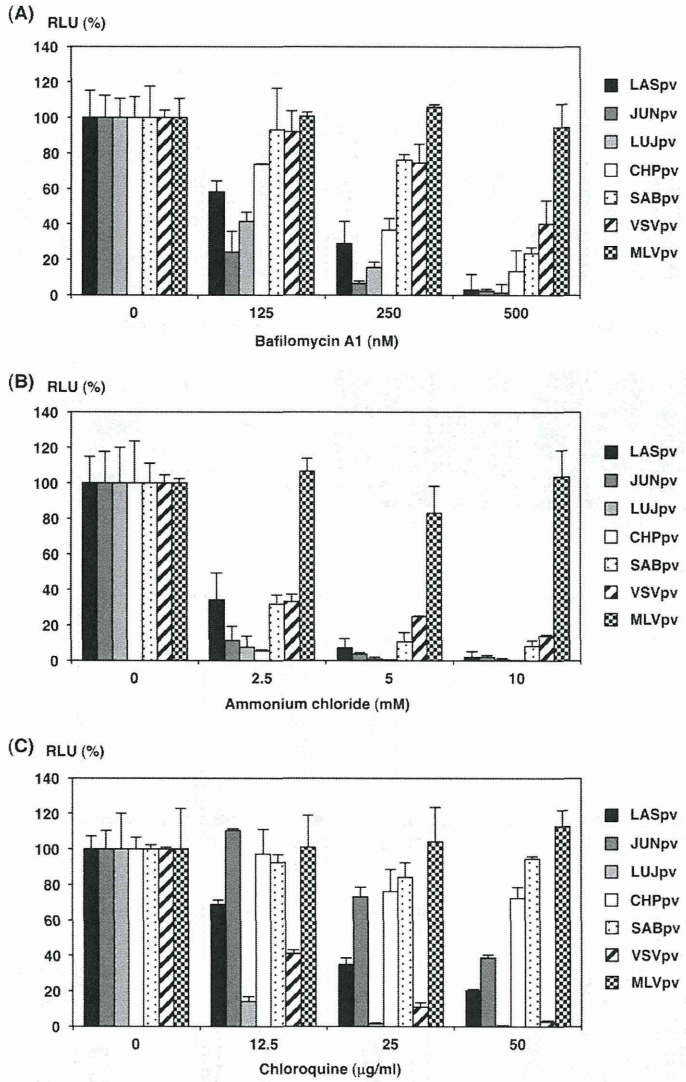


FIG 4 Inhibition of AREpv infection by H^+-ATPase inhibitors. AREpv (LASpv, JUNpv, LUJpv, CHPpv, and SABpv), VSVpv, and MLVpv were inoculated into Huh7 cells after treatment with various concentrations of bafilomycin A_1 (A), ammonium chloride (B), or chloroquine (C). Luciferase activities were determined at 24 h postinfection. The results shown are from three independent assays, with error bars representing standard deviations.

Syncytium formation and cell fusion activity in cells expressing arenaviral GPs. To examine syncytium formation in the arenaviral-GP-expressing cells, Huh7 cells were transfected with expression plasmids encoding various arenaviral GPs or VSV-G and cultured for 24 h. The cells were then treated with the indicated pH buffer for 2 min. The expression of each viral GP was confirmed by immunofluorescence assay with anti-FLAG antibody 24 h after the transfection of the expression plasmids. Synctia were observed after treatment with buffer at pH 4 in LASV-GP-expressing cells, below pH 5 in JUNV-GP- and CHPV-GP-expressing cells, and below pH 6 in VSV-G-expressing cells. In contrast, no syncytium formation was observed in LUJV-GP-expressing cells, even by treatment with buffer at pH 4 (Fig. 7A).

To further examine cell fusion activities of the arenaviral GPs, we utilized a previously established, highly sensitive, and quantitative reporter gene activation method (14). Cell fusion was induced in LASV-GP-expressing cells below pH 4, in JUNV-GP- and CHPV-GP-expressing cells below pH 5, and in VSV-G-ex-

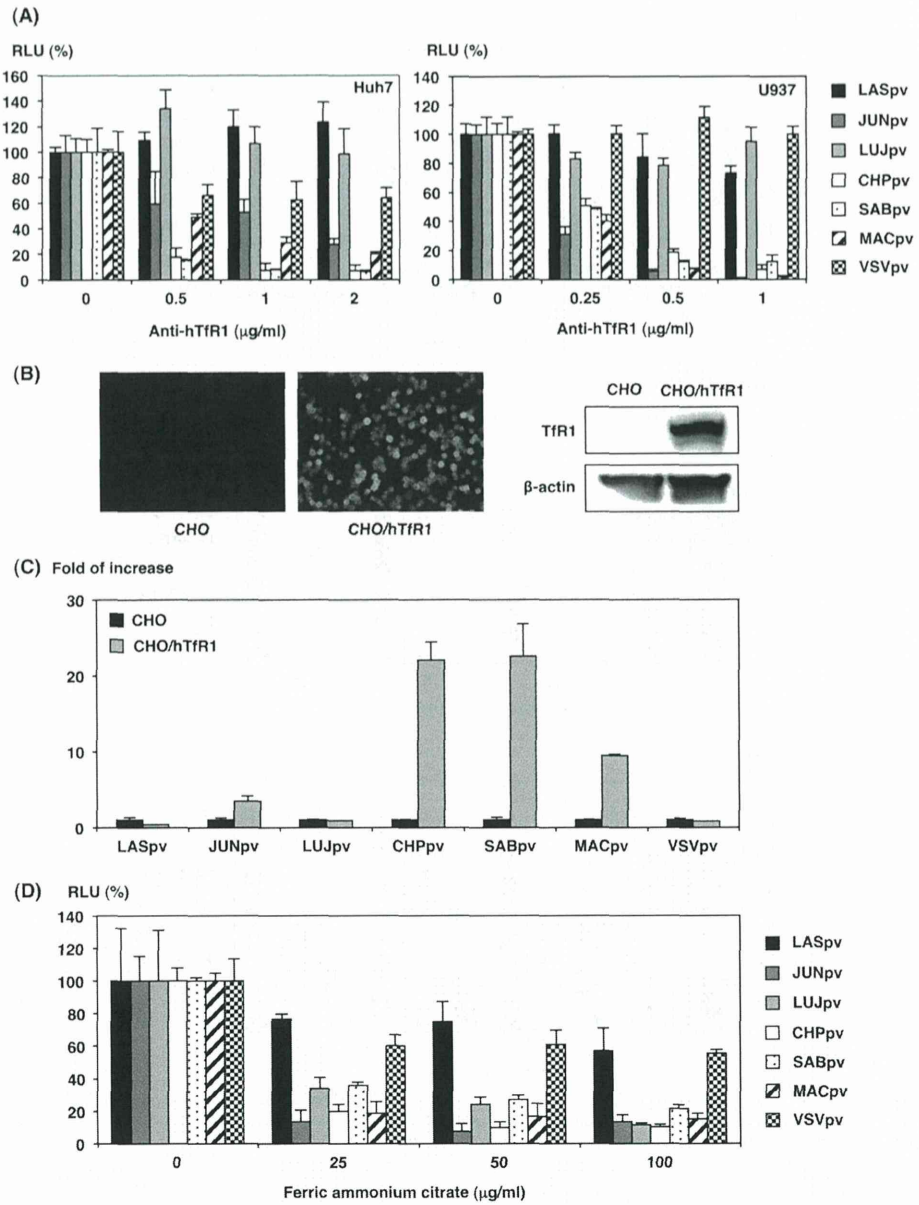


FIG 5 Involvement of human TfR1 in AREpv infection. (A) Inhibition of AREpv infection by anti-hTfR1 antibody. Huh7 or U937 cells were pretreated with various concentrations of anti-hTfR1 antibody for 1 h and inoculated with LASpv, JUNpv, LUJpv, CHPpv, SABpv, MACpv, or VSVpv, and infectivities were determined at 24 h postinfection by measuring luciferase activity. (B) Expression of hTfR1 on CHO cell lines constitutively expressing hTfR1 (CHO/hTfR1) was examined by immunofluorescence assay (left) or immunoblotting assay (right). (C) Infectivities of pseudotype immunoviruses for CHO cells expressing hTfR1. CHO or CHO/hTfR1 cells were infected with LASpv, JUNpv, LUJpv, CHPpv, SABpv, MACpv, or VSVpv, and infectivities were determined at 24 h postinfection by measuring luciferase activities. The infectivities of AREpv for CHO/hTfR1 cells were normalized to the infection of parental CHO cells. (D) Huh7 cells were pretreated with the indicated concentration of FAC and incubated at 37°C. After 1 h of incubation, the cells were inoculated with LASpv, JUNpv, LUJpv, CHPpv, SABpv, MACpv, or VSVpv, and infectivities were determined at 24 h postinfection by measuring luciferase activities. The results shown are from three independent assays, with error bars representing standard deviations.

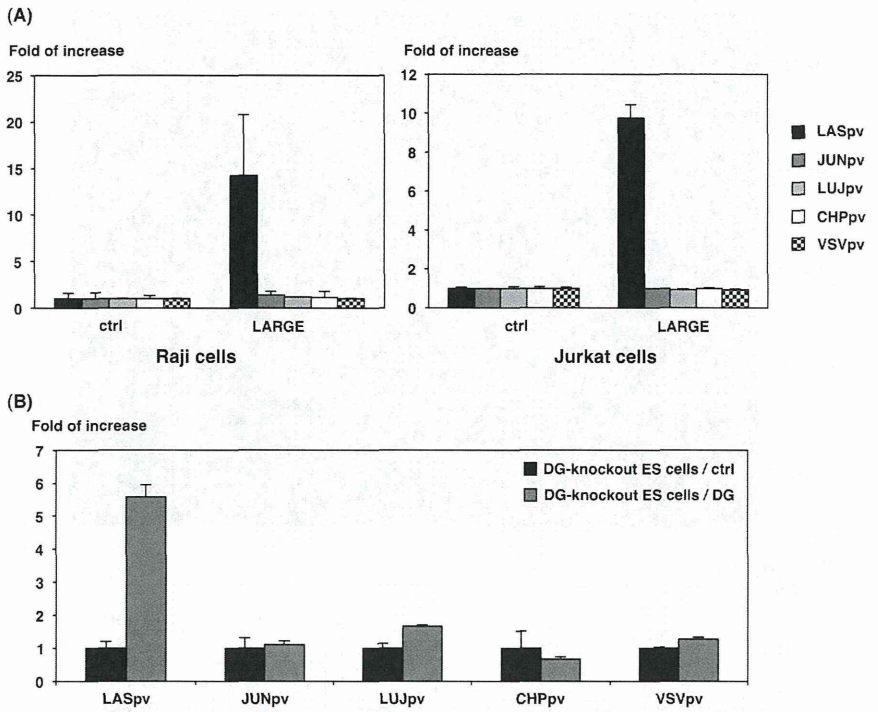
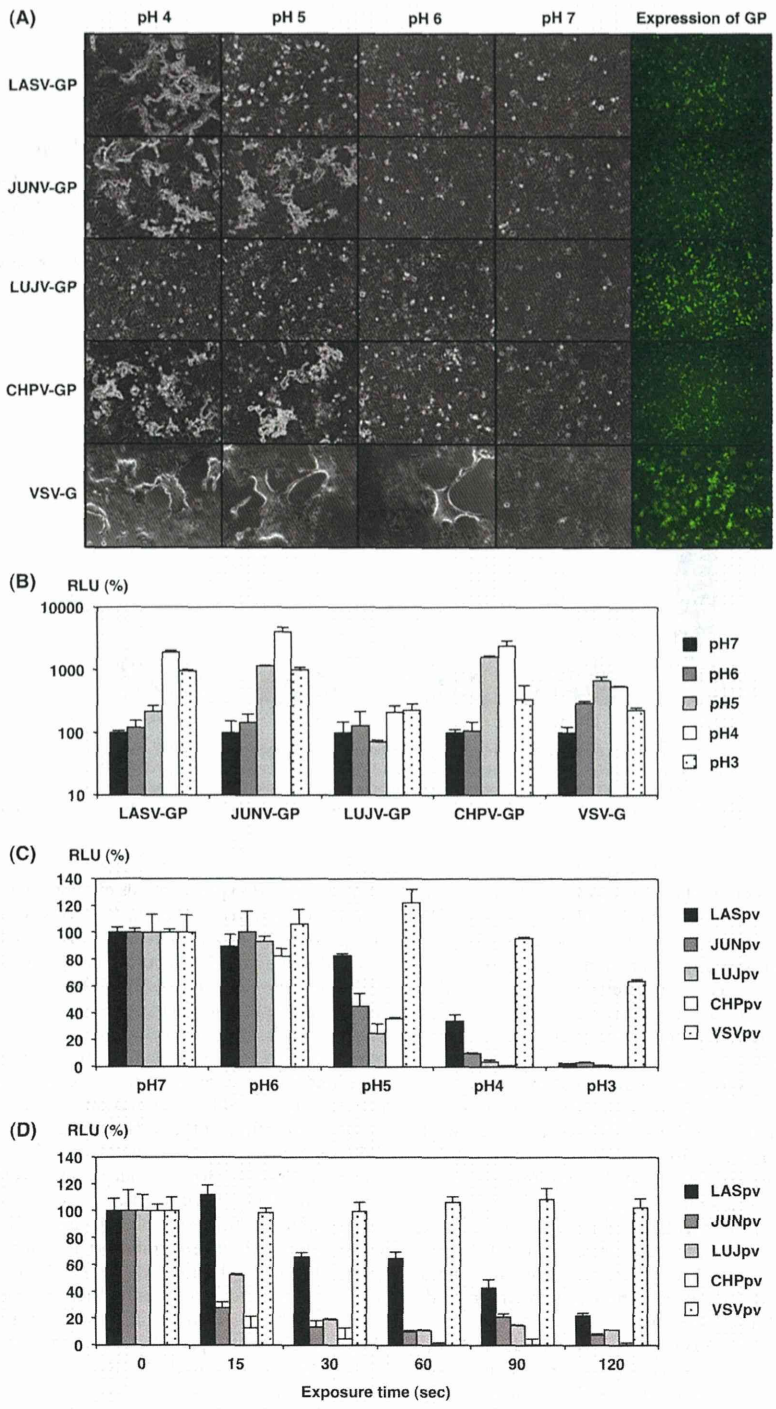


FIG 6 Involvement of αDG in AREpv infection. (A) Infectivities of AREpv in Jurkat or Raji cells expressing LARGE. Raji and Jurkat cells were transfected with the plasmid encoding LARGE or empty vector (ctrl). (B) Infectivities of AREpv in DG knockout ES cells expressing αDG. DG knockout ES cells were transfected with the plasmid encoding αDG or empty vector (ctrl). At 24 h posttransfection, the cells were infected with LASpv, JUNpv, LUJpv, CHPpv, or VSVpv, and infectivities were determined by measurement of luciferase activities. The results shown are from three independent assays, with error bars representing standard deviations.

pressing cells below pH 6. Cell fusion was not induced in cells expressing LUJV-GP, even by treatment with the buffer at pH 4 (Fig. 7B). The luciferase activities in the VSV-G-expressing cells exhibited moderate enhancement, even though syncytium formation was clearly observed. This may be due to cytotoxicity of the VSV-G in the expressing cells. Similarly, pH 3 treatment of the cells induced cytotoxicity, and thus, the expression of luciferase may have been affected.

In general, low-pH exposure is known to change the conformation of some viral GP in the absence of cellular receptors and then to abolish viral infectivity. To examine the effect of low-pH exposure on AREpv infection, the viruses were treated with the indicated pH buffer or for the indicated exposure times before infection. Infectivities of all of the AREpv, including LUJpv, were abolished after low-pH treatment and within 2 min of exposure (Fig. 7C and D). Notably, LASpv was relatively resistant to low-pH exposure in comparison to other AREpv, suggesting that conformational change of LASV-GP may be induced under more acidic pH conditions or by longer-duration exposure.

Involvement of cholesterol and sphingolipids in AREpv infection. Recently, it has been reported that Ebola virus infection requires cholesterol transporter protein, Niemann-Pick C1 (NPC1), and acid sphingomyelinase, which is the hydrolase enzyme involved in sphingolipid metabolism (31–33). In the present study, LUJV-GP-expressing cells did not exhibit cell fusion upon treatment at low pH, indicating the necessity for additional cellular factors for LUJV infection. To further examine the entry pathway and roles of lipid metabolism in AREpv infection (especially LUJpv infection), we examined the effect of chlorpromazine treatment of the cells. This chemical is widely known, not only as an inhibitor of clathrin-mediated endocytosis, but also as an inducer of lipidosis (34, 35). Even though infections by all of the AREpv were inhibited by chlorpromazine treatment in a dose-dependent manner, LUJpv and LASpv infections were drastically reduced in comparison with the other AREpv (Fig. 8A). The effects of other lipidosis-inducing drugs, imipramine, desipramine, and amitriptyline, which are known as antidepressant drugs and which cause lipid accumulation *in vitro* (33), were also examined. LUJpv and EBOpv were drastically inhibited by all of these drugs (Fig. 8B). Furthermore, a cholesterol transport inhibitor, U18666A, which is known to inhibit the function of NPC1 (36, 37), was utilized. Treatment with the chemical resulted in a dose-dependent decrease in LUJpv and EBOpv infectivities (Fig. 8C). Interestingly, these inhibitors also partially decreased LASpv infectivity, suggesting that the entry mechanisms of LASpv are similar, in part, to those of LUJpv (Fig. 8B and C). To further examine the involvement of NPC1, NPC1-deficient CHO mutant cells, CHO/A101 cells, were utilized. The infectivities of LUJpv and EBOpv were


(A)
pH 4
pH 5
pH 6
pH 7
Expression of GP
LASV-GP
JUNV-GP
LUJV-GP
CHPV-GP
VSV-G
(B)
RLU (%)
10000
1000
100
10
LASV-GP
JUNV-GP
LUJV-GP
CHPV-GP
VSV-G
pH7
pH6
pH5
pH4
pH3
(C)
RLU (%)
140
120
100
80
60
40
20
0
pH7
pH6
pH5
pH4
pH3
LASpv
JUNpv
LUJpv
CHPpv
VSVpv
(D)
RLU (%)
140
120
100
80
60
40
20
0
0
15
30
60
90
120
Exposure time (sec)
LASpv
JUNpv
LUJpv
CHPpv
VSVpv

decreased in CHO/A101 cells and were recovered by the replenishment of NPC1 in CHO/A101-expressing NPC1 cells (Fig. 8D).

DISCUSSION

The development of safe alternative research tools for pathogenic arenaviruses that cause VHF in humans is very important for research on arenavirus diagnosis and for the development of prevention systems because classification of the viruses as BSL4 pathogens impedes their use in many countries. Toward this end, pseudotype virus systems based on VSV and retroviruses have been established. In the present study, we generated pseudotype VSVs bearing various arenaviral GPs, including the GP of a novel arenavirus (Lujo virus), and analyzed the involvement of arenaviral receptor candidates, fusion activities, and entry-mediated molecules.

Both VSV and retroviruses normally bud from the plasma membranes of the infected cells. The foreign viral envelope proteins expressed on the cell surface are therefore thought to be incorporated into the pseudotype particles during their budding. In the present study, although the presence of G1 could not be detected due to the absence of detectable antibodies, all of the carboxyl-terminally FOS-tagged arenaviral GPCs and G2s were detected in the cell lysates and purified virions, respectively (Fig. 1A). Arenaviral GPs may have been efficiently incorporated by VSV because of the presence of mature arenaviral GPs on the plasma membrane, which would make all of the pseudotype VSVs possessing arenaviral GPs highly infectious to the target cells. The fact that mature G2, but not GPC, was detected in the AREpv is consistent with a previous report that showed that only cleaved G1 and G2 were incorporated into the virions (38).

Previous studies have demonstrated the glycosylation of both LASV-GP and JUNV-GP by N-linked high-mannose-type oligosaccharide (39, 40). The glycosylation of VSV-G by complex-type oligosaccharide has also been demonstrated (26, 41). In the present study, all of the arenaviral GPs expressed in the cells and incorporated into the virions were sensitive to Endo H and PNGase F treatment (Fig. 1A), showing that they were glycosylated by high-mannose-type oligosaccharide. Though analysis of the glycosylation of live LUJV is needed, these data indicate that LUJV-GP was modified by the same glycosylation as the AREpv in the above-mentioned reports.

Previous studies have also demonstrated that several arenaviruses, including LASV, JUNV, and CHPV, and pseudotype viruses bearing their GPs were able to infect various types of cell lines. The AREpv were also able to infect almost all of the cell lines examined in the present study. However, LUJpv failed to infect NIH 3T3, NMuLi, or Molt-4 cells, while LASpv failed to infect Jurkat cells. It is known that Jurkat cells are not susceptible to LASV infection because they lack the function of O-mannosylation of αDG (11, 42). These results indicate that LASpv generated in this study represents the tropism of live LASV. Although comparison with live viruses is difficult, the entry mechanisms and characteristics of the envelope proteins of other pseudotype viruses are also believed to mimic those of live viruses. LUJV is known to be able to propagate in VeroE6 cells (43). The present study showed that, in addition to VeroE6 cells, both COS7 and Huh7 cells were highly susceptible to LUJpv infection. In contrast, no susceptibility was observed in mouse-derived cell lines (NIH 3T3 and NMuLi), but hamster-derived cell lines (BHK and CHO) were susceptible. The receptors and reservoirs of LUJV remain unclear. Although the characteristics of established cell lines may differ from those of the primary host cells, these results provide a hint for identifying the LUJV receptor and allow us to speculate with regard to its reservoir, since the expression of TfR1 from the reservoir rodent was shown to confer the highest susceptibility to many New World clade B pathogenic arenaviruses (44).

In the present study, we demonstrated that LUJpv entry was drastically decreased by the treatment of cells with chloroquine or chlorpromazine. These chemicals are widely known, not only as inhibitors of H^+-ATPase activation and clathrin-mediated endocytosis, but also as inducers of lipidosis. These phenomena make sense, because our data suggest that lipid metabolism plays an important role in LUJpv cell entry. Furthermore, given the use of chloroquine in the prevention and treatment of malaria in some areas of the world, it might be also available as a medicine to patients infected with LUJV.

Several studies have revealed αDG and TfR1 to be major cellular receptors for pathogenic Old World and New World clade B arenaviruses, respectively. In the present study, we demonstrated that LUJpv cell entry occurred independently of both αDG and TfR1. Although further experiments utilizing live LUJV to examine the entry and/or propagation mechanisms are needed, our results suggest the possibility that LUJV utilizes one or more novel receptors, but not αDG or TfR1.

Observation of syncytium formation and the cell-cell fusion assay provide simple, quantitative, and versatile tools to study viral-glycoprotein-mediated cell fusion (14, 45, 46). Low-pH-induced membrane fusion by arenaviral GPs has been the subject of previous studies (47, 48). In the present study, with the exception of LUJV-GP, cell fusion induced by arenaviral GPs initiated by treatment with low-pH buffer was clearly observed, both in syncytium formation and in reporter gene activities. As for LUJV-GP, no syncytium formation or reporter gene activities were observed

FIG 7 pH dependence on arenavirus GP for cell fusion and entry. (A) Syncytium formation of Huh7 cells transiently expressing arenaviral GPs or VSV-G after treatment with low-pH buffer. Huh7 cells were transfected with pCAG-LASV-GP-FOS, pCAG-JUNV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-CHPV-GP-FOS, or pCAG-VSVG-FOS. At 24 h posttransfection, the cells were treated with citrate-phosphate buffer adjusted to the indicated pH value (pH 7, 6, 5, 4, or 3) for 2 min. Syncytium formation was determined by microscopic examination after 24 h (arenaviral GPs) or 8 h (VSV-G) incubation. Expression of arenaviral GPs or VSV-G at 24 h posttransfection was examined by immunofluorescence assay using anti-FLAG monoclonal antibody (right). (B) Quantitative cell fusion reporter assay. 293T cells transfected with pCAG-LASV-GP-FOS, pCAG-JUNV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-CHPV-GP-FOS, or pCAG-VSVG-FOS, together with a plasmid encoding T7 RNA polymerase, were cocultured with Huh7 cells, which were transfected with a plasmid carrying a luciferase gene under the control of the T7 promoter. Cell fusion activity after exposure at the indicated pH for 2 min was determined by measuring RLU. (C) The relative infectivities of AREpv after exposure at the indicated pHs. LASpv, JUNpv, LUJpv, CHPpv, or VSVpv was exposed at the indicated pHs for 2 min. After neutralization with DMEM containing 10% FCS, the remaining infectivities of the pseudotypes for Huh7 cells were measured. The luciferase activities were determined at 24 h postinfection. (D) Effects of exposure time in low-pH buffer on AREpv infectivity. LASpv, JUNpv, LUJpv, CHPpv, or VSVpv was pretreated with pH 4 citrate-phosphate buffer for the indicated time (0, 15, 30, 60, 90 or 120 s). After neutralization, the pseudotypes were inoculated into Huh7 cells. The luciferase activities were determined at 24 h postinfection. The results shown are from three independent assays, with error bars representing standard deviations.

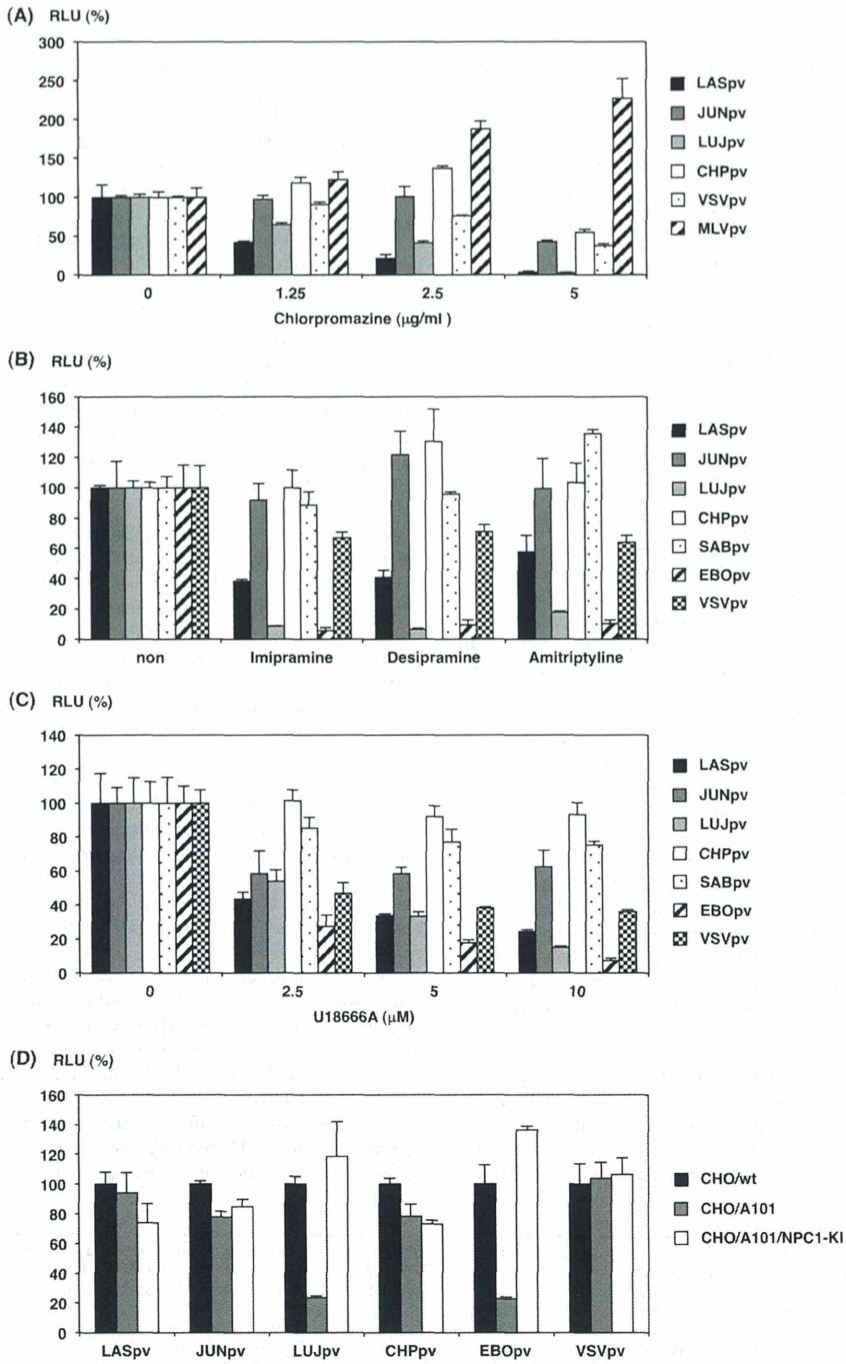


FIG 8 Involvement of cholesterol and sphingolipids in AREpv infection. (A to C) Infectivities of AREpv in Huh7 cells pretreated with the indicated concentrations of chlorpromazine (A); 10 μM imipramine, desipramine, or amitriptyline (B); or the indicated concentrations of U18666A (C). Huh7 cells were treated with each reagent and incubated at 37°C for 1 h. The cells were then inoculated with the pseudotype arenaviruses, EBOpv, MLVpv, or VSVpv, respectively. Their relative infectivities for the nontreated cells were determined at 24 h postinfection by measuring the luciferase activities. (D) Infectivities of pseudotype arenaviruses in wild-type CHO cells (CHO/wt), NPC1-deficient CHO cell mutants (CHO/A101), and CHO/A101 cells stably expressing FLAG-tagged NPC1 (CHO/A101/NPC1-KI). Their relative infectivities for the CHO/wt cells were obtained. The results shown are from three independent assays, with error bars representing standard deviations.

in the cells treated with low-pH buffer, despite the abundant expression of GP. Furthermore, immunofluorescence assays with and without permeabilized conditions revealed no difference between the cellular localization of GP in LUJV-GP and that of the other arenaviral GPs (data not shown). Moreover, LUJV-GP is considered to be present in the plasma membrane, because LUJV-GP was efficiently incorporated into virions and LUJpv, as well as other AREpv that exhibited high infectivity. In some of the enveloped viruses, such as Ebola virus or severe acute respiratory syndrome coronavirus (SARS-CoV), it is known that cleavage of the GP with the cysteine proteases cathepsins B and L is a prerequisite for membrane fusion (49–51). As far as we examined, the infectivity of LUJpv in Huh7 cells was not influenced by treatment with cathepsin B or L inhibitors (data not shown). Although we need to clarify whether extra cleavage or modification of LUJV-GP is necessary for membrane fusion, it is suggested that LUJV-GP shows unique characteristics among arenaviral GPs.

No visible syncytium formation was observed in the cells expressing Ebola virus GP under similar experimental conditions (data not shown). It was recently reported that in Ebola virus infection, the interaction of NPC1 with Ebola virus GP activated by the cathepsins is required for endosomal membrane fusion (31, 32). The results of the present study showed that LUJpv infection was inhibited, not only by lipidosis-inducing drugs, but also by the drug U18666A, which causes NPC1-like organelle defects. Furthermore, both LUJpv and EBOpv infections were abolished in NPC1-deficient cells, and these infectivities were recovered by the replenishment of NPC1 expression. Although it is currently not known whether LUJpv infection is directly involved in interaction with the NPC1 protein, the accumulation of cholesterol or some kinds of lipids may play important roles in the inhibition of LUJpv infection. Generally, NPC1 presents on cellular endosomes but not on the cell surface (52). If Lujo virus GP needs to interact with NPC1 in the same way as Ebola virus GP, this could be one of the reasons why cell-to-cell fusion did not occur in GP-expressing cells.

In conclusion, we generated replication-incompetent pseudotype arenaviruses possessing each arenaviral envelope glycoprotein, in particular, LUJpv, which is a novel surrogate model for the study of LUJV entry. In the present study, some of the analyses of LUJpv revealed that LUJV and its glycoprotein have unique characteristics for entry and fusion. Although LUJV is classified as a BSL4 pathogen, which makes it difficult to handle the authentic live virus, LUJpv and other pseudotype arenaviruses are quite useful for further detailed examination of arenaviral entry mechanisms.

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Sensitive and Specific PCR Systems for Detection of Both Chinese and Japanese Severe Fever with Thrombocytopenia Syndrome Virus Strains and Prediction of Patient Survival Based on Viral Load

Tomoki Yoshikawa,[a] Shuetsu Fukushi,[a] Hideki Tani,[a] Aiko Fukuma,[a] Satoshi Taniguchi,[a,b] Shoichi Toda,[c] Yukie Shimazu,[d] Koji Yano,[e] Toshiharu Morimitsu,[f] Katsuyuki Ando,[g] Akira Yoshikawa,[h] Miki Kan,[i] Nobuyuki Kato,[j] Takumi Motoya,[k] Tsuyoshi Kuzuguchi,[l] Yasuhiro Nishino,[m] Hideo Osako,[n] Takahiro Yumisashi,[o] Kouji Kida,[p] Fumie Suzuki,[q] Hirokazu Takimoto,[r] Hiroaki Kitamoto,[s] Ken Maeda,[t] Toru Takahashi,[u] Takuya Yamagishi,[v] Kazunori Oishi,[v] Shigeru Morikawa,[b] Masayuki Saijo,[a] Masayuki Shimojima[a]

Special Pathogens Laboratory, Department of Virology I, National Institute of Infectious Diseases, Musashimurayama-shi, Tokyo, Japan[a]; Department of Veterinary Science, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan[b]; Yamaguchi Prefectural Institute of Public Health and Environment, Yamaguchi, Japan[c]; Hiroshima Prefectural Technology Research Institute, Public Health and Environment Center, Minami-ku, Hiroshima-shi, Hiroshima, Japan[d]; Miyazaki Prefectural Institute for Public Health and Environment, Miyazaki-shi, Miyazaki, Japan[e]; The Public Institute of Kochi Prefecture, Kochi-shi, Kochi, Japan[f]; Saga Prefectural Institute of Public Health and Pharmaceutical Research, Saga-shi, Saga, Japan[g]; Nagasaki Prefectural Institute for Environmental Research and Public Health, Omura-shi, Nagasaki, Japan[h]; Ehime Prefectural Institute of Public Health and Environmental Science, Matsuyama-shi, Ehime, Japan[i]; Tottori Prefectural Institute of Public Health and Environmental Science, Yurihama-cho Tohaku-gun, Tottori, Japan[j]; Ibaraki Prefectural Institute of Public Health, Mito-shi, Ibaraki, Japan[k]; Gifu Prefectural Institute for Public Health and Environmental Science, Kakamigahara-shi, Gifu, Japan[l]; Tokushima Prefectural Public Health, Pharmaceutical and Environmental Sciences Centre, Tokushima-shi, Tokushima, Japan[m]; Kumamoto Prefectural Institute of Public Health and Environmental Science, Uto-shi, Kumamoto, Japan[n]; Osaka Prefectural Institute of Public Health, Higashinari-ku Osaka-shi, Osaka, Japan[o]; Okayama Prefectural Institute for Public Health and Environmental Science, Minami-ku Okayama-shi, Okayama, Japan[p]; Shizuoka City Institute of Environmental Sciences and Public Health, Suruga-ku Shizuoka-shi, Shizuoka, Japan[q]; Shimane Prefectural Institute of Public Health and Environmental Science, Matsue-shi, Shimane, Japan[r]; Public Health Science Research Center, Hyogo Prefectural Institute of Public Health and Consumer Sciences, Hyogo-ku Kobe-shi, Hyogo, Japan[s]; Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan[t]; Department of Hematology, Yamaguchi Grand Medical Center, Hofu, Yamaguchi, Japan[u]; Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan[v]

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease with a high case fatality risk and is caused by the SFTS virus (SFTSV). A retrospective study conducted after the first identification of an SFTS patient in Japan revealed that SFTS is endemic to the region, and the virus exists indigenously in Japan. Since the nucleotide sequence of Japanese SFTSV strains contains considerable differences compared with that of Chinese strains, there is an urgent need to establish a sensitive and specific method capable of detecting the Chinese and Japanese strains of SFTSV. A conventional one-step reverse transcription-PCR (RT-PCR) (cvPCR) method and a quantitative one-step RT-PCR (qPCR) method were developed to detect the SFTSV genome. Both cvPCR and qPCR detected a Chinese SFTSV strain. Forty-one of 108 Japanese patients suspected of having SFTS showed a positive reaction by cvPCR. The results from the samples of 108 Japanese patients determined by the qPCR method were in almost complete agreement with those determined by cvPCR. The analyses of the viral copy number level in the patient blood samples at the acute phase determined by qPCR in association with the patient outcome confirmed that the SFTSV RNA load in the blood of the nonsurviving patients was significantly higher than that of the surviving patients. Therefore, the cvPCR and qPCR methods developed in this study can provide a powerful means for diagnosing SFTS. In addition, the detection of the SFTSV genome level by qPCR in the blood of the patients at the acute phase may serve as an indicator to predict the outcome of SFTS.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease with a high case mortality rate (approximately 12%) that is caused by the SFTS virus (SFTSV). The disease was first reported to be endemic to some parts of China in 2011 (1–3). The virus is classified within the *Phlebovirus* genus of the *Bunyaviridae* family.

In January 2013, a female patient who lived in western Japan died, and her death was confirmed to be due to SFTSV infection (4). Additionally, 10 SFTS patients were retrospectively identified (4). A phylogenetic study revealed that all eight strains isolated from Japanese patients were clustered in a lineage that was independent from the other lineages of the Chinese strains. Given that an isolate obtained from a South Korean patient with SFTS belongs to a Chinese lineage (5), Japanese SFTSV appears to be an indigenous virus.

For the rapid diagnosis of viral infectious diseases, a method of pathogen detection with both high sensitivity and high specificity is needed. Based on the nucleotide sequences of the Chinese SFTSV isolates, there have been some reports describing a reverse transcription-PCR (RT-PCR)-based method, a reverse transcription-loop-mediated isothermal amplification assay (RT-LAMP), reverse transcription-cross-priming amplification coupled (RT-CPA) with vertical flow (VF) visualization, and TaqMan-based

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Address correspondence to Masayuki Shimojima, shimoji-@nih.go.jp.
T. Yoshikawa and S. Fukushi contributed equally to this work.

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TABLE 1 The primer-probe sets used for qPCR

Primer set target (segment)	Primer sequence			Target region (positions)
	Forward	Reverse	Probe sequence[a]	
N (S)	TGTCAGAGTGGTCCAGGATT	ACCTGTCTCCTTCAGCTTCT	FAM-TGGAGTTTGGTGAGCAGCAGC-BHQ1	1566–1702
GPC (M)	GGCAGCTACATGCAGACATA	CCTATCACCCCCAGAATCCA	TexasRed-GCC**C**TGTTTGGCAATGGGCT-BHQ2	236–357
			TexasRed-GCC**T**TGTTTGGCAATGGGCT-BHQ2	
RdRp (L)	AACATCCTGGACCTTGCATC	CAATGTGGCCATCTTCTCCA	Cy5-TGGGAGCT**C**TACTCAGAA**G**TCCA-BHQ3	3710–3832
			Cy5-TGGGAGCT**C**TACTCAGA**G**GTCCA-BHQ3	
			Cy5-TGGGAG**A**T**C**TACTCAGAA**G**TCCA-BHQ3	
			Cy5-TGGGAGCT**T**TACTCAGAA**G**TCCA-BHQ3	
Contamplicon			JOE (HEX)-AGTAGCTTGCTCTTTCATCTGTTACG-BHQ1	

[a] The bases in bold type indicate the mismatch of the sequences between the probes. FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1; JOE, 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein; HEX, hexacholoro-6-carboxyfluorescein.

quantitative real-time PCR for SFTSV detection (3, 6–8). However, since there are considerable sequence mismatches between the Chinese and Japanese lineages, especially in the reported TaqMan probe target regions, these primers/probes might not be suitable for use with the Japanese linage (4).

In this study, we developed a conventional one-step RT-PCR (cvPCR) and a quantitative one-step RT-PCR (qPCR) capable of detecting both the Chinese and Japanese SFTSV lineages. The efficacies of these methods were evaluated using clinical specimens collected from Japanese patients suspected of having SFTS. Furthermore, it was demonstrated that the SFTS viral RNA levels in the acute-phase peripheral blood samples were significantly higher from the patients who died than those from the patients who survived, as reported previously (9). On the other hand, there was a contradictory finding that the viral copy number in the serum during the initial 1 to 7 days after the onset was comparable between those from the patients who died and survived (10). We therefore also analyzed the association of the viral copy number in the peripheral blood specimens as determined by qPCR with the prognosis of the patients. The results support the report by Zhang et al. (9), indicating that the viral copy number in the acute-phase patient blood samples correlated with the outcome, with a higher level being associated with a poorer prognosis.

MATERIALS AND METHODS

Clinical specimens from patients suspected of having SFTS. We asked medical personnel in Japan to inform us on a voluntary basis if they had seen any patients with symptoms similar to those of SFTS, as summarized by Takahashi et al. (4), from 30 January to 30 September 2013. Through the courtesy of prefectural and municipal public health institutes, 149 specimens consisting of serum, plasma, urine, and cerebrospinal fluid from 108 patients were collected and used in this study.

Viruses. SFTSV strains YG1 and SPL005, previously isolated from serum samples from Japanese patients (4), and a Chinese strain of HB29 were utilized to develop the SFTSV genome amplification systems with RT-PCR. The supernatant of Vero cells infected with Rift Valley fever virus strain MP-12 (RVFV) and Forecariah virus (11, 12), which was a kind gift from Robert Tesh (University of Texas Medical Branch), was used in this study. The infectious dose of each of the SFTSV, RVFV, and Forecariah virus stock solutions was determined by calculating the 50% tissue culture infectious dose ($TCID_{50}$) in Vero cells either through the visualization of infection by an indirect immunofluorescence assay, as described previously (4), or by observing the cytopathic effects (CPE). Briefly, cells infected with SFTSV were reacted with a rabbit anti-SFTSV NP antibody, followed by staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H+L) (ZyMax Grade; Life Technologies, Carlsbad, CA).

Development of PCR references. Positive-control plasmids for cvPCR and the RNA reference for qPCR that contained the target sequences of the cvPCR and qPCR primer-probe sets were prepared. To differentiate positive-control contamination of the samples during the cvPCR reaction, a pCR-SFTSV-posicon was constructed. Briefly, portions of two regions (positions 1044 to 1386 and 1267 to 1665) in the SFTSV YG1 small (S) segment were amplified with the addition of EcoRI site at each side (next to positions 1386 and 1267). These PCR products were ligated by a Rapid DNA ligation kit (Roche Applied Science, Penzberg, Germany) after EcoRI digestion. The ligated PCR product was then further ligated into pCR-Blunt II-TOPO using a Zero Blunt TOPO PCR cloning kit (Life Technologies), according to the manufacturer's protocol. The qPCR references for the primer-probe sets targeting the large (L), medium (M), or S segments were constructed with the insertion of the contamplicon sequence, which is, using a method described by Atkinson et al. (13), for the detection of RNA reference contamination by the contamplicon probe. Briefly, each of the virus genomes from positions 3710 to 3832 in the L segment, positions 236 to 357 in the M segment, and positions 1566 to 1702 in the S segment was cloned into pCR2.1-TOPO, which contains the T7 promoter sequence upstream of the cloning site. The complementary sequence of the contamplicon probe shown in Table 1 was replaced by positions 3732 to 3757 in the L segment, positions 291 to 316 in the M segment, or positions 1591 to 1616 in the S segment. The RNA references were produced from the plasmids containing partial L, M, or S segments using the MEGAscript T7 kit (Life Technologies) after XhoI digestion of the pUC plasmids. The copy numbers of the RNA references were determined based on the optical density (OD) values, and samples were diluted to an appropriate concentration in Tris-EDTA (TE) buffer containing 100 μg/ml of yeast tRNA (Life Technologies).

The synthetic RNA sequences of the Heartland virus (used as a reference) (14) were made from an RNA solution extracted from the virus-infected culture supernatant, which was a kind gift from Hideki Ebihara (National Institute of Allergy and Infectious Diseases). Briefly, portions of three regions that contain potential target sequences for the cvPCR and qPCR (positions 2392 to 2947 in the L segment, 138 to 596 in the M segment, and 896 to 1749 in the S segment of Heartland virus patient 1) were amplified with the addition of a T7 promoter sequence. The synthesized RNA references were produced from the PCR products using the MEGAscript T7 kit (Life Technologies).

Total RNA extraction. Total RNAs were extracted from 200 μl of the specimens using a High Pure viral RNA kit (Roche Applied Science) according to the manufacturer's protocol. The elution volume for RNA extraction was 50 μl.

Conventional one-step RT-PCR. SFTSV genome-specific primer sets 1 and 2 (Table 2) were selected using the following procedures. First,