Simian Retrovirus 4 Induces Lethal Acute Thrombocytopenia in Japanese Macaques

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ABSTRACT

In 2001–2002, six of seven Japanese macaques (*Macaca fuscata*) died after developing hemorrhagic syndrome at the Kyoto University Primate Research Institute (KUPRI). While the cause of death was unknown at the time, we detected simian retrovirus 4 (SRV-4) in samples obtained from a similar outbreak in 2008–2011, during which 42 of 43 Japanese macaques died after exhibiting hemorrhagic syndrome. In this study, we isolated SRV-4 strain PRI-172 from a Japanese macaque showing severe thrombocytopenia. When inoculated into four Japanese macaques, the isolate induced severe thrombocytopenia in all within 37 days. We then constructed an infectious molecular clone of strain PRI-172, termed pSR415, and inoculated the clone-derived virus into two Japanese macaques. These animals also developed severe thrombocytopenia in just 31 days after inoculation, and the virus was reisolated from blood, bone marrow, and stool. At necropsy, we observed bleeding from the gingivae and subcutaneous bleeding in all animals. SRV-4 infected a variety of tissues, especially in digestive organs, including colon and stomach, as determined by real-time reverse transcription-PCR (RT-PCR) and immunohistochemical staining. Furthermore, we identified the SRV-4 receptor as ASCT2, a neutral amino acid transporter. ASCT2 mRNA was expressed in a variety of tissues, and the distribution of SRV-4 proviruses in infected Japanese macaques correlated well with the expression levels of ASCT2 mRNA. From these results, we conclude that the causative agent of hemorrhagic syndrome in KUPRI Japanese macaques was SRV-4, and its receptor is ASCT2.

IMPORTANCE

During two separate outbreaks at the KUPRI, in 2001–2002 and 2008–2011, 96% of Japanese macaques (JM) that developed an unknown hemorrhagic syndrome died. Here, we isolated SRV-4 from a JM developing thrombocytopenia. The SRV-4 isolate and a molecularly cloned SRV-4 induced severe thrombocytopenia in virus-inoculated JMs within 37 days. At necropsy, we observed bleeding from gingivae and subcutaneous bleeding in all affected JMs and reisolated SRV-4 from blood, bone marrow, and stool. The distribution of SRV-4 proviruses in tissues correlated with the mRNA expression levels of ASCT2, which we identified as the SRV-4 receptor. From these results, we conclude that SRV-4 was the causative agent of hemorrhagic syndrome in JMs in KUPRI.

Macaque monkeys are commonly used as experimental animals for biomedical research around the world, especially in the field of neuroscience. Japanese macaques (*Macaca fuscata*) have been frequently employed in Japan because they are endemic (1). They have a reputation for being relatively tame and gentle and show considerably less genetic variation than other macaque species, such as rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (long-tailed macaques) (*Macaca fascicularis*) (2). Presumably, a small population of Japanese macaques initially colonized Japan during the Ice Age (about 400,000 years ago), when these islands were connected to the Eurasian continent, and the current population is derived from the limited numbers of founders (2). Currently, the use of Japanese macaques caught in the wild for research is restricted, and to meet the demands of the biomedical research field for Japanese macaques, the Kyoto University Primate Research Institute (KUPRI) breeds these monkeys as a part of national bioresource projects.

In 2001–2002, seven Japanese macaques kept in the KUPRI developed hemorrhagic syndrome, resulting in 6 deaths. After the incident, there were no further cases of Japanese macaques exhibiting hemorrhagic syndrome for 6 years. However, a new outbreak

occurred in 2008–2011 in which 43 Japanese macaques developed hemorrhagic syndrome and 42 died (17 were euthanized after diagnosis based on the number of platelets and clinical observations). Most Japanese macaques affected by the disease showed facial pallor, bleeding from nasal mucosa and/or the alveolar ridge, subcutaneous hemorrhaging, and mucous and bloody stool, with high mortality rates. In each outbreak, only one infected Japanese macaque survived. Severe thrombocytopenia followed by erythropenia and/or panleukopenia was commonly ob-

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served. At the time of death, none of the Japanese macaques had detectable platelets (3).

Previously, we detected simian retrovirus 4 (SRV-4), which is a simian betaretrovirus, in Japanese macaques developing severe thrombocytopenia (4). In this study, we isolated SRV-4 from a Japanese macaque exhibiting thrombocytopenia and inoculated the SRV-4 isolate into Japanese macaques. Severe thrombocytopenia was induced in all virus-inoculated Japanese macaques within 37 days. We then constructed an infectious molecular clone of the SRV-4 isolate and, after Japanese macaques were inoculated with the clone-derived virus, observed hemorrhagic symptoms within just 31 days.

MATERIALS AND METHODS

Ethics statement. Animal experiments were carried out in the Institute for Virus Research in Kyoto University (IVRKU) (authorization numbers D10-32, D11-21, and R12-12) after approval by the Committee on the Ethics of Animal Experiments of IVRKU in accordance with the guidelines for animal experiments at IVRKU. To prevent viral transmission, animals were housed in individual cages, allowing them to make sight and sound contact with one another; the temperature was kept at 24°C, with light for 14 h per day. Animals were fed apples and commercial monkey diet (type 5048; LabDiet, St. Louis, MO). Blood collection and SRV-4 challenge were performed under ketamine (5 to 10 mg/kg of body weight)-xylazine (0.25 to 2 mg/kg) anesthesia. The endpoint for euthanasia was determined by blood platelet counts (less than 20,000/ μ l). At euthanasia, animals were given a bolus intravenous administration of pentobarbital under ketamine (5 to 10 mg/kg)/xylazine (0.25 to 2 mg/kg) anesthesia, and then whole blood and tissues were collected.

Cells. Human embryonic kidney (HEK) 293T cells (ATCC, CRL-11268), TE671 cells (human rhabdomyosarcoma) (5), *Mus dunni* tail fibroblasts (MDTFs), and TELCeB6 cells (6) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μ g/ml) (Invitrogen, Carlsbad, CA). Peripheral blood mononuclear cells (PBMCs) of Japanese macaques were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 100 units/ml of recombinant human interleukin 2, 50 μ M 2-mercaptoethanol, L-glutamine (2 mM), minimum essential medium (MEM) nonessential amino acid solution (Invitrogen), sodium pyruvate (1 mM) (Invitrogen), and penicillin (100 units/ml) and streptomycin (100 μ g/ml) (Invitrogen).

Japanese macaques and sampling for virus isolation. Heparinized blood samples were taken by venipuncture from eight SRV-4 proviral-DNA-positive Japanese macaques (JM-a [KUPRI ID: N162], JM-b [TM2196], JM-c [AR1944], JM-d [TH2245], JM-e [TH1102], JM-f [TH2187], JM-g [TH2188], and JM-h [TH1495]) and one Japanese macaque (JM-i [N172]) which developed hemorrhagic syndrome (severe thrombocytopenia). In addition, bone marrow (BM) cells and stool were taken from macaque JM-i. The stool was suspended in phosphate-buffered saline (PBS) and centrifuged at 1,500 rpm for 15 min. The supernatant was filtered through 0.8- μ m and then 0.2- μ m filter units (Acrodisc; Pall Co., Ann Arbor, MI).

Isolation of SRV-4 from SRV-4 proviral DNA-positive Japanese macaques. Concanavalin A-stimulated PBMCs were cocultured with HEK293T cells. Two weeks after cocultivation, supernatants of the cocultured cells were filtered and then inoculated into uninfected HEK293T cells. In parallel, the plasma samples were inoculated into HEK293T cells and cultured. Two weeks after inoculation, genomic DNAs were isolated from the inoculated cells using a QIAamp DNA blood minikit (Qiagen, Valencia, CA) and then subjected to PCR analysis as described below.

Isolation of SRV-4 from a Japanese macaque exhibiting thrombocytopenia. PBMCs or BM cells were cocultured with TELCeB6 cells. In parallel, the plasma sample or stool suspension was inoculated into TELCeB6 cells. Two weeks after inoculation or cocultivation, each culture

supernatant containing 8 μ g/ml of Polybrene (hexadimethrine bromide) (Sigma-Aldrich) was filtered through a 0.45- μ m filter unit (Pall) and then inoculated into uninfected TE671 cells. Two days after inoculation, cells were stained with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). In addition, the genomic DNAs were isolated from all inoculated cells using a QIAamp DNA blood minikit (Qiagen) and then subjected to PCR analysis as described below.

PCR to detect SRV-4 proviruses. To detect SRV-4, partial SRV-4 proviral DNAs were amplified using primers corresponding to a part of the *gag* gene (forward primer, 5'-CAAAGGAGGAAGTCAAAGAA-3'; reverse primer, 5'-CCGGTCAGCATGTCAAAT-3') and the *env* gene (forward primer, 5'-TTCTCACTGCATACCAACCTAC-3'; reverse primer, 5'-AGGGTGACAAGTACACACCTTC-3'). The PCR was carried out using ExTaq polymerase (TaKaRa, Ohtsu, Shiga, Japan) according to the manufacturer's instructions. The PCR conditions were 94°C for 5 min, followed by 30 or 45 cycles of amplification, consisting of denaturation at 94°C for 30 s, annealing at 56°C (*gag*) or 65°C (*env*) for 30 s, and extension at 72°C for 30 s, and then a final extension at 72°C for 5 min. PCR was carried out in 200- μ l thin-walled tubes using a thermal cycler (C1000 thermal cycler; Bio-Rad, Hercules, CA).

Construction of an infectious molecular clone of SRV-4. To amplify the 5' and 3' halves of SRV-4 (designated SRV-4a and SRV-4b, respectively), we designed primers based on the deposited nucleotide sequence of SRV-4 in GenBank (accession number [NC_014474](#)). Total genomic DNA was isolated from TELCeB6 cells persistently infected with SRV-4, isolated from plasma of a Japanese macaque exhibiting thrombocytopenia, using a QIAamp DNA blood minikit (Qiagen). To clone fragments SRV-4a and SRV-4b from total genomic DNA, genomic DNA was amplified using primers corresponding to the fragment SRV-4a (forward primer, 5'-TGTCCGAGCCGTCGCGCCC-3'; reverse primer, 5'-CCG AACAGAGACGGATATATCCAG-3') and the fragment SRV-4b (forward primer, 5'-GACCCAGGAGCTTCGCTCAC-3'; reverse primer, 5'-TGTCCCGTCCCGCGGGATCAAC-3'). The PCR was carried out using PrimeSTAR GXL DNA polymerase (TaKaRa) according to the manufacturer's instructions. The amplification settings for SRV-4a were 98°C for 5 min followed by 30 cycles of amplification, consisting of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, and extension at 68°C for 20 s, and then final extension at 68°C for 5 min. The amplification settings for SRV-4b were 98°C for 5 min followed by 35 cycles of amplification, consisting of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, and extension at 68°C for 6 min, and then final extension at 68°C for 5 min. PCR was carried out in 200- μ l thin-walled tubes using a thermal cycler (C1000 Thermal Cycler; Bio-Rad). To reconstitute SRV-4a and SRV-4b fragments as a complete provirus, we used the EcoRI site present in the middle of the SRV-4 genome. Fragment SRV-4a was digested with EcoRI and cloned into the EcoRI/EcoRV sites of the pSP73 vector (Promega, Madison, WI) to produce pSP73/SRV-4a. Fragment SRV-4b was also digested with EcoRI and inserted into the EcoRI/PvuII sites of pSP73/SRV-4a. We designated the reconstituted plasmid clones pSR414, pSR415, pSR416, and pSR424.

Phylogenetic analyses. We obtained the complete genome sequences of SRVs from the NCBI database: Mason-Pfizer monkey virus (MPMV), [M12349.1](#); simian endogenous retrovirus (SERV), [U85505.1](#); SRV-1, [M11841.1](#); SRV-2, [AF126467.1](#); SRV-4 V1, [FJ971077.1](#); SRV-4 V2, [FJ979638.1](#); SRV-4 V3, [FJ979639.1](#); SRV-5, [AB611707.1](#). These entire nucleotide sequences were aligned using an L-INS-i program in MAFFT version 7 (7). Then, a maximum-likelihood phylogenetic tree was constructed using RAXML version 7.2.8 (8) with the general time-reversible (GTR) model of nucleotide substitutions with gamma-distributed rate heterogeneity and an estimated proportion of invariable sites.

Confirmation of infectivity of SRV-4 clones by PCR and LacZ marker rescue assay. To confirm the infectivity of viruses derived from pSR414, pSR415, pSR416, and pSR424, TE671 cells were transfected with 1 μ g of pSR414, pSR415, pSR416, or pSRV424 using Lipofectamine 2000

(Invitrogen) according to the manufacturer's instructions. Two days after transfection, each culture supernatants with 8 µg/ml of Polybrene (Sigma-Aldrich) was filtered through 0.45-µm filter units (Pall) and then inoculated into TELCeB6 cells. Two weeks after inoculation, total genomic DNA was isolated from the inoculated cells, and culture supernatants were collected from the inoculated cells and filtered through a 0.45-µm filter unit (Pall). The presence of SRV-4 proviruses was confirmed by PCR using SRV-4 *gag*-specific primers. In parallel, the presence of infectious SRV-4 particles was confirmed by LacZ marker rescue assay (9) using TE671 cells as target cells as described below.

LacZ marker rescue assay using TELCeB6 cells. The LacZ marker rescue assay was performed using TELCeB6 cells as described previously (9). TELCeB6 cells contain the *nls-lacZ* gene (nuclear localization signal fused to *lacZ*) with a packaging signal of murine leukemia virus (MLV) and MLV *gag-pol* genes in the genome (6). The SRV-4-infected TELCeB6 cells produce SRV-4 pseudotype viruses, which harbor envelope (Env) of SRV-4 and the core of MLV and contain the *nls-lacZ* gene as a viral genome. To monitor SRV-4 proliferation in TELCeB6 cells, the virus was inoculated into TELCeB6 cells. Several days after inoculation, culture supernatants were filtered through 0.45-µm membrane filters (Pall), and serially diluted samples were immediately inoculated into naive TE671 cells. Two days after inoculation, cells were fixed with 1% glutaraldehyde and stained with 1 mg/ml X-Gal, and *lacZ*-positive foci were counted.

Quantitative real-time PCR. Total genomic DNA was extracted from various tissues of Japanese macaques using a QIAamp DNA minikit (Qiagen). Real-time PCR was performed by using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. PCR and real-time monitoring of the resulting increase in reporter fluorescent dye emission were conducted using an ABI Prism 7000 real-time PCR system (Applied Biosystems). The primer pairs used were as follows: forward primer, 5'-TGCGAGTGCAAAGGAGGATAT-3', and reverse primer, 5'-CGCAAGAAATGGCTGCAA-3', for SRV-4; forward primer, 5'-GGCATCCTGGGCTACACTGA-3', and reverse primer, 5'-AAGAGTGGGTGTCGCTGTTG-3', for Japanese macaque GAPDH; forward primer, 5'-ACGGCCAGGTCATCACTATTG-3', and reverse primer, 5'-CAAGAAGGAAGGCTGGAAAAGA-3', for mouse Actb. To quantify the DNA concentration, standard curves were generated for each gene by serial dilution of the plasmids containing their DNAs. The copy ratio of each gene was normalized relative to the abundance of a validated endogenous control Japanese macaque's GAPDH DNA or mouse Actb DNA to adjust for variation in the PCR. Quantitation was performed in triplicate for each sample, and all values are presented as means ± standard errors of the means (SEM).

Quantitative real-time RT-PCR. Total RNA was extracted from various tissues of Japanese macaques using RNeasy minikit (Qiagen). To exclude contamination of cellular DNA from RNA samples, the samples were treated with DNase I (Roche Diagnostics GmbH, Mannheim, Germany). Real-time reverse transcription PCR (RT-PCR) was performed by using the Power SYBR green RNA-to-CT 1-step kit (<https://www.lifetechologies.com/jp/ja/home/life-science/pcr/real-time-pcr/real-time-pcr-reagents/one-step-real-time-rt-master-mix/power-sybr-rna-to-ct-1-step.html>; Applied Biosystems) according to the manufacturer's instructions. PCR and real-time monitoring of the resulting increase in reporter fluorescent dye emission were conducted using an ABI Prism 7000 real-time PCR system (Applied Biosystems). The primer pairs used were as follows: forward primer, 5'-TGCGAGTGCAAAGGAGGATAT-3', and reverse primer, 5'-CGCAAGAAATGGCTGCAA-3', for SRV-4; forward primer, 5'-CTGGCTGTGGACTGGATTGTG-3', and reverse primer, 5'-AGTTCCTGCTCGCCCTTCTTC-3', for Japanese macaque ASCT1; forward primer, 5'-ACTTCCTCTTCACCCGCAAA-3', and reverse primer, 5'-TCTCTCCACGCACCTTCATC-3', for Japanese macaque ASCT2; forward, 5'-GAAATCCCATCACCATCTTCCAGG-3', and reverse primer, 5'-GAGCCCCAGCCTTCTCCATG-3', for Japanese macaque GAPDH. To quantify the cDNA concentration, standard curves were generated for each gene by the serial dilution of the plasmids containing their DNAs.

The expression ratio of each gene was normalized relative to the abundance of a validated endogenous control Japanese macaque's GAPDH RNA to adjust for variation in the PCRs. Quantitation was performed in triplicate for each sample, and all values are presented as means ± SEM.

Quantification of SRV-4 RNA in plasma. To determine SRV-4 viral RNA copy numbers in plasma, SRV-4 viral RNA was amplified by real-time RT-PCR using a TaqMan probe. To exclude contamination of cellular DNA from plasma, the samples were treated with DNase I (Roche). PCR was carried out using a One Step PrimeScript RT-PCR kit (TaKaRa) according to the manufacturer's instructions. The primer pair used was 5'-TGCGAGTGCAAAGGAGGATAT-3' (forward) and 5'-CGCAAGAAATGGCTGCAA-3' (reverse), with the TaqMan probe FAM-TCCACCTAGTCCCC-MGB. Quantitation was performed in triplicate for each sample, and all values are presented as means ± SEM.

Experimental infection of Japanese macaques with SRV-4. Female (JM1 and JM2) and male (JM3, JM4, JM6, and JM7) Japanese macaques were used for experimental infection. Virus inoculation was carried out under anesthesia by intramuscular injection of a mixture of ketamine chloride (Ketalar; Daiichi Sankyo, Tokyo, Japan) at 5 to 10 mg/kg and xylazine chloride (Celactal; Bayer Healthcare, Leverkusen, Germany) at 1.5 to 2.0 mg/kg. Animal experiments were conducted in a biosafety level 3 animal facility, in compliance with institutional regulations approved by the Committee on Experimental Use of Nonhuman Primates of the Institute for Virus Research, Kyoto University, Kyoto, Japan.

(i) Experiment 1. To prepare SRV-4 inocula, strain PRI-172 isolated from a Japanese macaque (JM-i) exhibiting thrombocytopenia was inoculated into uninfected TE671 cells. Two weeks after inoculation, the culture supernatants were collected from the inoculated cells and filtered through a 0.45-µm filter unit (Pall). To exclude contamination of cellular DNA from culture supernatants, the samples were treated with DNase I (Roche). Four Japanese macaques (JM1, JM2, JM3, and JM4) were inoculated with SRV-4 strain PRI-172 (3.6×10^8 50% tissue culture infective doses [TCID₅₀]/shot) intraperitoneally and intravenously. Before inoculation and 4, 11, 15, 18, 25, 32, 35, and 37 days postinoculation (dpi), blood was collected from animals by venipuncture. Various tissues (cerebrum, epencephalon, lung, heart, thymus, stomach, liver, kidney, pancreas, spleen, small intestine, large intestine, testis, ovary, prostate gland, uterus, skin, muscle, and lymph nodes [mesentery, axillary, mandibular, and inguinal]) were collected at necropsy (32, 35, and 37 dpi).

(ii) Experiment 2. To prepare SRV-4 derived from pSR415, HEK293T cells were transfected with pSR415. Two days after transfection, the culture supernatant was inoculated into TE671 cells (TE671/SR415 cells). Two weeks after inoculation, the culture supernatant was collected from the inoculated cells and filtered through a 0.45-µm filter unit (Pall). To exclude contamination of cellular DNA from the culture supernatant, the sample was treated with DNase I (Roche). Two Japanese macaques (JM6 and JM7) were inoculated with SR415 (1.4×10^8 TCID₅₀/shot) intraperitoneally and intravenously. Before inoculation and 3, 8, 14, 18, 21, 23, 29, and 31 dpi, blood was collected from animals by venipuncture. Various tissues (cerebrum, epencephalon, thyroid, amygdala, lung, heart, thymus, stomach, liver, kidney, pancreas, spleen, small intestine, large intestine, testis, ovary, prostate gland, uterus, skin, muscle, and lymph nodes [mesentery, axillary, mandibular, and inguinal]) were collected at necropsy (31 dpi).

SRV-4 LacZ marker rescue assay. To make a LacZ reporter plasmid with a SRV-4 packaging signal, named pSRV4ψLacZ, the nucleotides from positions 801 to 7594 of pSR415 (the transcription initiation site was defined as position 1) were replaced by the simian virus 40 (SV40) promoter and *nls-lacZ* using Infusion (Clontech, Mountain View, CA). PBMCs or BM cells were cocultured with HEK293T cells. In parallel, the plasma samples were inoculated into HEK293T cells. Two weeks after inoculation or cocultivation, the inoculated or cocultured cells were transfected with pSRV4ψLacZ using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two days after transfection, each culture supernatant with 8 µg/ml of Polybrene (Sigma-Aldrich) was

FIG 1 Isolation of SRV-4 from Japanese macaques at KUPRI. PCR amplification of genomic DNAs of HEK293T cells inoculated with plasma (A) and cocultured with PBMCs (B) from Japanese macaques JM-a (lane 1), JM-b (lane 2), JM-c (lane 3), JM-d (lane 4), JM-e (lane 5), JM-f (lane 6), JM-g (lane 7), and JM-h (lane 8). (C) PCR amplification of genomic DNAs of TELCeB6 cells inoculated with plasma or stool suspension and cocultured with PBMCs or BM cells from a Japanese macaque exhibiting thrombocytopenia. (D) Confirmation of the infectivity of the virus derived from pSR414, pSR415, pSR416, and pSR424. PCR was conducted to amplify the partial *gag* gene of SRV-4 from genomic DNA isolated from TELCeB6 cells inoculated with viruses derived from each molecular clone. The results of the LacZ marker rescue assay (LMRA) are shown as negative (–) and positive (+). M, 1-kb ladder marker; N.C., negative control; *env*, amplicon of partial SRV-4 *env*; *gag*, amplicon of partial SRV-4 *gag*. (E) Unrooted tree of the entire nucleic acid sequences of SRV-4 isolates and other SRVs. Numbers at the nodes indicate the rapid bootstrap values (percentages of 1,000 replicates). MPMV, Mason-Pfizer monkey virus (SRV-3); SERV, simian endogenous retrovirus.

filtered through a 0.45- μ m filter unit (Pall) and then inoculated into TE671 cells. Two days after infection, all inoculated cells were stained with X-Gal.

Antibodies. For anti-envelope (Env) antibodies, rabbits were immunized with a mixture of two peptides ([C]KKFEELHKNLFPEL of the Env surface [SU] subunit and [C]GVVRDKIKRLQDDL of the Env transmembrane [TM] subunit; [C] indicates an additional cysteine residue for peptide purification). For anticapsid (CA) antibodies, rabbits were immunized with a mixture of two peptides ([C]TVDGQGQAWRHHNG and [C]TKAWRKLPVKGDPG of CA). Their sera were collected after six immunization steps.

Detection of anti-SRV-4 antibodies in plasma. To detect anti-SRV-4 antibodies in plasma, we conducted immunoblotting analysis using viral proteins prepared from the culture supernatant of HEK293T/SR415 cells. Viruses in the culture supernatant were collected by centrifugation ($6,300 \times g$) for 20 h, and the concentrated viral proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking with 10% skim milk in PBS containing 0.05% Tween 20 (PBS-T), the membrane was incubated with 1:100-diluted plasma sample in 1% bovine serum albumin (BSA)-PBS-T at 4°C overnight. After washing with PBS-T, the membrane was inoculated with 1:10,000-diluted horseradish peroxidase (HRP)-conjugated anti-human IgG antibody (GE Healthcare, Buckinghamshire, United Kingdom) and detected with a Super Signal West Femto maximum-sensitivity system (Thermoscientific, Rockford, IL).

IHS of SRV-4 in tissues. Fixed necropsy organs of the two experimentally infected Japanese macaques (JM6 and TM7) were embedded in paraffin. Tissue sections of 5 μ m were immunohistochemically examined; expression of SRV-4 CA protein was examined by immunohistochemical staining (IHS). Tissue sections were deparaffinized and rehydrated, fol-

lowed by blocking of endogenous peroxidase with 3% (vol/vol) H_2O_2 for 5 min and then with 10% (vol/vol) normal goat serum-PBS solution at room temperature for 30 min to minimize nonspecific staining. The sections were incubated at 4°C overnight with a 1:10,000 dilution of rabbit anti-SRV-4 CA antiserum, followed by incubation for 1 h at room temperature with a secondary antibody conjugated with horseradish peroxidase-labeled polymer (Envision kit; Dako). Streptavidin-peroxidase complex reagent (Vector Laboratories) was then added and incubated at room temperature for 30 min. Color development was performed by using 3,3'-diaminobenzidine (DAB), and samples were counterstained with Meyer's hematoxylin.

Cloning of ASCT1 and ASCT2 cDNA from Japanese and cynomolgus macaques. cDNAs encoding ASCT1 and ASCT2 molecules of Japanese macaques and cynomolgus macaques were cloned by RT-PCR. RNAs from PBMCs of a Japanese macaque and a cynomolgus macaque were isolated with an RNeasy minikit (Qiagen). cDNAs were synthesized using a SuperScript III first-strand synthesis kit (Invitrogen). cDNAs were amplified using primers corresponding to ASCT1 (forward primer, 5'-AAAAGCTTCCACCATGGA GAAGAGCAACGA-3'; reverse primer, 5'-AAGGTACCCACAGAACCGA CTCCTTGGA-3') and ASCT2 (forward primer, 5'-AAAAGCTTCCACCAT GGTGGCCGATCCTCC-3'; reverse primer, 5'-AAGGTACCCACATGACT GATTCCTTCTCAG-3'). The PCR was carried out using PrimeSTAR GXL polymerase (TaKaRa) according to the manufacturer's instruction. The amplification conditions for both ASCT1 and ASCT2 were 98°C for 5 min followed by 30 cycles of amplification, consisting of denaturation at 98°C for 10 s, annealing at 55°C for 15 s and extension at 68°C for 60 s, and then final extension at 68°C for 5 min. PCR was carried out in 200- μ l thin-walled tubes using a C1000 thermal cycler (Bio-Rad, Hercules, CA). The amplicons, corresponding to the entire ASCT1 and ASCT2 genes, were inserted into

FIG 2 Experimental infection of four Japanese macaques (JM1, JM2, JM3, and JM4) with SRV-4 strain PRI-172. (A to C) Hematological analyses. Blood was routinely collected, and the numbers of platelets (A), leukocytes (B), and erythrocytes (C) were measured by hematology. (D) Copy numbers of SRV-4 RNA in plasma samples. SRV-4 viral RNAs were quantified by real-time RT-PCR using a TaqMan probe. Values are means $\pm$ standard errors of data from three independent experiments.

pACGFP-N1 (Clontech, Mountain View, CA) to produce pJmASCT1/GFP, pJmASCT2/GFP, pCyASCT1/GFP, and pCyASCT2/GFP.

Functional assay of ASCT1 and ASCT2 as SRV-4 virus receptors. To confirm whether Japanese macaque and cynomolgus macaque ASCT1 and ASCT2 function as SRV-4 receptors, pJmASCT1/GFP, pJmASCT2/GFP, pCyASCT1/GFP, pCyASCT2/GFP, or pACGFP-N1 was transfected into MDTFs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. One day after transfection, the expression of green fluorescent protein (GFP) was observed with a fluorescence microscope (IX72 UV microscope; Olympus). MDTFs expressing Japanese macaque ASCT1-GFP, Japanese macaque ASCT2-GFP, cynomolgus macaque ASCT1-GFP, cynomolgus macaque ASCT2-GFP, or GFP only (MDTF/JmASCT1, MDTF/JmASCT2, MDTF/CyASCT1, MDTF/CyASCT2 or MDTF/GFP cells, respectively) were seeded in 12-well plates at 10^5 cells per well 1 day before infection. Infectivity of SRV-4 to MDTF/JmASCT1, MDTF/JmASCT2, MDTF/CyASCT1, MDTF/CyASCT2, or MDTF/GFP cells was confirmed by real-time PCR using SRV-4-specific primers.

Nucleotide sequence accession numbers. pSR415, pSR416, JmASCT1, JmASCT2, CyASCT1, and CyASCT2 have been deposited in GenBank with accession numbers [AB920339](#), [AB920340](#), [AB920376](#), [AB920377](#), [AB920378](#), and [AB920379](#), respectively.

RESULTS

Isolation of SRV-4 from SRV-4 provirus-positive Japanese macaques. Metagenomic analysis of RNA isolated from the plasma of a Japanese macaque with hemorrhagic syndrome revealed the presence of RNA sequences that were highly homologous to SRV (4). By PCR, using SRV-4 specific primers, we found a correlation between SRV-4 infection and hemorrhagic illness in Japanese macaques in KUPRI (4). Next, we attempted to isolate SRV-4 from

the blood of eight SRV-4-positive Japanese macaques by using genomic DNA obtained from samples as PCR templates. HEK293T cells and cells of the human rhabdomyosarcoma line TE671 are known to be susceptible to SRVs (10). Using HEK 293T cells, we succeeded in isolating SRV-4 from 5 of 8 plasma samples and from 7 of 8 PBMC samples (Fig. 1A and B). We then tried to isolate SRV-4 from plasma, PBMCs, stool, and BM cells of a Japanese macaque (KUPRI ID, N172) exhibiting thrombocytopenia. PBMCs or BM cells were cocultured with TELCeB6 cells (a derivative of TE671) (6). In parallel, the plasma samples or stool suspensions were inoculated into TELCeB6 cells. Two weeks after cocultivation or inoculation, virus isolation was confirmed by PCR and the LacZ marker rescue assay (9). As a result, we isolated SRV-4 from plasma, PBMCs, stool, and BM samples (Fig. 1C). We designated SRV-4 isolated from plasma of the Japanese macaque as strain PRI-172.

Experimental infection of Japanese macaques by SRV-4 strain PRI-172. Four Japanese macaques (JM1, JM2, JM3, and JM4) were inoculated with SRV-4 strain PRI-172 intraperitoneally and intravenously at a high dose (3.6×10^8 TCID₅₀/shot). JM3 died at 32 dpi following blood sampling. At necropsy, we found bleeding from the gingiva, subcutaneous bleeding, and hepatoma within the cavitas. The platelet numbers dropped severely in JM1 and JM2 at 32 dpi (Fig. 2A) and we euthanized these animals at 35 dpi. Similarly, the platelet number of JM4 dropped severely at 36 dpi, and we euthanized the animal at 37 dpi (Fig. 2A). Leukocyte numbers were also markedly reduced in all SRV-4-inoculated Japanese macaques (Fig. 2B). Erythrocyte numbers

FIG 3 Detection and isolation of SRV-4 in four Japanese macaques (JM1, JM2, JM3, and JM4) experimentally infected with SRV-4 strain PRI-172. (A) Genomic DNAs were isolated from blood and subjected to PCR to amplify the gag region of the SRV-4. Numbers above lanes indicate days postinoculation. (B) Isolation of SRV-4 from Japanese macaques. Data for PCR amplification of genomic DNAs of HEK293T cells inoculated with plasma and those cocultured with PBMCs or BM cells isolated from JM1, JM2, JM3, and JM4 are shown. (C and D) Detection and quantification of SRV-4 proviruses in various tissues by PCR (in JM1) (C) and real-time PCR (in JM1 and JM3) (D) analyses using SRV-4 *env*-specific primers. Lane M, 1-kb ladder marker; lane N. C., negative control; Ly, lymph node.

were decreased only in JM1 (Fig. 2C). By PCR, we detected proviruses in blood cells from 4 to 11 dpi in all SRV-4-inoculated Japanese macaques (Fig. 3A). We also detected viral RNA in plasma from 4 or 11 dpi by real-time RT-PCR (Fig. 2D). The viral copy numbers in plasma plateaued at a high level from 11 dpi (10^4 to 10^7 copies/ml) (Fig. 2D). At necropsy, we reisolated infectious SRV-4 from plasma samples, BM cells, and PBMCs of all SRV-4-inoculated Japanese macaques (Fig. 3B).

Construction of an infectious molecular clone of SRV-4 strain PRI-172. Total genomic DNA was isolated from TELCeB6 cells persistently infected with SRV-4 strain PRI-172. We amplified the 5' and 3' halves of SRV-4 and reconstituted the clones as a complete provirus. We designated these clones pSR414, pSR415, pSR416, and pSR424. To confirm the infectivity of the derived viruses, TE671 cells were transfected with these clones. Two days after transfection, each culture supernatant was used to inoculate uninfected TELCeB6 cells. Two weeks after inoculation, the presence of SRV-4 proviruses was confirmed by PCR using SRV-4 *gag*-specific primers. In parallel, the presence of infectious SRV-4 particles was confirmed by the LacZ marker rescue assay using TE671 cells as target cells. Consequently, we confirmed the viral infectivity of clones pSR415 and pSR416 (Fig. 1D). Using the genome sequences of pSR415 and pSR416, we inferred their phylogeny among SRVs (Fig. 1E). As shown in the phylogenetic tree,

pSR415 and pSR416 made a cluster with SRV-4 strains, which was supported by its relatively long branch and a high bootstrap value (100%). These results confirmed that the viruses we obtained were SRV-4.

Experimental infection of Japanese macaques with SRV-4 clone SR415. Two Japanese macaques (JM6 and JM7) were inoculated with SRV-4 clone SR415 derived from pSR415 (1.4×10^8 TCID₅₀/shot) intraperitoneally and intravenously. The platelet numbers dropped steeply in JM6 and JM7 at 29 dpi (Fig. 4A), and these animals were euthanized at 31 dpi. Necropsy revealed bleeding from the gingivae and subcutaneous bleeding in both animals. Leukocyte numbers were also markedly reduced in the virus-inoculated Japanese macaques (Fig. 4B). Erythrocyte numbers were slightly decreased in both animals (Fig. 4C). By PCR, we detected proviruses in blood cells from 8 and 3 dpi in JM6 and JM7, respectively (Fig. 5A). Real-time PCR supported the PCR results (Fig. 5B). The viral copy numbers in plasma stayed at a high level from 8 dpi (10^5 to 10^7 copies/ml) (Fig. 4D). At necropsy, we reisolated infectious SRV-4 from plasma, PBMCs, and BM cells of the virus-inoculated Japanese macaques (Fig. 5C).

Antibody responses. We examined antibody responses against SRV-4 in Japanese macaques inoculated with SRV-4 by immunoblot analysis. We generated rabbit anti-CA and anti-Env antibodies using synthetic peptides. We clearly detected CA, SU, and TM

FIG 4 Experimental infection of two Japanese macaques (JM6 and JM7) with SRV-4 clone SR415. (A to C) Hematological analyses. Blood was routinely collected, and the numbers of platelets (A), leukocytes (B), and erythrocytes (C) were measured by hematology. (D) Copy numbers of SRV-4 RNA in plasma samples. SRV-4 viral RNA was quantified by real-time RT-PCR using a TaqMan probe. Values are means $\pm$ standard errors of data from three independent experiments.

Env of SRV-4 by using the positive rabbit sera (Fig. 6). The band observed between 50 and 75 kDa was the result of a nonspecific reaction. Positive serum of a Japanese macaque (KUPRI ID, N162) detected SU, TM Env, and CA proteins of SRV-4 (Fig. 6). In JM1 and JM2, the antibodies against SRV-4 TM Env were detected by immunoblot analysis. In JM4, we detected TM Env and CA antibodies. In JM3, JM6, and JM7, we could not detect any antibodies against SRV-4.

Tissue distribution of SRV-4. We flushed out body fluids with phosphate-buffered saline to exclude blood cells in all Japanese macaques except JM3 at necropsy. In SRV-4-inoculated animals, we detected SRV-4 proviruses in all tissues examined except the cerebellum of JM7 (Fig. 3C and 7A). Real-time PCR to detect SRV-4 proviruses was also carried out in tissues of JM1, JM3, JM6, and JM7. We detected relatively high copy numbers of SRV-4 proviruses in stomach, cecum, and colon (Fig. 3D and 7B). Real-time RT-PCR to detect SRV-4 RNA was carried out in various tissues in JM7, and we detected relatively high copy numbers of SRV-4 mRNA in stomach, small intestine, cecum, colon, lung, thymus, spleen, and lymph nodes (Fig. 7C). IHS of JM7 tissues with the rabbit anti-SRV-4 CA antibody revealed that SRV-4 antigens were expressed in mucosal epithelium and glands in several sections: mucosal epithelium in the nasal pharynx and pharyngeal glands, tracheal mucosal epithelium, and absorptive epithelium in the small intestine (Fig. 7D to G).

Identification of the SRV-4 receptor as ASCT2 and tissue distribution of ASCT2. In humans, receptors for SRVs were reported to be ASCT2, a neutral amino acid transporter (10, 11). We investi-

gated the receptors for SRV-4 in Japanese macaques and cynomolgus macaques. It is known that the carboxyl-terminal region in extracellular loop 2 (ECL2) (termed region C) of human ASCT2 plays critical roles in entry of SRV (12). The amino acid sequence of region C of human ASCT2 showed 100% similarity with those of Japanese macaque and cynomolgus macaque ASCT2s. We expressed ASCT1 or ASCT2 in *Mus dunni* tail fibroblasts (MDTFs), which are not susceptible to infection by SRVs (13) (Fig. 8A). Consequently, we found that ASCT2 molecules derived from both Japanese macaques and cynomolgus macaques function as receptors for SRV-4 (Fig. 8B). In contrast, ASCT1 molecules did not function as receptors for SRV-4 (Fig. 8B). Finally, we examined the expression of ASCT1 and ASCT2 mRNAs in various tissues in a Japanese macaque (JM7) (Fig. 8C and D). ASCT1 and ASCT2 mRNAs were expressed in various tissues, and we detected relatively high expressions of ASCT2 mRNA in stomach, colon, lung thymus, spleen, lymph nodes, testis, and prostate.

DISCUSSION

Four Japanese macaques inoculated with SRV-4 strain PRI-172 developed severe thrombocytopenia from 32 to 37 dpi. We then examined whether a single replication-competent SRV-4 was sufficient for inducing severe acute thrombocytopenia in Japanese macaques. We inoculated two Japanese macaques with SRV-4 clone SR415 and found that these animals showed severe thrombocytopenia within 31 dpi. We reisolated SRV-4 from plasma, BM cells, and PBMCs of SR415-inoculated Japanese macaques. From

FIG 5 Detection and isolation of SRV-4 in two Japanese macaques (JM6 and JM7) experimentally infected with SRV-4 clone SR415. To prepare SRV-4 inocula, HEK293T cells were transfected with pSR415. Two days after transfection, the culture supernatant was inoculated into TE671 cells, and the cells were further incubated for 2 weeks. Then the culture supernatant was collected and filtered through a 0.45- μ m filter unit to make stock virus. To exclude contamination of cellular DNA, the stock virus was treated with DNase I. (A) Genomic DNAs were isolated from blood and subjected to PCR to amplify the Env region of the SRV-4. Numbers above lanes indicate days postinoculation. (B) Quantification of SRV-4 proviral DNA copy numbers in collected blood samples by real-time PCR. The copy numbers of SRV-4 viral DNA were normalized to one copy of GAPDH. Values are the means $\pm$ standard errors of data from three independent experiments. (C) Isolation of SRV-4 from Japanese macaques using the PCR assay and the SRV-4 LacZ marker rescue assay (SLMRA). Data for PCR amplification of genomic DNAs of HEK293T cells inoculated with plasma and cocultured with PBMCs or BM cells from JM6 and JM7 are shown. The results of the SLMRA are shown as negative (–) and positive (+). Lane M, 1-kb ladder marker; lane N.C., negative control.

these data, we conclude that SRV-4 was the causative agent of hemorrhagic syndrome in Japanese macaques.

To the best of our knowledge, fatal severe acute thrombocytopenia caused by SRVs in macaques had not been reported until we reported this incidence. Currently, there are five serotypes (SRV-1 to SRV-5) and two additional genotypes (SRV-6 and SRV-7) of SRVs reported (14–16). SRV-1, -2, and -3 induce immunodeficiency-like diseases in macaques; however, symptoms are generally mild and chronic (14). Although one study implied that SRV-2 infection causes thrombocytopenia in Japanese macaques (17), no clear evidence was shown. Moreover, there has been no

report of such acute severe thrombocytopenia caused by betaretroviruses. In general, acute lethal retroviral infections are rare. Among lentiviruses, a simian immunodeficiency virus from sooty mangabeys (SIVsmm), clone PBj-14, killed pig-tailed macaques within several weeks (18). Among gammaretroviruses, a molecularly cloned feline leukemia virus (FeLV) variant, termed EECC, induced fatal acquired immunodeficiency in cats (called feline AIDS) within 3 months (19, 20).

Many cynomolgus macaques reared in breeding and experimental facilities in Japan are infected with SRV-4. In the Tsukuba Primate Research Center, National Institute of Biomed-

FIG 6 Immunoblot assay using concentrated SRV-4 as the antigen. (A) Detection of anti-SRV-4 antibodies in plasma samples of JM1, JM2, JM3, and JM4. (B) Detection of anti-SRV-4 antibodies in plasma samples of JM6 and JM7. Numbers shown above the lanes indicate dpi. Rb, rabbit immune serum; CA, rabbit anti-SRV-4 CA antibody; Env, rabbit anti-SRV-4 Env antibody. Dots indicate bands specific to SRV-4. N162, SRV-4-positive serum of a Japanese macaque (KUPRI ID, N162).

FIG 7 Detection of SRV-4 proviruses, RNA, and proteins in Japanese macaques (JM6 and JM7) infected with SRV-4 clone SR415. (A and B) Detection and quantification of SRV-4 proviruses in various tissues by PCR (A) and real-time PCR analysis (B) using SRV-4 *env*-specific primers. (C) Quantification of SRV-4 viral RNA copy numbers in various tissues of JM7 by real-time RT-PCR. The copy numbers of SRV-4 RNA were normalized to one copy of GAPDH. Values are means $\pm$ standard errors of data from three independent experiments. (D to G) Immunohistochemical staining using anti-SRV-4 CA antibody. Mucosal epithelium on nasal pharynx (D), pharyngeal glands (E), tracheal mucosal epithelium (F), and absorptive epithelium in the small intestine (G) are shown. Ly, lymph node.

cal Innovation (Tsukuba, Japan), 95% of laboratory-bred cynomolgus macaques tested positive for either anti-SRV antibodies or viral RNA; however, they do not usually develop profound symptoms and are supplied for biomedical research use (21). In KUPRI, SRV-4-positive cynomolgus macaques and Japanese macaques are housed transiently in the same room for medical treatments. Presumably, SRV-4 may have been transmitted from cynomolgus macaques to Japanese macaques when they were in the same room. The routes of the SRV-4 infection in the colonies are under investigation. SRV-4 infected a variety of tissues, especially digestive tissues, including colon and stomach. By IHS, we detected SRV-4 antigens in digestive and respiratory tissues and SRV-4 virions in stool samples. Therefore, it is possible that the virus was transmitted via feces or airborne droplet nuclei containing SRV-4 from infected cynomolgus macaques and then spread among Japanese macaques rapidly in free-ranging spaces.

The reason why Japanese macaques are extremely sensitive to SRV-4 is not clear at present. Importantly, most Japanese macaques which developed hemorrhagic syndrome did not produce any detectable antibodies against SRV-4 (4). In this study, we de-

tected weak antibody responses against SRV-4 TM Env and/or CA in 3 of 6 Japanese macaques inoculated with SRV-4, but we could not detect any anti-SU Env antibodies (Fig. 6). It is possible that the majority of Japanese macaques are tolerant of SRV-4 and cannot produce antibodies against it, especially for SU Env. Alternatively, SRV-4 may impair the function of B cells, as observed in SRV-2 infection in rhesus macaques (22). Primates have antiretroviral restriction factors, such as APOBEC3, TRIM5 α , and tetherin, that serve as an intrinsic line of defense (23). Certain SRVs are sensitive to inhibition by these factors (24–26), and it is possible that there is a defect in the Japanese macaque defense system. It is necessary to compare restriction factors between Japanese macaques and cynomolgus macaques to resolve this question. Both Japanese and cynomolgus macaques belong to the genus *Macaca*, and they are genetically quite similar to each other. Subtle genetic differences may explain the different outcomes in these macaque species after SRV-4 infection.

In KUPRI, by isolating Japanese macaques positive for SRV-4 provirus DNA and/or SRV-4 antibodies from uninfected Japanese macaques, we succeeded in establishing SRV-4-free colonies. We have had no cases of hemorrhagic symptoms in KUPRI since the

FIG 8 Functional assay of Japanese macaque and cynomolgus macaque ASCT1 and ASCT2 as SRV-4 receptors. (A) Expression of ASCT1-GFP, ASCT2-GFP, and GFP in MDTFs detected by fluorescence microscopy. (B) An SRV-4 infection assay was performed in MDTFs transiently expressing ASCTs from Japanese macaques (JmASCT1 and JmASCT2) and cynomolgus macaques (CyASCT1 and CyASCT2). Infectivity of SRV-4 was quantified by real-time PCR. The copy numbers of SRV-4 DNA were normalized to one copy of mouse Actb. (C) Expression of JmASCT1 in various tissues in JM7. (D) Expression of JmASCT2 in various tissues in JM7. The copy numbers of JmASCT1 and JmASCT2 RNA were normalized to one copy of GAPDH. Values are means $\pm$ standard errors of data from three independent experiments. Ly, lymph node.

last case occurred in 2012. In the facilities dealing with primates, some primates, including rhesus and cynomolgus macaques, may have been infected with SRV-4 or other SRVs. Measures to avoid SRV-4 infection in Japanese macaques should be taken if Japanese macaques are housed with other macaques in primate facilities and zoos.

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