave no digestion products (data not shown). The presence of this novel allele was confirmed by using other methods, that is,

PCR-RFLP and sequence-based typing, for the Exon 9-specific clones.

Analysis of recombinant GPIIIa allelic isoforms

To examine the involvement of Amino Acid 407 in the formation of the Hit antigenic determinants, we transfected allele-specific expression vectors coding for 407serine and 407proline into 293T cells. Stable
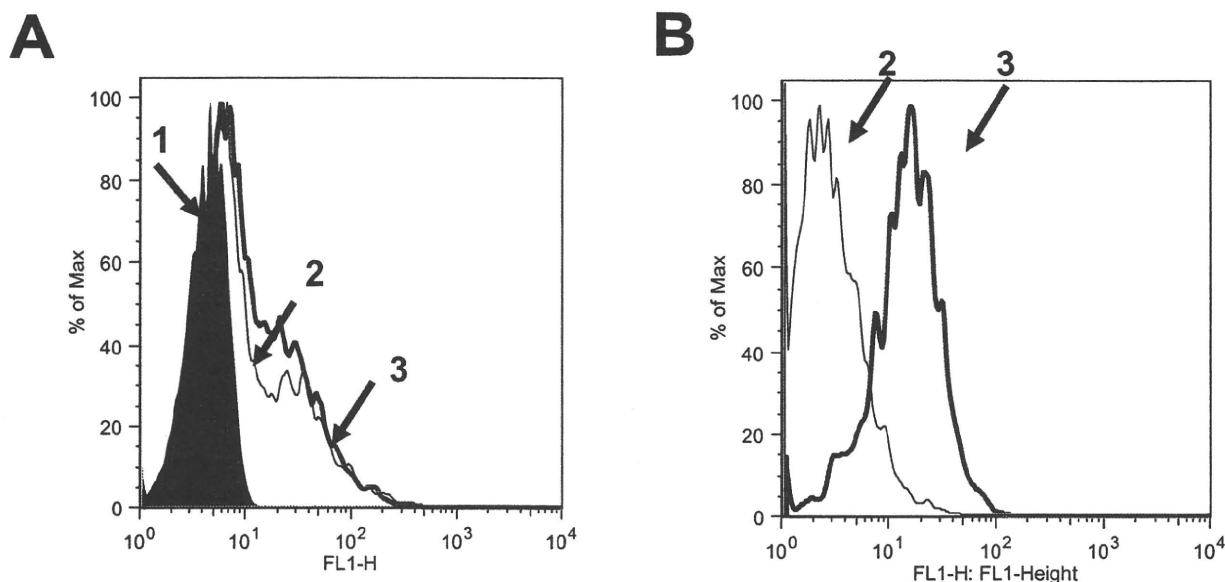
A

B

Fig. 5. Reactions of serum samples from the Hit mother with 293T cells expressing mutated GPIIIa (407Ser) or nonmutated GPIIb/IIIa. (A) Anti-GPIIb/IIIa MoAb reacted equally well with both constructs but not with non–GPIIb/IIIa-transfected cells (shaded peak). (B) The mother's serum sample reacted with 293T cells expressing GPIIb/IIIa containing the new HPA-7 mutation (bold lines) but not with cells expressing nonmutated GPIIb/IIIa (thin lines). Numbers 1, 2, and 3 indicate samples from non–GPIIb/IIIa-transfected cells, nonmutated GPIIb/IIIa-transfected cells, and mutated GPIIb/IIIa-transfected cells, respectively.

Donor number	HPA genotype
TABLE 2. HPA genotypes of the Hit-positive blood donors	
739	1a/a, 2a/a, 3a/b, 4a/a, 5a/a, 6a/a, 7a/new,* 15a/a
743	1a/a, 2a/a, 3b/b, 4a/a, 5a/a, 6a/a, 7a/new, 15a/b
744	1a/a, 2a/a, 3a/b, 4a/a, 5a/a, 6a/a, 7a/new, 15a/b
751	1a/a, 2a/b, 3b/b, 4a/a, 5a/a, 6a/a, 7a/new, 15b/b
752	1a/a, 2a/a, 3b/b, 4a/a, 5a/a, 6a/a, 7a/new, 15a/b
754	1a/a, 2a/a, 3b/b, 4a/a, 5a/a, 6a/a, 7a/new, 15b/b
786	1a/a, 2a/b, 3a/b, 4a/a, 5a/b, 6a/a, 7a/new, 15b/b
* "New" indicates the new third allele of HPA-7.	

transfectants expressing GPIIIa recombinant proteins were analyzed by FCM. The mother's serum reacted only with the transfected cells containing the mutated GPIIIa allele (Fig. 5A). In control experiments, the MoAb to GPIIb/IIIa reacted with cells containing both the mutated and the nonmutated GPIIIa alleles (Fig. 5B), whereas normal human serum reacted with neither type of transfected cells (data not shown).

Serologic screening test with Hit using MPHA and DNA typing for Hit[a]-positive blood donors

Using MPHA, we screened 4536 Osaka blood donors with the Hit maternal serum. Of these donors, seven were found to be Hit[a]-positive. Table 2 shows the HPA genotypes (HPA-1, -2, -3, -4, -5, -6, and -15) of the seven donors.

The presence of a heterozygous new third allele in each of the seven donors was confirmed by DNA typing. This implies that Hit[a] has a 0.15% (1/648) frequency in the Osaka area.

DISCUSSION

We experienced a typical case of NAIT in the second child of a Japanese woman, which was caused by an unknown antibody in the mother's serum that did not react with any known HPA antigen, but exhibited a clear reaction with the father's PLTs. We suspected a new HPA antigen and investigated the cause of the NAIT by analyzing the mother's serum using various serologic methods. Furthermore, a screening study in 4536 unrelated Japanese blood donors identified other Hit-positive individuals, showing that this low-frequency antigen is present in the Japanese population outside the propositus family.

The first routine serologic study using MPHA showed that the Hit[a] antigen localized on the cells including PLTs. Using the five-cell-lineage immunofluorescence test, we confirmed that it localized only on the PLTs and not other WBCs. The next immunochemical analysis, MAIPA, and an inhibition test using MoAb to GP proteins revealed that the antigen resided on the GPIIIa molecule. Additionally, genetic analysis, including sequence analysis of the GPIIIa gene of the Hit father, showed a mutation (C>T) at np 1297 in the heterozygous state. The 1297C>T mutation, which changes a CCC codon for proline into a TCC for serine at

Amino Acid 407 of the GPIIIa protein, occurs at the same nucleotide position as the C > G mutation of HPA-7b. Thus, we identified a new third allele of the HPA-7 antigen. The seven Hit-positive blood donors also had the same heterozygous mutation in the GPIIIa gene (Table 2). The mutated molecule was strongly associated with the Hit[a] antibody. We also confirmed indirectly that the new antigen was not related to the HPA-15 antigens, which are polymorphic in many countries including Japan. First, Hit[a]-positive members in the Hit family all have the same HPA-15 type (HPA-15a/b), as determined by genotyping (Table 1). Second, the seven Hit[a]-positive donors have different HPA-15 types: three donors have HPA-15b/b, three have HPA-15a/b, and one has HPA-15a/a (Table 2).

To confirm that the epitope of the Hit antibody was on the mutated GPIIIa molecule, we performed transfection expression experiments. Anti-Hit[a] reacted only with the transfected 293T cells expressing the mutated GPIIIa molecule (Fig. 5).

The corresponding Hit antibody was found in the serum of the healthy mother of the NAIT infant. The new antigen has a serine at the 407th amino acid of the GPIIIa molecule, whereas HPA-7b has an alanine at the same position. As serine and alanine have similar structures, antibodies raised against the HPA-7b and HPA-7 new antigens might crossreact. Indeed, subsequent to this study, Morita and colleagues (personal communication, 2007) confirmed that anti-HPA-7b (Mo) reacted with the father's PLTs. However, since we do not have the HPA-7b PLTs, we have been unable to perform the crossmatch test between the Hit maternal serum and the HPA-7b PLTs. This test will be conducted when the HPA-7b PLTs become available.

The Hit serum reacted with the PLTs from the affected child, his father, and the child's grandfather (Fig. 1). DNA analysis, both direct sequencing and PCR-RFLP analysis, revealed that the grandmother and the seven positive donors have a 1297C>T mutation in the GPIIIa gene. These results also strongly support the contention that an epitope of the Hit[a] antigen is characterized by a 407proline > serine substitution in the GPIIIa protein. All 10 Hit[a]-positive individuals (seven donors and three members of the Hit family) have the new third HPA-7 allele. The phenotypic frequency of Hit[a] in the Japanese population is 0.15% and Hit[a]-homozygous individuals are extremely rare. Furthermore, to date, we have failed to detect HPA-7b in the Japanese population. The new third HPA-7 allele might therefore be characteristic of Japanese populations.

In the NAIT case we describe, the mother's serum clearly reacted with the infant's and father's PLTs, whereas it failed to react with any of the known HPA-1 to -6 antigens. The crossmatch test using maternal serum and paternal PLTs unexpectedly led to the discovery of a new HPA antigen. This finding clearly emphasizes the neces-sity to perform crossmatches for analysis of an unknown antigen of a clear NAIT case, because all the low-frequency HPA antigens (HPA-7 to -14 and -16) were discovered by similar analyses of NAIT cases.[10-18]

In summary, we have identified a third HPA-7 allele from a NAIT infant. This allele encodes a serine at the 407th residue of the GPIIIa molecule, contrasting with the 407th residue in HPA-7b, which is alanine. From a serologic screening of 4536 Japanese blood donors, we identified seven individuals (0.15%) positive for the new allele.

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

REFERENCES

1. Metcalfe P, Watkins NA, Ouwehand WH, Kaplan C, Newman P, Kekomaki R, deHaas M, Aster R, Shibata Y, Smith J, Kiefel V, Santoso S. Nomenclature of human platelet antigens. Vox Sang 2003;85:240-5.
2. Juji T, Saji H, Satake M, Tokunaga K. Typing for human platelet alloantigens. Rev Immunogenet 1999;1:239-54.
3. Kroll H, Santoso S, Bohringer M, Ernst Schlegel M, Adam K, Mueller-Eckhardt C. Neonatal alloimmune thrombocy-topenia caused by immunization against Oe[a], a new low frequency alloantigen on platelet glycoprotein IIIa. Blood 1995;86:540a.
4. Liu TC, Shin MC, Lin CL, Lin SF, Chen CM, Chang JG. Gene frequencies of the HPA-1 to HPA-8w platelet alleles in Taiwanese, Indonesian and Thai. Ann Hematol 2002;81:244-8.
5. Shibata Y, Matsuda I, Miyaji T, Ichikawa Y. Yuk[a], a new platelet antigen involved in two cases of neonatal alloim-mune thrombocytopenia. Vox Sang 1985;50:177-80.
6. Goldman M, Trudel E, Richard L, Khalife S, Spurll GM. Neonatal alloimmune thrombocytopenia due to anti-HPA-2b (anti-Koa). Immunohematology 2003;19:43-6.
7. von dem Borne AE, Simsek S, Vlekke AB, Kuijpers RW, Goldschmeding R. Bak[a], a new platelet-specific antigen involved in neonatal allo-immune thrombocytopenia. Vox Sang 1980;39:113-20.
8. Kaplan C, Morel-Kopp MC, Kroll H, Kiefel V, Schlegel N, Chesnel N, Mueller-Eckhardt C. HPA-5b (Br(a)) neonatal

alloimmune thrombocytopenia: clinical and immunological analysis of 39 cases. Br J Haematol 1991;78:425-9.

9. Kekomäki R, Jouhikainen T, Ollikainen J, Westman P, Laes MA. A new platelet alloantigen, Tua, on glycoprotein IIIa associated with neonatal alloimmune thrombocytopenia in two families. Br J Haematol 1993;83:306-10.

10. Kuijpers RW, Simsek S, Faber NM, Goldschmeding R, Van Wermerkerken RK, von dem Borne AE. Single point mutation in human glycoprotein IIIa is associated with a new platelet-specific alloantigen (Mo) involved in neonatal alloimmune thrombocytopenia. Blood 1993;83: 70-6.

11. Kroll H, Kiefel V, Santoso S, Mueller-Eckhardt C. Sr^a, a private platelet antigen on glycoprotein IIIa associated with neonatal alloimmune thrombocytopenia. Blood 1990; 76:2296-302.

12. Noris P, Simsek S, de Bruijne-Admiraal LG, Porcelijn L, Huiskes E, van der Vlist GJ, van Leeuwen EF, van der Schoot CE, von dem Borne AE. Max(a), a new low-frequency platelet-specific antigen localized on glycoprotein IIb, is associated with neonatal alloimmune thrombocytopenia. Blood 1995;86:1019-26.

13. Peyruchaud O, Bourre F, Morel-Kopp MC, Reviron D, Mercier P, Nurden A, Kaplan C. HPA-10w(b) (La(a)): genetic determination of a new platelet-specific alloantigen on glycoprotein IIIa and its expression in COS-7 cells. Blood 1997;89:2422-8.

14. Simsek S, Vlekke AB, Kuijpers RW, Goldschmeding R, von dem Borne AE. A new private platelet antigen, Groa, localized on glycoprotein IIIa, involved in neonatal alloimmune thrombocytopenia. Vox Sang 1994;67:302-6.

15. Kiefel V, Vicariot M, Giovangrandi Y, Kroll H, Böhringer M, Greinacher A, Breitfeld C, Santoso S, Mueller-Eckhardt C. Alloimmunization against Iy, a low-frequency antigen on platelet glycoprotein Ib/IX as a cause of severe neonatal alloimmune thrombocytopenic purpura. Vox Sang 1995;69: 250-4.

16. Santoso S, Amrhein J, Hofmann HA, Sachs UJ, Walka MM, Kroll H, Kiefel V. A point mutation Thr799Met on the α_2 integrin leads to the formation of new human platelet alloantigen Sit^a and affects collagen-induced aggregation. Blood 1999;94:4103-11.

17. Kelton JG, Smith JW, Horsewood P, Humbert JR, Hayward CP, Warkentin TE. Gova/b alloantigen system on human platelets. Blood 1990;75:2172-6.

18. Jallu V, Meunier M, Brement M, Kaplan C. A new platelet polymorphism Duv(a+), localized within the RGD binding domain of glycoprotein IIIa, is associated with neonatal thrombocytopenia. Blood 2002;99:4449-56.

19. Davoren A, Curtis BR, Aster RH, McFarland JG. Human platelet-specific alloantibodies implicated in 1162 cases of neonatal alloimmune thrombocytopenia. Transfusion 2004;44:1220-5.

20. Morel-Kopp MC, Blanchard B, Kiefel V, Joly C, Mueller-Eckhardt C, Kaplan C. Anti-HPA-4b (anti-Yuk^a) neonatal

21. Puig N, Muniz-Diaz E, Monteagudo E, Ribera A, Montro JA. A second case of neonatal alloimmune thrombocytopenia by anti-HPA-4b (anti-Yuk^a) in a Caucasian family. Transfus Med 1993;3:164-5.

22. Peterson JA, Balthazor SM, Curtis BR, McFarland JG, Aster RH. Maternal alloimmunization against the rare platelet-specific antigen HPA-9b (Max) is an important cause of neonatal alloimmune thrombocytopenia. Transfusion 2005;45:1487-95.

23. Kaplan C, Porcelijn L, Vanlieferinghen P, Julien E, Bianchi F, Martageix C, Bertrand G, Jallu V. Anti-HPA-9bw (Max^a) fetomaternal alloimmunization, a clinically severe neonatal thrombocytopenia: difficulties in diagnosis and therapy and report on eight families. Transfusion 2005;45: 1799-803.

24. Taniue A, Amakishi E, Kou Y, Fukumori Y, Tani Y. Cases of neonatal alloimmune thrombocytopenia detected with HPA (HLA) antibodies (in Japanese). Jpn J Obstet Gynecol Neonat Hematol 2007;(Suppl):31-2.

25. Fujiwara K, Isa K, Oka T, Maekawajiri S, Yamane A, Akaza T, Tadokoro K, Juji T, Shibata Y, Tokunaga K. Large-scale DNA typing for human platelet alloantigens by PCR-PHFA (preferential homoduplex formation assay). Br J Haematol 1996;95:198-203.

26. Shibata Y, Juji T, Tohyama H, Sakamoto H, Ozawa N, Kano K. Mixed passive hemagglutination with soluble platelet antigens. Int Arch Allergy Appl Immunol 1984;74:93-6.

27. Shibata Y, Juji T, Nishizawa Y, Sakamoto H, Ozawa N. Detection of platelet antibodies by a newly developed mixed agglutination with platelets. Vox Sang 1981;41:25-31.

28. Matsuyama N, Kojima Y, Hirayama F, Yasui K, Taniue A, Fukumori Y, Yoshimura K, Tabata N, Sakata N, Tani Y, Shibata H. Simultaneous five cell-lineage flow cytometric analysis system for detection of leucocyte antibodies. Transfus Med 2006;16:111-8.

29. Matsuyama N, Hirayama F, Yasui K, Kojima Y, Furuta RA, Kimura T, Taniue A, Fukumori Y, Tani Y, Shibata H. Non-HLA white cell antibodies in nonhemolytic transfusion reaction. Transfusion 2008;48:1527-8.

30. Cambell K, Rishi K, Howkins G, Gilby D, Mushens R, Ghevaert C, Metcalfe P, Ouwehand WH, Lucas G. A modified fast MAIPA for the detection of HPA antibodies: a multi-centre evaluation of a rapid monoclonal antibody specific immobilization of platelet antigen (MAIPA) assay. Vox Sang 2007;93:289-97.

31. Tanaka S, Ohnoki S, Shibata H, Okubo Y, Yamaguchi H, Shibata Y. Gene frequencies of human platelet antigens on glycoprotein IIIa in Japan. Transfusion 1996;36:813-7.

32. Tanaka S, Taniue A, Nagao N, Tomita T, Ohnoki S, Shibata H, Okubo Y, Yamaguchi H, Shibata Y. Genotype frequencies of the human platelet antigen, Ca/Tu, in Japanese, determined by a PCR-RFLP method. Vox Sang 1996;70: 40-4.

33. Ertel K, Tawil MA, Santoso S, Kroll H. Relevance of the HPA-15 (Gov) polymorphism on CD109 in alloimmune thrombocytopenic syndromes. Transfusion 2005;45:366-73.

34. Tanaka S, Hayashi T, Yoshimura K, Nakayama M, Fujita T, Amano T, Tani Y. Double heterozygosity for a novel missense mutation of Ile304 to Asn in addition to the missense mutation His280 to Pro in the integrin beta3 gene as a cause of the absence of platelet alphaIIbbeta3 in Glanzmann's thrombasthenia. J Thromb Haemost 2005;3:68-73. ◨

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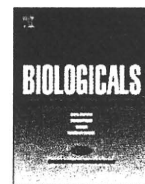
Endogenous retroviruses as potential hazards for vaccines

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ARTICLE INFO

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.

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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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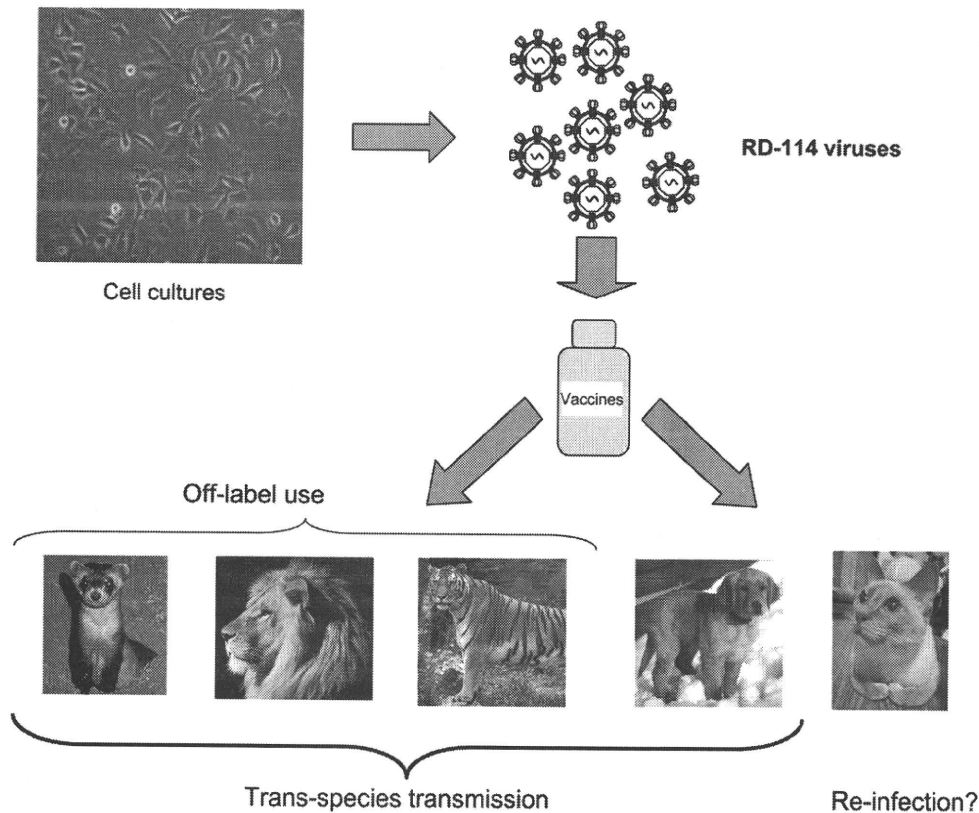*E-mail address:* takavet@gmail.com

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
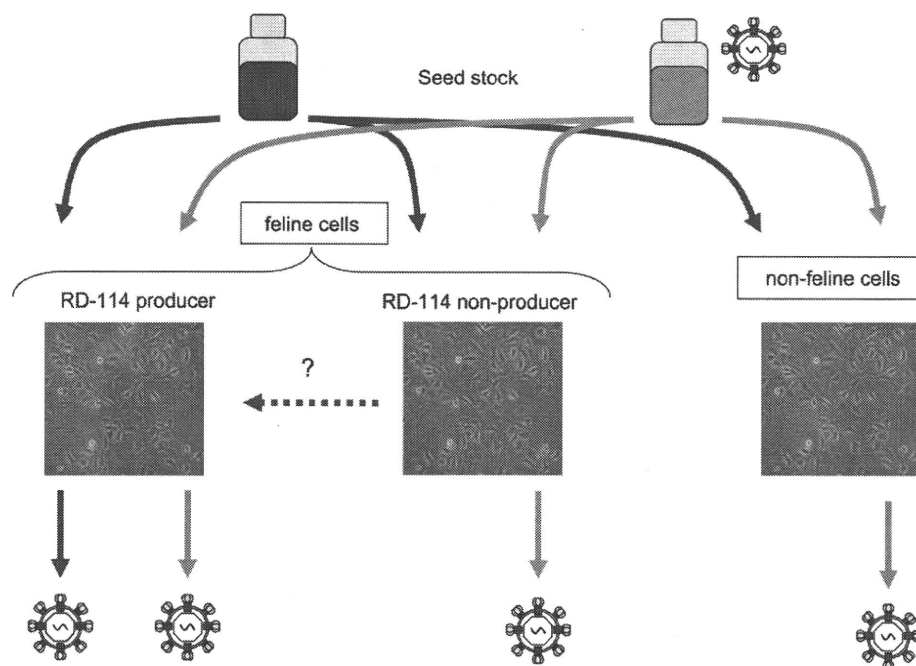

Fig. 2. Possible routes of contamination of RD-114 virus in vaccines. RD-114 virus grows efficiently in feline and canine cell lines and some viral seed stocks are prepared by using feline cells which produce RD-114 virus. When seed stocks are contaminated with RD-114 virus, vaccines may be contaminated 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.
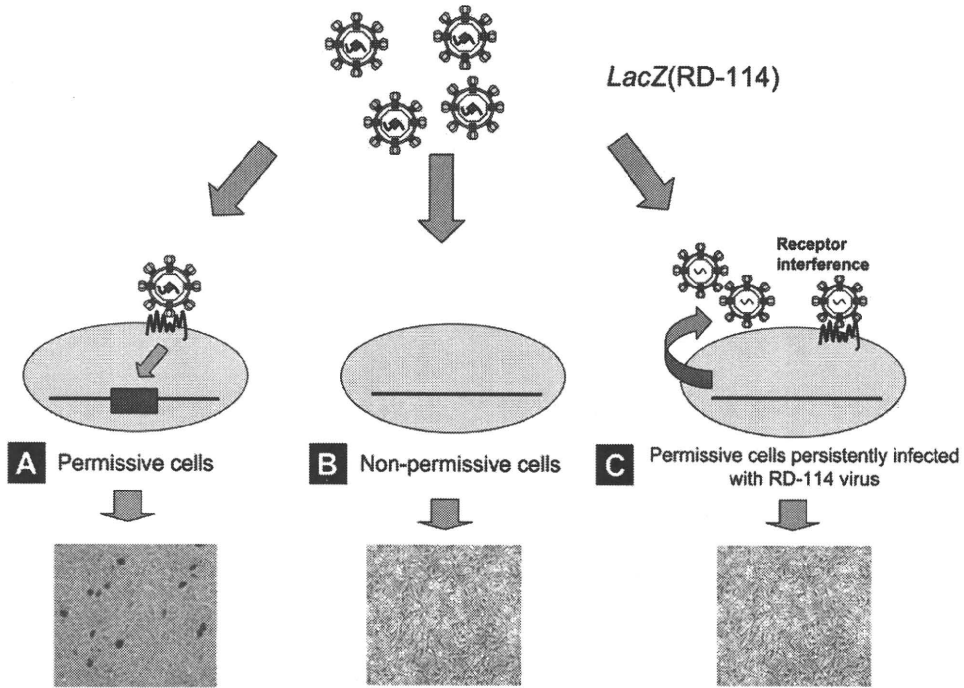
Fig. 3. Principles of LacZ assay and receptor interference. Details are described in the text.

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;
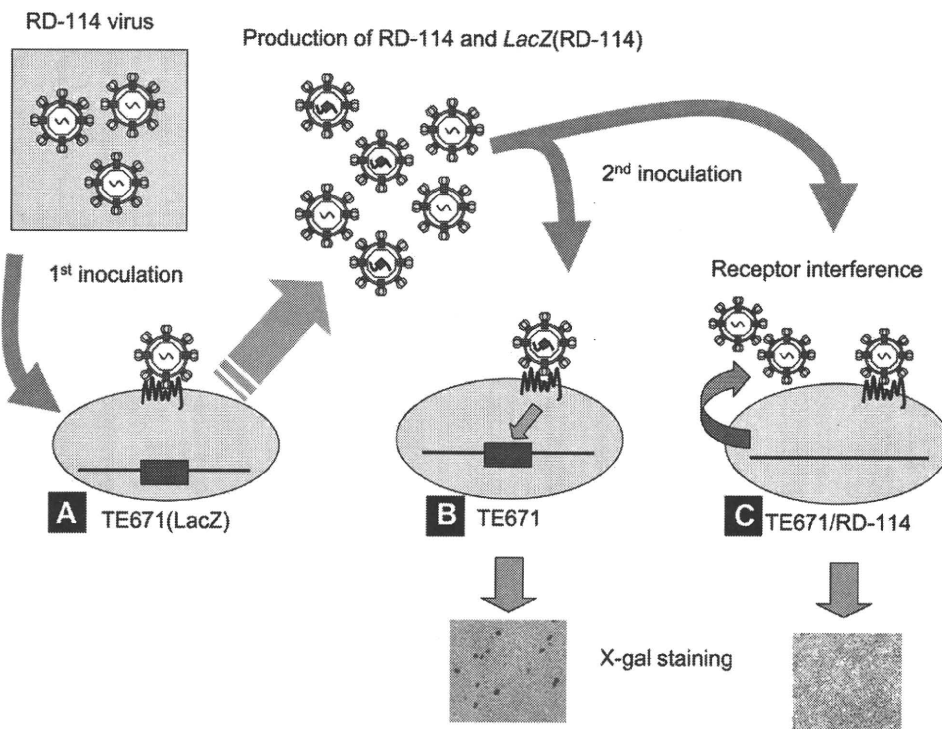

Fig. 4. Principle of LacZ marker rescue assay. Details are described in the text (modified from Sakaguchi et al. [15] with permission from the publisher).
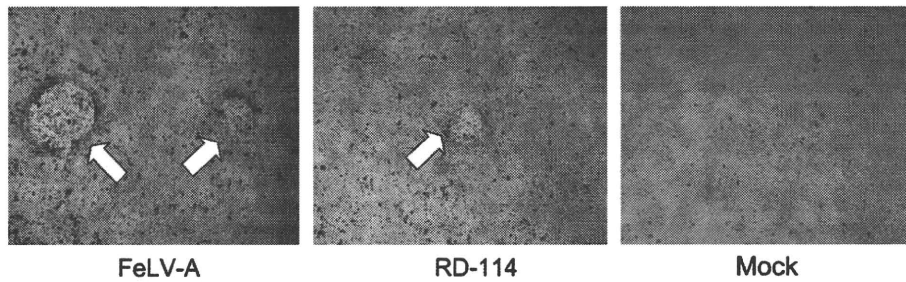
FeLV-A   **RD-114**   **Mock**

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.

Acknowledgements

References

[1] Baumann JG, Günzburg WH, Salmons B. CrFK feline kidney cells produce an RD114-like endogenous virus that can package murine leukemia virus-based vectors. J Virol 1998;72:7685–7.

[2] Bonner TI, Todaro GJ. Carnivores have sequences in their cellular DNA distantly related to the primate endogenous virus, MAC-1. Virology 1979;94:224–7.

[3] Cheney CM, Rojko JL, Kociba GJ, Wellman ML, Di Bartola SP, Rezanka LJ, et al. A feline large granular lymphoma and its derived cell line. In Vitro Cell Dev Biol 1990;26:455–63.

[4] Dunn KJ, Yuan CC, Blair DG. A phenotypic host range alteration determines RD114 virus restriction in feline embryonic cells. J Virol 1993;67:4704–11.

[5] Gifford R, Tristem M. The evolution, distribution and diversity of endogenous retroviruses. Virus Genes 2003;26:291–315.

[6] Haapala DK, Robey WG, Oroszlan SD, Tsai WP. Isolation from cats of an endogenous type C virus with a novel envelope glycoprotein. J Virol 1985;53:827–33.

[7] Kumar DV, Berry BT, Roy-Burman P. Nucleotide sequence and distinctive characteristics of the *env* gene of endogenous feline leukemia provirus. J Virol 1989;63:2379–84.

[8] Lombardi VC, Ruscetti FW, Das Gupta J, Pfost MA, Hagen KS, Peterson DL, et al. Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. Science 2009;326:585–9.

[9] Marin M, Tailor CS, Nouri A, Kabat D. Sodium-dependent neutral amino acid transporter type 1 is an auxiliary receptor for baboon endogenous retrovirus. J Virol 2000;74:8085–93.

[10] McDougall AS, Terry A, Tzavaras T, Cheney C, Rojko J, Neil JC. Defective endogenous proviruses are expressed in feline lymphoid cells: evidence for a role in natural resistance to subgroup B feline leukemia viruses. J Virol 1994;68:2151–60.

[11] Nowinski RC, Hays EF. Oncogenicity of AKR endogenous leukemia viruses. J Virol 1978;27:13–8.

[12] Porter CD, Collins MK, Tailor CS, Parkar MH, Cosset FL, Weiss RA, et al. Comparison of efficiency of infection of human gene therapy target cells via four different retroviral receptors. Hum Gene Ther 1996;7:913–9.

[13] Rasko JEJ, Battini J-L, Gottschalk RJ, Mazo I, Miller DA. The RD114/simian type D retrovirus receptor is a neutral amino acid transporter. Proc Natl Acad Sci U S A 1999;96:2129–34.

[14] Reeves RH, Nash WG, O'Brien SJ. Genetic mapping of endogenous RD-114 retroviral sequences of domestic cats. J Virol 1985;56:303–6.

[15] Sakaguchi S, Okada M, Shojima T, Baba K, Miyazawa T. Establishment of a LacZ marker rescue assay to detect infectious RD114 virus. J Vet Med Sci 2008;70:785–90.

[16] Sakaguchi S, Baba K, Ishikawa M, Yoshikawa R, Shojima T, Miyazawa T. Focus assay on RD114 virus in QN10S cells. J Vet Med Sci 2008;70:1383–6.

[17] Scobie L, Takeuchi Y. Porcine endogenous retrovirus and other viruses in xenotransplantation. Curr Opin Organ Transplant 2009;14:175–9.

[18] Tailor C, Nouri A, Zhao Y, Takeuchi Y, Kabat D. A sodium-dependent neutral-amino-acid transporter mediates infections of feline and baboon endogenous retroviruses and simian type D retroviruses. J Virol 1999;73:4470–4.

[19] Tarlinton R, Meers J, Young P. Biology and evolution of the endogenous koala retrovirus. Cell Mol Life Sci 2008;65:3413–21.

[20] Ting-De Ravin SS, Kennedy DR, Naumann N, Kennedy JS, Choi U, Hartnett BJ, et al. Correction of canine X-linked severe combined immunodeficiency by in vivo retroviral gene therapy. Blood 2006;107:3091–7.

[21] Urisman A, Molinaro RJ, Fischer N, Plummer SJ, Casey G, Klein EA, et al. Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. PLoS Pathog 2006;2:e25.

[22] van der Kuyl AC, Dekker JT, Goudsmit J. Discovery of a new endogenous type C retrovirus (FcEV) in cats: evidence for RD-114 being an FcEV-$^{Gag-Pol}$/baboon endogenous virus BaEV^{Env} recombinant. J Virol 1999;73:7994–8002.