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Rapid report

JNK and PI3k/Akt signaling pathways are required for establishing persistent SARS-CoV infection in Vero E6 cells

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Abstract

Persistence was established after most of the SARS-CoV-infected Vero E6 cells died. RNA of the defective interfering virus was not observed in the persistently infected cells by Northern blot analysis. SARS-CoV diluted to 2 PFU failed to establish persistence, suggesting that some particular viruses in the seed virus did not induce persistent infection. Interestingly, a viral receptor, angiotensin converting enzyme (ACE)-2, was down-regulated in persistently infected cells. G418-selected clones established from parent Vero E6 cells, which were transfected with a plasmid containing the neomycin resistance gene, were infected with SARS-CoV, resulting in a potential cell population capable of persistence in Vero E6 cells. Our previous studies demonstrated that signaling pathways of extracellular signal-related kinase (ERK1/2), c-*Jun* N-terminal protein kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3′-kinase (PI3K)/Akt were activated in SARS-CoV-infected Vero E6 cells. Previous studies also showed that the activation of p38 MAPK by viral infection-induced apoptosis, and a weak activation of Akt was not sufficient to protect from apoptosis. In the present study, we showed that the inhibitors of JNK and PI3K/Akt inhibited the establishment of persistence, but those of MAPK/ERK kinase (MEK; as an inhibitor for ERK1/2) and p38 MAPK did not. These results indicated that two signaling pathways of JNK and PI3K/Akt were important for the establishment of persistence in Vero E6 cells.

Keywords: SARS-CoV; Persistent infection; c-*Jun* N-terminal protein kinase; Phosphatidylinositol 3′-kinase/Akt

Severe acute respiratory syndrome (SARS) is a newly found infectious disease caused by a novel coronavirus, SARS coronavirus (SARS-CoV) [11,17]. SARS spread from Guangdong Province in China to more than 30 countries in late 2002, causing severe outbreaks of atypical pneumonia. SARS-CoV is an enveloped, single-stranded positive-sense RNA virus with an RNA genome of approximately 30,000 nucleotides encoding at least 15 open reading frames [11]. As the viral virulence and mortality rate of the patients are very high, understanding the mechanisms of the pathogenicity of SARS-CoV is important for the prevention of SARS.

The gene organization of SARS-CoV is similar to those of other coronaviruses. Coronaviruses exhibit a unique ability to establish persistent infections in vivo and in vitro. Mouse hepatitis virus (MHV), a prototype coronavirus, causes central nervous system diseases in rodents. Astrocytes are the predominant cells that harbor persistent viruses in the central nervous system (CNS) [16]. A previous in vitro study also indicated that the astrocytoma cell line DBT has a potential to establish persistent infection after MHV infection [10]. However, the mechanism of establishment of coronavirus persistence has not been yet well understood. Recently, the down-regulation of the pro-apoptotic protein BNip-3 was found in MHV-infected DBT cells by DNA microarray methodologies [2]. In MHV-infected cells, BNip3 levels were significantly decreased at the transcriptional level, and this down-regulation was mediated by the fusion between the viral envelope and the cell membrane. This observation suggested that the anti-apoptotic effect by the down-regulation of BNip3 in MHV-infected DBT cells allows persistent infection.

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Recently, a human intestinal cell line, LoVo cells, was shown to permit SARS-CoV infection, resulting in the establishment of persistent infection [4]. However, the mechanism of persistence in LoVo cell is still unclear. The monkey kidney cell line, Vero E6, is widely used in the SARS-CoV research because of a high sensitivity to the virus infection. Vero E6 cells express a viral receptor, angiotensin converting enzyme (ACE)-2 [9], at high levels, and SARS-CoV infection of Vero E6 causes cytopathic effects after 24 h [13]. We showed that SARS-CoV infection of Vero E6 cells induced apoptosis via caspase-3 [13]. Several signaling pathways are activated in SARS-CoV-infected Vero E6 cells. One of the mitogen-activated protein kinases (MAPKs), p38, was shown to be phosphorylated and to have pro-apoptotic roles, including tyrosine dephosphorylation of signal transducer and activator of transcription (STAT) 3 [13,15]. Although Akt, which is known to act as an anti-apoptosis factor, was also phosphorylated in virus-infected cells, the activity of Akt is not enough to prevent apoptosis in the SARS-CoV-infected cells [14].

In the present study, Vero E6 cells were subcultured routinely in 75-cm^3 flasks in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 0.2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% (v/v) fetal bovine serum (FBS) and maintained at 37 °C in an atmosphere of 5% CO_2. For use in the experiments, the cells were split once they reached 90% confluence and were seeded onto 24-well tissue culture plate inserts or 25-cm^3 (T-25) flasks. The 100% confluent cells were used in the present and previous studies [13–15]. The culture medium was changed to 2% FBS containing DMEM prior to virus infection. We used a SARS-CoV isolate, Frankfurt 1, kindly provided by Dr. J. Ziebuhr.

Our recent studies indicated that SARS-CoV induces apoptosis in Vero E6 cells after 24-h post infection (h.p.i.) [13]. Although almost all of the virus-infected cells showed morphological changes indicative of cell death until 48 h.p.i., very few surviving cells were observed, and these cells grew and formed colonies on 4 days p.i. (d.p.i.). To investigate whether SARS-CoV replicates in these surviving cells, indirect immunofluorescence (IF) staining was performed to detect intracellular viral antigens. As shown in Fig. 1, all the cells showed viral antigens in the cytoplasm at 10 d.p.i. The persistently infected cell culture at 7 passages produced infectious viral particles at 1.26×10^5 PFU/ml. These results indicated that the persistently infected Vero E6 cells produce infectious viruses.

Persistent infection of many RNA viruses in cell culture has been studied. In these studies, it has been demonstrated that the establishment of RNA virus persistent infection in a cell culture is often involved in the generation of defective interfering (DI) particles and reduction of interferons. In the case of measles virus, persistent viral infected Vero cells produced DI particles [3]. Non-

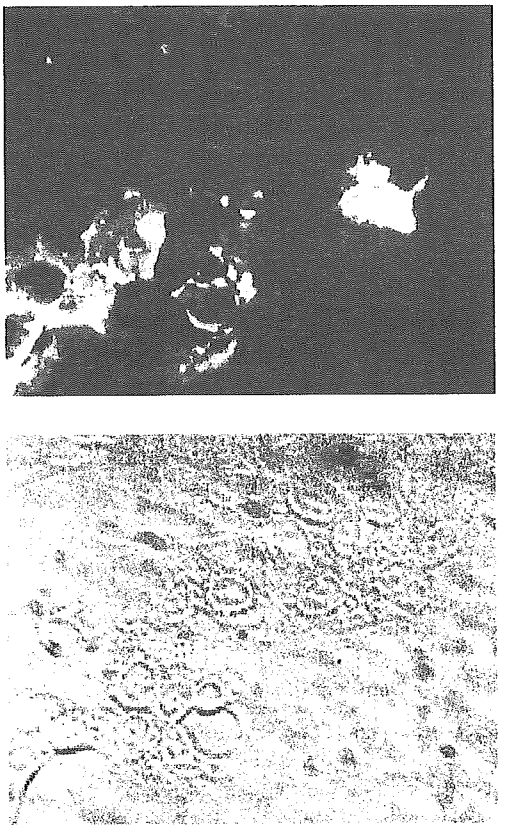

Fig. 1. Indirect immunofluorescence staining of SARS-CoV proteins. For the establishment of persistent infection, the virus was infected to Vero E6 cells at 10 m.o.i. At 10 days p.i., intracellular viral antigens were detected by indirect immunofluorescence staining using rabbit anti-SARS-CoV antibody, which recognizes SARS-CoV proteins (unpublished data). Briefly, cells were spotted onto a 12-well Teflon-coated slideglass, air dried in a biosafety cabinet under UV irradiation, and fixed with 100% pre-chilled acetone. Aliquots of 25 μl of 1:160 diluted anti-SARS-CoV antibody were placed on the coated wells and incubated at 37 °C for 30 min in a moist chamber. After washing with PBS, a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody was added at a dilution of 1:40, and incubated for 30 min at 37 °C.

cytopathic (ncp) bovine viral diarrhea virus (BVDV) does not induce type I interferon (IFN), whereas cytopathic (cp) BVDV is able to induce type I IFN [1,6], suggesting that the different capabilities of cp and ncpBVDV to establish persistent infections relates to the difference in their ability to induce IFN [1]. Because Vero E6 cells are known to lack type I interferon genes [5], the involvement of interferon was unlikely in the establishment of SARS-CoV persistence in Vero E6 cells. To investigate whether DI RNAs exist in the persistent cells, Northern blot analysis was performed using a probe complementary to mRNA9 for detecting virus standard genome and all (m)RNAs of SARS-CoV. As shown in Fig. 2, we could not detect any signals of additional viral mRNA in the viral persistent cells at 11 days p.i. This result suggested that the DI virus was not involved in the establishment of persistence in the case of SARS-CoV. However, we cannot

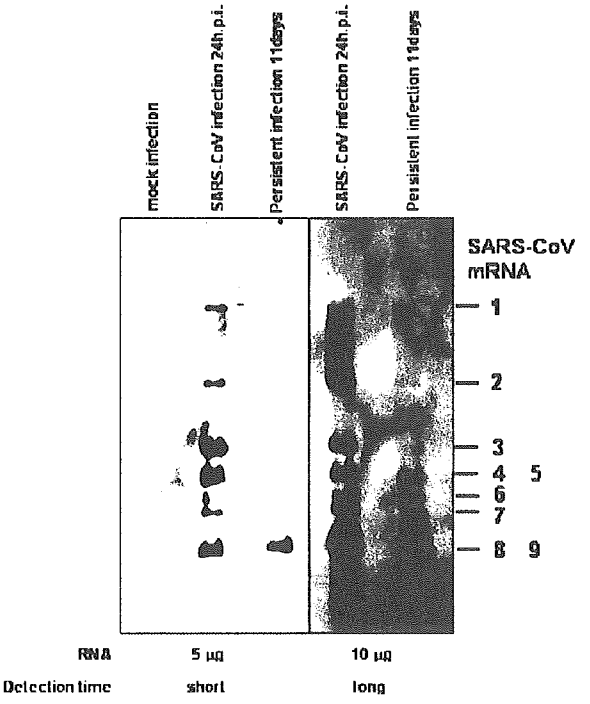

Fig. 2. Northern blot analysis of viral mRNAs. Vero E6 cells were inoculated with SARS-CoV at 10 m.o.i. Total RNA was extracted with Isogen (Nippon Gene, Tokyo, Japan) from SARS-CoV-infected cells at 24 h and 11 days p.i. and from mock-infected cells. RNAs were electrophoresed on 1% agarose gels in the presence of formaldehyde, blotted onto the nitrocellulose membrane. The probe was constructed based on the PCR product of mRNA9. Briefly, viral RNA was reverse-transcribed using SuperScriptIII (Invitrogen, Carlsbad, CA, USA) and the reverse primer, 5′-AGTCAGTCTAATACGACTCACTATAGGGTCCTAAGAAGCTAT-TAAAATTAGC-3′(29,687 to 29,721 nt of GenBank accession numberNC_004718). The underline indicates the promoter sequence of T7 RNA polymerase. PCR amplification was performed using High Fidelity Platinum Taq DNA polymerase (Invitrogen) using the reverse primer and forward primer, 5′-TGGCTAGCGGAGGTGGTGAAACTGCCC-3′(28,751 to 28,777 nt). The RNA probe was transcribed from the PCR product using T7 RNA polymerase (Ambion, Austin, TX, USA) under incorporating digoxigenin (DIG RNA labeling kit, Boehringer Mannheim GmbH, Mannheim, Germany). Hybridization and wash were performed as described previously [12]. Left and right panels indicated short and long detection times using the LAS-3000 mini system (Fuji Photo Film Co. Ltd, Tokyo, Japan), respectively.

rule out a possibility that a small amount of DI RNAs appeared in the persistent cells. We measured the densities of mRNA8, 9 and 4, 5 using the LAS-3000 mini system (Fuji Photo Film Co. Ltd, Tokyo, Japan). The amount of mRNA8 and 9 of the persistent cells are 81.4% of the acute infected cells. On the other hand, mRNA4 and 5 of the persistent cells were 12.29% of acute infected cells. In addition, a new round of infection did not induce cell death into persistent infected cells (data not shown). Therefore, we investigated whether persistent cells express a functional SARS-CoV receptor, ACE-2. On Western blotting analysis, ACE-2 was not detected in the persistent cell line (Fig. 3). Interestingly, ACE-2 was also decreased in the acute infection of SARS-CoV. This result suggested that virus particles produced by persistently infected cells could not infect other cells due to a lack of the receptor.

We were interested in determining whether the virus or the host cell is responsible for the establishment of persistent SARS-CoV infection. As shown above, small numbers of Vero E6 cells survived and then continued to grow after SARS-CoV infection. These cells were infected and produced cytopathic viruses (data not shown). The majority of the virus in a seed could induce the apoptotic cell death of Vero E6 cells [13]. If some species of the seed SARS-CoV are involved in an establishment of persistence, seed virus with a limited dilution should not permit persistent infection. The seed SARS-CoV in the present study contained 2×10^8 plaque forming unit (PFU)/ml on Vero E6 cells. The seed virus was diluted from 2×10^{-2} to 2×10^{-11} PFU, and these virus suspensions were used to inoculate aliquots of 1.5×10^5 cells in T-25 flasks. As shown in Fig. 4, no cell death was observed in cells infected with virus at a dilution of 2×10^{-11} PFU, indicating that viral dilution had been performed correctly. We found that 2×10^0 (=2) PFU-infected cells had fewer plaques than did the 2×10^{-2} (=200) PFU-infected cells at 36 h.p.i. However, all flasks other than those infected at 2×10^{-11} PFU showed death of almost all cells at 4 d.p.i. and surviving colonies at 7 d.p.i. These colonies became larger at 13 d.p.i. From these results, it seems most likely that some particular viruses in the seed virus did not induce persistent infection.

If a certain Vero E6 cell has the ability to establish persistence after viral infection, such a cell should survive without cell death following infection. To address this possibility, clones from parent Vero E6 cells were established by geneticin-selection (500 μg/ml) after the transfection of pcDNA3 (Invitrogen). Although a total of 115 clones was obtained, 24 clones showed morphological changes or grew very slowly. Therefore, 91 clones that showed similar growth rates in 24-well plates were infected with SARS-CoV at 10 m.o.i. The parental Vero E6 cells

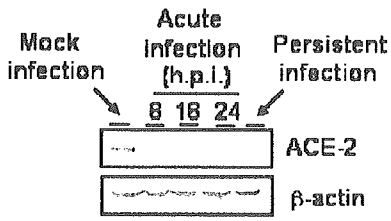

Fig. 3. Down-regulation of ACE-2 by SARS-CoV infection. Western blot of acute infection (8, 18, and 24 h.p.i.) and persistent infection (passage 7) were performed using anti-ACE-2 antibody. Whole-cell extracts were electrophoresed in 10–20% gradient polyacrylamide gel and transferred onto PVDF membranes (Immobilon-P, Millipore, Bedford, MA, USA). We applied two sets of samples to the polyacrylamide gels, and the blots were divided into two sheets for detection by a ProtoBlot II AP system (Promega Co., Madison, WI, USA), as described previously [13–15]. Mouse anti-human ACE-2 ectodomain monoclonal antibody was purchased from R and D systems Inc (Minneapolis, MN, USA) and used at a dilution of 1:1000. Mouse anti-beta actin antibody was purchased from Sigma (St. Louis, MO, USA) and used at a dilution of 1:5000.

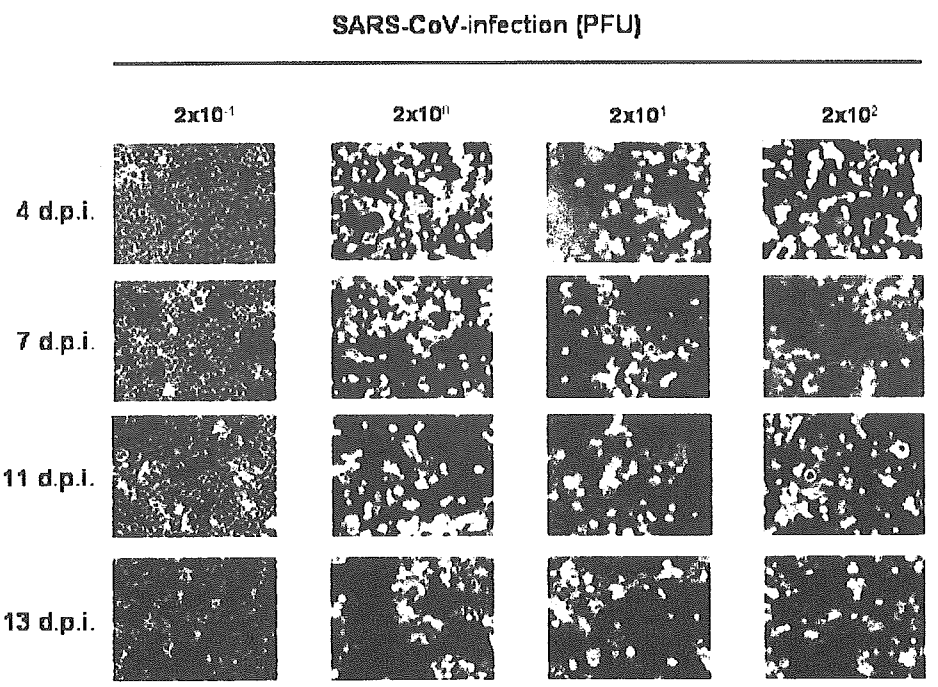

Fig. 4. Establishment of persistent SARS-CoV-infected cells by diluted viruses. Aliquots of 1.5×10^5 Vero E6 cells were inoculated with 2×10^{-1}, 2×10^0, 2×10^1, and 2×10^2 PFU of SARS-CoV. The cells were stained with 0.05% crystal violet after fixing with 20% formaldehyde.

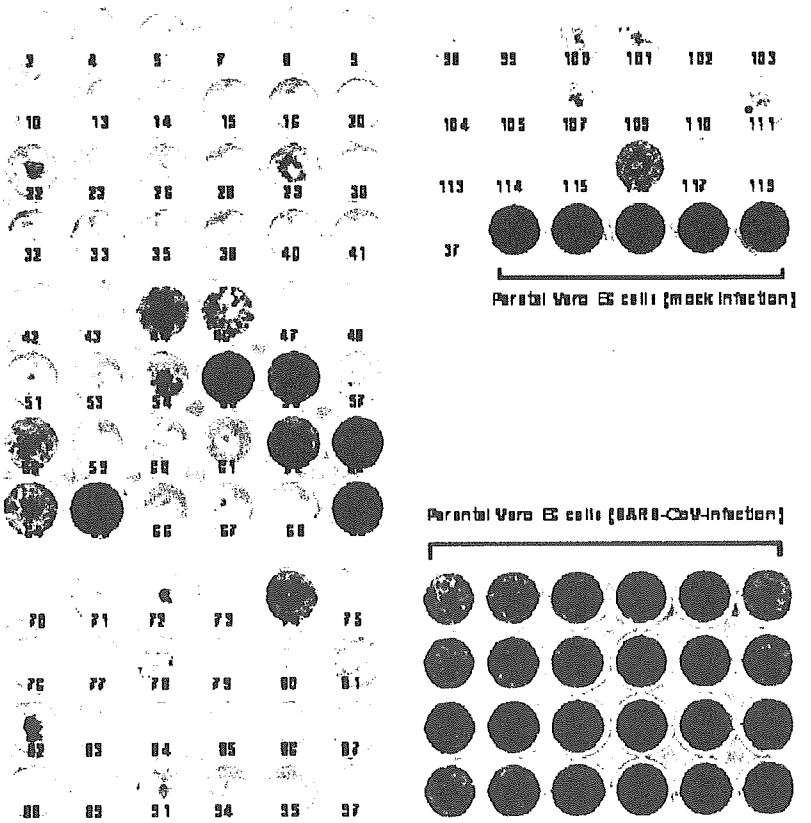

Fig. 5. Persistent SARS-CoV infection of cloned Vero E6 cells. A plasmid, pcDNA3, was transfected into Vero E6 cells using FuGene6 (Roche Diagnostics, Penzberg, Germany), and cloned cells were picked after neomycin selection (500 μg/ml). The 91 cloned cells were inoculated with SARS-CoV at 10 m.o.i. At 20 days p.i., the 24-well plates were fixed and stained.

were used as a control. As shown in Fig. 5, 16 of the 91 clones established persistent infection at 20 d.p.i. Especially, the growth rate of persistently infected clone number 65 was higher than those of the other 15 persistently infected clones. Thus, growth rate was different among the persistently infected clones. This may be due to a difference in a number of survived cells among these 16 cell clones. Interestingly, 75 of the 91 clones failed to establish persistent infection. These results suggested that parental Vero E6 cells always produce a minor population of cells with a potential to allow persistence.

Recently, we reported that extracellular signal-related kinase (ERK1/2), c-*Jun* N-terminal protein kinase (JNK), p38 MAPK, and Akt are phosphorylated in SARS-CoV-infected Vero E6 cells and that viral replication was not affected by the activation of signaling pathways [13–15]. To clarify the signaling pathway important for establishing persistent infection, Vero E6 cells were pre-treated with several concentrations of inhibitors of MAPKs and PI3K/Akt, and then infected with 2 m.o.i. of SARS-CoV. As shown in Fig. 6, SB203580 as an inhibitor of p38 MAPK and PD98059 as an inhibitor of MEK1/2 did not affect the establishment of persistent infection, whereas no surviving cells were observed following treatment with SP600125, an inhibitor of JNK, or LY294002, an inhibitor of PI3K/Akt. This result suggested that JNK and PI3K/Akt are necessary to establish persistence. Our previous study and Fig. 7A indicated that virus-induced Akt phosphorylation was not strong, and it was significantly down-regulated at 24 h.p.i.[14] Therefore, we concluded that the weak Akt activation was not sufficient to protect the viral infected Vero E6 cells from apoptosis. To confirm that the phosphorylation of Akt is necessary to establish persistence, SARS-CoV was infected to clone cell lines of Vero E6, which were showed in Fig. 5. At 20 h.p.i, phosphorylated Akt was not detected in viral infected clone cell lines 43, 48, and 80, which could not establish persistence in Fig. 5, while phosphorylated Akt was detected in viral

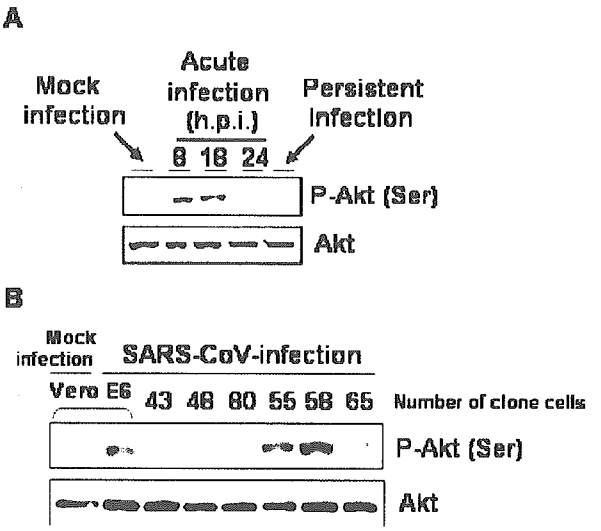

Fig. 7. Phosphorylation status of Akt in viral infected cells. Western blot of acute infection (8, 18, and 24 h.p.i.) and persistent infection (passage 7) were performed using anti-phospho Akt antibody. The rabbit anti-phospho Akt (Ser473) antibody was purchased from Cell Signaling Technology Inc. and used at a dilution of 1:1000. SARS-CoV was infected to cloned Vero E6 cells at 10 m.o.i. The Western blot of the protein samples at 20 h.p.i. was performed using anti-phospho Akt antibody.

infected parental Vero E6, clone cell lines 55, 58, and 65 (Fig. 7B). This result suggested that, at least, the phosphorylation of Akt in viral infected cells were necessary to establish persistence. However, Akt was not phosphorylated in persistent cell lines (Fig. 7A). The phosphorylation of Akt may not be necessary to maintain persistence after establishment.

The present study showed that Vero E6 cells persistently infected with SARS-CoV were established and maintained after multiple passages. A population of cells produced from parental Vero E6 cells may have the potential to support persistent infection. The present study also showed that JNK or PI3K/Akt activation during SARS-CoV infection was

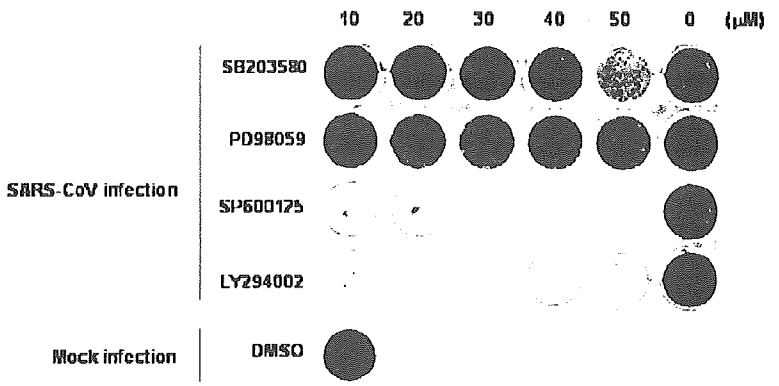

Fig. 6. Effects of signaling pathways on establishing the persistence of Vero E6 cells. SB203580 as a p38 MAPK inhibitor, PD098059 as a MEK inhibitor, and SP600125 as a JNK inhibitor were purchased from Calbiochem (San Diego, CA, USA). LY294002 as a PI3K inhibitor was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). All reagents were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 or 20 mM. All test wells, including mock-treated controls, were treated with DMSO. Vero E6 cells were pre-treated with SB203580, PD98059, SP600125, and LY294002 at concentrations from 10 to 50 μM for 1 h, and then inoculated with SARS-CoV at 2 m.o.i. The plates were fixed and stained at 20 days p.i.

necessary for the establishment of persistence, or for surviving the cells. Both JNK and PI3K/Akt are known to be involved in anti-apoptotic signaling pathways. Especially, our previous study indicated that the activation of Akt signaling pathways is important for anti-apoptosis in SARS-CoV-infected Vero E6 cells [14]. Pro-apoptotic signals, including p38 MAPK, induced cells to undergo apoptosis [13]. In some cell population in Vero E6 cells, anti-apoptotic signals may be stronger than pro-apoptotic signals, resulting in survival.

In the majority of patients infected with hepatitis C virus (HCV), acute infection results in persistent viral replication and the establishment of chronic infection. One viral protein, NS5A, which interacts with receptor binding protein 2 (Grb2), inhibits the activation of ERK1/2 by epidermal growth factor (EGF) [19]. Moreover, NS5A interacts with PI3K (p85), resulting in the promotion of the PI3K/Akt anti-apoptotic signaling pathway [8]. This mechanism may lead to persistent HCV infection in vivo. Tumor necrosis factor-α and interleukin-1β expression, as well as the activities of JNK and activator protein-1 (AP-1), were increased in transgenic mice constitutively expressing HCV core protein [20]. The alternation of cytokine expression and the activation of signaling pathways by core protein may contribute to hepatocarcinogenesis in persistent HCV infection. Thus, viral proteins involve in the activation or inactivation of signaling pathways. Recently, the expression of the nucleocapsid (N) protein of SARS-CoV was shown to induce the up-regulation of AP-1 [7], p38, and JNK, and the down-regulation of ERK and Akt [18]. Although these reports included results obtained in unnatural hosts in vitro, the N protein of SARS-CoV is a candidate for interaction with anti- and pro-apoptotic proteins of host cells. Thus, N protein may also play an important role in the establishment of persistent infection, even though a further experiment is necessary to confirm this hypothesis.

In this paper, we showed a possible mechanism of the establishment of SARS-CoV persistent infection The activation of JNK and PI3K/Akt signaling pathways helps a minor cell population with the potential for persistent infection, to establish persistence. Host gene expression and/or signaling pathways could play important roles in the establishment of persistence. Further investigations are needed to determine the up- or down-regulation of host mRNAs under signaling pathways of JNK and PI3K/Akt in SARS-CoV-infected Vero E6 cells.

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Short communication

Inhibitory effect of mizoribine and ribavirin on the replication of severe acute respiratory syndrome (SARS)-associated coronavirus

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Abstract

The activity of inosine-5′-monophosphate dehydrogenase (IMPDH) inhibitors, mizoribine and ribavirin, against severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) was determined by plaque reduction and yield reduction assays. Mizoribine and ribavirin selectively inhibited replication of SARS-CoV. The 50% inhibitory concentration (IC_{50}) of mizoribine for SARS-CoV Frankfurt-1 and SARS-CoV HKU39849, as determined by plaque reduction was 3.5 μg/ml and 16 μg/ml, respectively, and the IC_{50} of ribavirin for SARS-CoV Frankfurt-1 and SARS-CoV HKU39849 was 20 μg/ml and 80 μg/ml, while the 50% cytotoxic concentration of mizoribine and ribavirin for Vero E6 cells exceeded 200 μg/ml. In a yield reduction assay, mizoribine (10 μg/ml) and ribavirin (40 μg/ml) inhibited the replication of SARS-CoV and reduced the infectious SARS-CoV titers to one-tenth or less. Mizoribine inhibited replication of SARS-CoV more strongly than ribavirin. However, neither drug could completely inhibit replication of SARS-CoV even at concentrations up to 100 μg/ml.

Keywords: SARS; Coronavirus; Ribavirin; Mizoribine

The first outbreak of severe acute respiratory syndrome (SARS) occurred in the Guangdong Province, in Southern China, in November 2002, and then spread through human-to-human infection from there to other areas of China, as well as Vietnam, Singapore, Canada and some 30 countries (Lee et al., 2003; Poutanen et al., 2003; Tsang et al., 2003). Approximately 8000 SARS patients have been reported and about 800 patients died in the outbreak from November 2002 and July 2003. SARS-associated coronavirus (SARS-CoV) has been identified as the causative agent for SARS (Drosten et al., 2003; Ksiazek et al., 2003).

In the present study, inhibitory effect of mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate, M_w 259.22) and ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, M_w 244.2) on the replication of SARS-CoV was evaluated. Mizoribine, an imidazole nucleoside, is an immunosuppressive agent used for renal transplantation, autoimmune diseases and steroid-resistant nephrotic syndrome in Japan. Mizoribine is phosphorylated by adenosine kinase and converted its active form, mizoribine 5′-monophosphate. This activated form of mizoribine acts as an inhibitor of the enzyme, inosine 5′-monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase, both enzymes being essential to the synthesis of guanosine monophosphate from inosine monophosphate through the de novo pathway (Yokota, 2002). Furthermore, mizoribine possesses in vitro anti-viral activities against herpes simplex virus, cytomegalovirus, respiratory syncytial virus, influenza viruses, and bovine viral diarrhea virus (Shiraki et al., 1990; Hosoya et al., 1993; Shigeta, 2000; Pancheva et al., 2002; Stuyver et al., 2002). Ribavirin is a well-known broad-spectrum antiviral agent. Ribavirin 5′-monophosphate, a metabolite of ribavirin, also acts as an inhibitor of IMPDH.

Mizoribine and ribavirin were supplied from Yamasashouyu Co., Ltd., Choshi, Chiba, Japan. The cytotoxic effect, cytotoxic concentration (CC) of mizoribine and ribavirin for Vero E6 cells (American Type Cell Collection, Manas-

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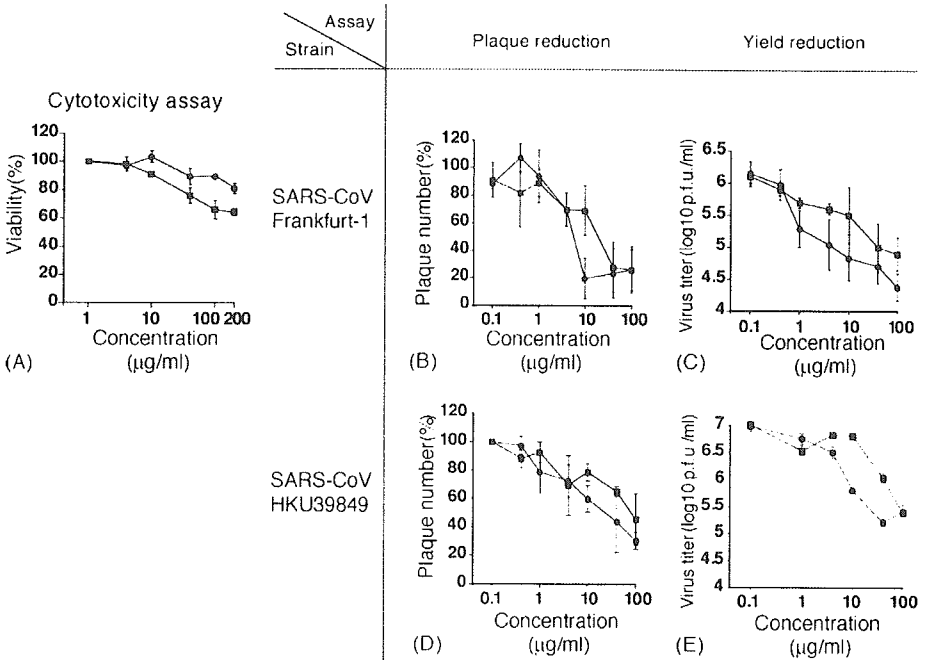

Fig. 1. Cytotoxicity (A), inhibitory effect on plaque formation of SARS-CoV Frankfurt-1 (B) and HKU39849 (D) in a plaque reduction assay and inhibitory effect on replication of SARS-CoV Frankfurt-1 (C) and HKU39849 (E) in yield reduction assay. The symbols, "●" and "■" represent mizoribine and ribavirin, respectively. The vertical bar indicates 1 S.D. One hundred percent of plaque numbers in (B) and (D) correspond to the control (no compound). The SARS-CoV titers at 0 μg/ml of drugs are not shown in these figures, as these titers were not significantly different from those at a concentration of 0.1 μg/ml.

sas, VA.) was measured using a WST-1 cytotoxicity assay kit (Roche Diagnositics, Mannheim, Germany), according to the manufacturer's instructions. The CC_{50} and CC_{20} were defined as the concentration at which the viability of Vero E6 cells decreased to 50% and 80% of that of cells cultured without the addition of antiviral drugs, respectively. In the cytotoxicity assay, Vero E6 cells were cultured in Eagle's minimum essential medium (MEM) containing 2% fetal bovine serum (FBS), penicillin G, and streptomycin (MEM-2FBS), which was the same medium for growth of SARS-CoV. Briefly, approximately 10^4 of Vero E6 cells per well were inoculated in each well in a 96-well microplate and let stand for 4 h to allow the cells to adhere to the bottom of the wells. The culture medium was then replaced by the MEM-2FBS with or without drug. The cells were cultured for 3 days in a CO_2 incubator under humidified condition, and then evaluated for cytotoxicity using the WST-1 kit. The level of Vero E6 cells' viability at different concentration of either drug is shown in Fig. 1A. The CC_{50} of mizoribine and ribavirin exceed at 200 μg/ml, and the CC_{20} of mizoribine and ribavirin was 200 and 40 μg/ml, respectively.

In the present study, two strains of SARS-CoV (Frankfurt-1 and HKU39849) were used. SARS-CoV (Frankfurt-1) and SARS-CoV (HKU39849) were kindly provided by Dr. John Ziebuhr of the Institute of Virology and Immunology, University of Wuerzburg, Wuerzburg, Germany, and Dr. J.S. Malik Peiris, Department of Microbiology, Hong Kong University, HKSAR, respectively. The anti-SARS-CoV activity of these drugs was firstly measured by a plaque reduction assay in Vero E6 cells. Vero E6 cell monolayers seeded in 24-well microplates were inoculated with 0.2 ml of SARS-CoV stock solution at 100 plaque formation unit (p.f.u.)/ml. After a 1-h incubation, the inocula were removed and the cells were washed with phosphate-buffered saline solution (PBS). The cells were then cultured in MEM-2FBS with a designated concentration of each compound and 0.5% methylcellulose for 48 h in a CO_2 incubator under humidified conditions. The culture medium was then removed and the cells in the culture plate were fixed with a 10% formalin solution and stained with crystal violet solution. The plaque number was then counted. The incubation time was set at 48 h, because the plaque size became too large to be counted when the incubation time was set at 3 days or more. The experiment was performed in duplicate. The 50% inhibitory concentration (IC_{50}) was defined as the concentration at which the plaque number decreased to half of that in cells cultured without addition of antiviral drugs. The same experiment was performed three times independently for each drug and the IC_{50} values were calculated as the average ± standard deviation (SD) of the three experiments. The 20% inhibitory concentration (IC_{20}) and 80% inhibitory concentration (IC_{80}) were determined as well. The inhibitory effect of mizoribine and ribavirin on SARS-CoV (Frankfurt-1 and HKU39849)-plaque formation is shown in Fig. 1B and D, respectively. The IC_{50}, IC_{20} and IC_{80} values of mizoribine and ribavirin for SARS-CoV are shown in Table 1, and so are the cytotoxic concentrations. Re-

Table 1
Inhibitory concentrations of mizoribine and ribavirin on SARS-CoV Frankfurt-1 and HKU39849 strains

Drug	SARS-CoV strain	Inhibitory concentration (μg/ml)			Cytotoxic concentration (μg/ml)	
		IC_{20}	IC_{50}	IC_{80}	CC_{20}	CC_{50}
Mizoribine	Frankfurt-1	2.2 ± 2.0	3.5 ± 2.9	>100	200	>200
	HKU39849	1.4 ± 0.6	16 ± 2.8	>100		
Ribavirin	Frankfurt-1	8.9 ± 6.3	20 ± 15	>100	40	>200
	HKU39849	15 ± 1.4	80 ± 28	>100		

duction in the number of plaques was demonstrated in cells cultured in medium to which mizoribine or ribavirn were added. However, IC_{80} values of both drugs could not be determined, because plaque formation by SARS-CoV Frankfurt-1 and HKU39849 was not completely inhibited even at a concentration of 100 μg/ml of each drug (Fig. 1B and D). As shown in Fig. 1B and D, mizoribine inhibited plaque formation by SARS-CoV more strongly than ribavirin in plaque reduction assay. The size of plaques was significantly decreased at a concentration of 10 μg/ml or greater for each drug (data not shown).

The inhibitory effects of mizoribine and ribavirin on SARS-CoV replication were further evaluated by a yield reduction assay. Vero E6 cells seeded in 6-well microplates were inoculated with SARS-CoV solution for 1 h to allow infection with SARS-CoV at a multiplicity of infection (m.o.i.) of 0.01 p.f.u./cell. After 1 h, the inocula were removed and the cells were washed twice with PBS and cultured in MEM-2FBS with or without drug for 20 h. The time between inoculation of SARS-CoV and harvesting samples was 20 h, because the preliminary study revealed that the growth of SARS-CoV was so fast that 2 or more days-incubation made it difficult to assess an inhibitory effect of mizoribine and ribavirin on SARS-CoV replication. Therefore, a 20-h incubation time was set in the yield reduction assays. The medium was then collected and stored in −80 °C until use. At this stage, no obvious specific cytopathic effect was observed (data not shown). In each experiment, two wells were used at each concentration of the antiviral drugs. The infectious dose of SARS-CoV was determined by a plaque assay in Vero E6 cells. The same experiment was conducted three times independently and the infectious dose was calculated as the average ± S.D. of the three independent experiments. Mizoribine inhibited replication of SARS-CoV (Frankfurt-1 and HKU39849), and the infectious viral dose at the concentration of 10 μg/ml decreased to one-tenth or less of the control (Fig. 1C and E). The inhibitory effect of mizoribine on SARS-CoV replication was greater than that of ribavirin (Fig. 1C and E).

In both plaque reduction and yield reduction assays, it was demonstrated that mizoribine inhibited replication of SARS-CoV more strongly than ribavirin. In order to determine whether the difference in the degree of inhibitory effect between mizoribine and ribavirin on SARS-CoV was a phenomenon observed only for SARS-CoV, the inhibitory effect of these drugs on replication of vaccinia virus (Lister strain) and herpes simplex virus type 1 (VR-3 strain), which were stored in the National Institute of Infectious Diseases, Tokyo, Japan, was measured as a control by plaque reduction assay in Vero E6 cells. It has been reported that ribavirin inhibits replication of vaccinia virus (Kirsi et al., 1983). The plaque reduction assay for vaccinia virus and HSV-1 was carried out as described above for SARS-CoV except for the length of the culture period. In the plaque reduction assay for vaccinia virus and HSV-1, the cells were cultured for 4 days. As shown in Fig. 2A, ribavirin inhibited replication of vaccinia virus to the same extent as mizoribine. However, ribavirin did not show anti-HSV-1 activity in Vero E6 cells, while mizoribine showed an inhibitory effect on the replication of HSV-1 (Fig. 2B). These results suggested that the difference in degree of inhibitory effect on viral replication between mizoribine and ribavirin was dependent on the type of virus and that the mode of antiviral action of mizoribine was not the same as that of ribavirin, although both drugs act as IMPDH inhibitors.

During the outbreak of SARS in 2003, several therapeutic strategies for the treatment of SARS were tried, such as administration of ribavirin, lopinavir/ritonavir (a protease inhibitor), interferon, steroids, and so on, alone or in combination with each other (Ho et al., 2003; Loutfy et al., 2003; Zhao et al., 2003; Zhaori, 2003; Chu et al., 2004). However, no efficacious treatments of SARS with antiviral agents have been developed. Several compounds, such as ribavirin, lopinavir/ritonavir, 6-azauridine, pyrazofurin, interferon-α, interferon-β, glycyrrhizin, niclosamide (anthelminthic drug), aurintricarboxylic acid, nelfinavir (HIV protease inhibitor), *S*-nitroso-*N*-acetylpenicillamine (a nitric oxide donor), and chloroquine (antimalaria drug) were demonstrated to show anti-SARS-CoV activity in vitro (Cinatl et al., 2003; Chu et al., 2004; He et al., 2004; Hensley et al., 2004; Keyaerts et al., 2004; Sainz et al., 2004; Stroher et al., 2004; Wu et al., 2004; Yamamoto et al., 2004).

Cinatl et al. (2003) reported that ribavirin did not have anti-SARS-CoV activity in vitro. Also in the present study ribavirin did not completely inhibit replication of SARS-CoV even at a concentration of 100 μg/ml. However, ribavirin did possess an inhibitory effect on replication of SARS-CoV, as demonstrated in our present study as well as that of Chu et al. (2004). Scoring of cytopathogenicity was performed in the former study to determine the antiviral activity of the tested compounds (Cinatl et al., 2003), while plaque reduction assay was performed in the present studies and those of Chu et al.

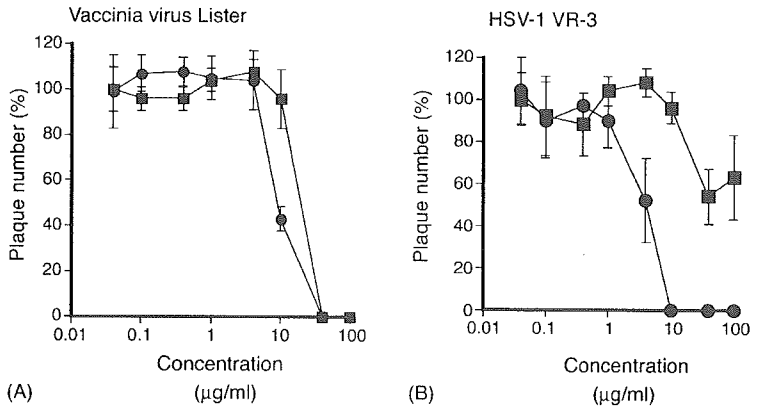

Fig. 2. Inhibitory effect of mizoribine (●) and ribavirin (■) on vaccinia virus (Lister strain) (A) and HSV-1 VR-3 (B) determined by plaque reduction assay. The vertical bar indicates 1 S.D. calculated from the data obtained by independent three experiments. One hundred percent of plaque numbers correspond to the control (no compound).

(2004). The reason why an inhibitory effect of ribavirin was demonstrated in the present study and not in the previous study by Cinatl et al. (2003) may be attributed to difference in the procedures used, and, in particular, to the duration of the incubation times of the cells in the presence of ribavirin.

Ribavirin is phosphorylated to form ribavirin mono-, di- and triphosphate. Ribavirin-monophosphate inhibits the enzymatic activity of IMPDH, resulting in decreased intracellular guanosine triphosphate and deoxyguanosine triphosphate pool levels (Streeter et al., 1973). This alteration in guanosine triphosphate pool levels causes suppression in cellular DNA, mRNA and protein synthesis. Possible mechanisms of antiviral activity of ribavirin were reported for rotavirus, vesicular stomatitis virus, influenza virus and vaccinia virus (Eriksson et al., 1977; Lowe et al., 1977; Muller et al., 1977; Goswami et al., 1979; Smee et al., 1982; Kirsi et al., 1983; Toltzis et al., 1988). As described above, the mode of antiviral action of mizoribine is considered to be similar, but not identical to that of ribavirin, although both drugs act as IMPDH inhibitors. The mechanism of anti-SARS-CoV activity of mizoribine and ribavirin should be addressed in future studies.

In summary, mizoribine and ribavirin possess an inhibitory effect on the replication of SARS-CoV in vitro, but their anti-SARS-CoV activity is virustatic rather than virucidal. The findings obtained in the present study do not legitimate the use of ribavirin or mizoribine for the treatment of patients with SARS, but may contribute to the further development of antiviral agents for SARS-CoV and of therapeutic strategies for SARS.

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Genotypic Characterization of the DNA Polymerase and Sensitivity to Antiviral Compounds of Foscarnet-Resistant Herpes Simplex Virus Type 1 (HSV-1) Derived from a Foscarnet-Sensitive HSV-1 Strain

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Foscarnet is widely used for the treatment of acyclovir-resistant herpesvirus infections, and foscarnet-resistant herpesvirus infections are a serious concern in immunocompromised patients. Twenty-seven single-plaque isolates of herpes simplex virus type 1 (HSV-1) resistant to foscarnet were selected from foscarnet- and acyclovir-sensitive HSV-1 strain TAS by exposure to foscarnet, and the DNA polymerase genes were analyzed. The sensitivities of these mutants to foscarnet, cidofovir, S2242, acyclovir, ganciclovir, and penciclovir were determined. A single amino acid substitution, double amino acid substitutions, and a combination of a single amino acid substitution with a deletion or insertion of amino acid residues in the viral DNA polymerase were demonstrated in 21, 4, and 2 isolates, respectively. Of the 27 isolates, an amino acid substitution of serine for asparagine at amino acid position 724 in the DNA polymerase (724 S-N) was detected in 8 isolates. An amino acid substitution in conserved region II was demonstrated in these eight isolates as well as four other isolates. The mutation in the DNA polymerase responsible for resistance to foscarnet was located between the pre-IV region and conserved region V, especially within conserved region II. All the isolates were sensitive or hypersensitive to cidofovir and ganciclovir. Seven, 5, and 15 of the 27 isolates were also sensitive to S2242, acyclovir, and penciclovir, respectively. Thus, most of the foscarnet-resistant HSV-1 isolates were sensitive or hypersensitive to cidofovir and ganciclovir.

The number of immunodeficient patients, such as those with bone marrow transplants or AIDS, has been increasing. Treatment strategies for herpes simplex virus (HSV) infections in these patients have become a major concern, because immunocompromised patients often experience recurrent or chronic herpesvirus infections. Among the herpesvirus infections, HSV type 1 (HSV-1), HSV-2, and varicella-zoster virus infections are usually treated with acyclovir (ACV). ACV treatment sometimes induces ACV-resistant (ACV^r) infections in these patients. ACV^r herpesvirus infections are treated with foscarnet (PFA), a pyrophosphate analogue (1, 3, 5, 7, 10–12, 14, 16, 17, 20, 22, 28, 29, 32, 35), and ACV^r and/or PFA-resistant (PFA^r) herpesvirus infections are also treated with cidofovir (CDV), an acyclic nucleoside analogue (4, 5, 19, 31). These two drugs are commonly prescribed for the treatment of herpesvirus infections.

There have been many reports on PFA^r HSV-1 and HSV-2 infections (4, 6, 8–10, 18, 19, 23, 28, 30, 33). Therefore, it is important to study the sensitivities of PFA^r HSV-1 strains to other antiviral agents with different mechanisms of action against viral strains and to characterize PFA^r viruses genetically. In the present study, we independently selected 27 PFA^r HSV-1 isolates to clarify their genotypic characterizations and also determined their sensitivities to other antiherpesvirus drugs. The therapeutic strategies used for the treatment of PFA^r HSV-1 infections are also discussed in light of the results of the present study as well as those of previous studies.

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MATERIALS AND METHODS

Virus and cells. An ACV-sensitive and PFA-sensitive HSV-1 strain, strain TAS, isolated from a patient with Wiskott-Aldrich syndrome (25, 26) was used in this study. An African green monkey kidney cell line (Vero cell line; American Type Cell Collection) was used for the selection of PFA^r HSV-1 isolates and the plaque reduction assay. Vero cells were grown in Eagle's minimum essential medium (MEM) containing streptomycin and penicillin G and 5% fetal bovine serum (FBS) (MEM-5FBS). Vero cells were cultured in MEM containing the two antibiotics and 2% FBS (MEM-2FBS) when they were used for virus growth.

Antiviral agents. The antiviral agents used in the present study were selected on the basis of their different mechanisms of action for inhibition of virus replication. The antiviral agents used were PFA, ACV, and ganciclovir (GCV) (Sigma-Aldrich Chemical Company, St. Louis, Mo.); penciclovir (PCV; Smith-Kline Beecham, West Sussex, United Kingdom); CDV (Gilead Sciences, Foster City, Calif.); and 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl] purine (S2242; Hoechst, Frankfurt, Germany). PFA is a direct DNA polymerase inhibitor (2). ACV, GCV, and PCV are viral thymidine kinase (TK)-associated antiherpesvirus drugs (2, 34). S2242 possesses anti-HSV-1 and anti-HSV-2 activities, but it displays no TK-associated activity (2, 21). Except for S2242, all the drugs are available in the clinical setting.

Selection of PFA^r HSV-1 isolates. HSV-1 isolates were selected by exposure of wild-type HSV-1 strain TAS to PFA, as summarized in Fig. 1. First, 30 plaque-purified wild-type HSV-1 TAS isolates were obtained. Plaque purification was carried out by selecting viruses that formed plaques in cell culture overlaid with MEM-2FBS with 1% agarose. The plaque purification was performed twice at this step. Next, each of the plaque-purified HSV-1 isolates was propagated independently in Vero cells in the presence of PFA at a concentration of 4 μg/ml, and the virus produced was then inoculated onto cells cultured in MEM-2FBS with PFA at a concentration of 10 μg/ml. The concentration of PFA was gradually increased from 4 to 10, 40, 100, and finally, 200 μg/ml. Plaque purification was carried out for each of the HSV-1 TAS isolates propagated in the presence of PFA at 200 μg/ml in order to obtain a single isolate from each of the virus solutions. Plaque purification was also performed twice at this step. The plaque-

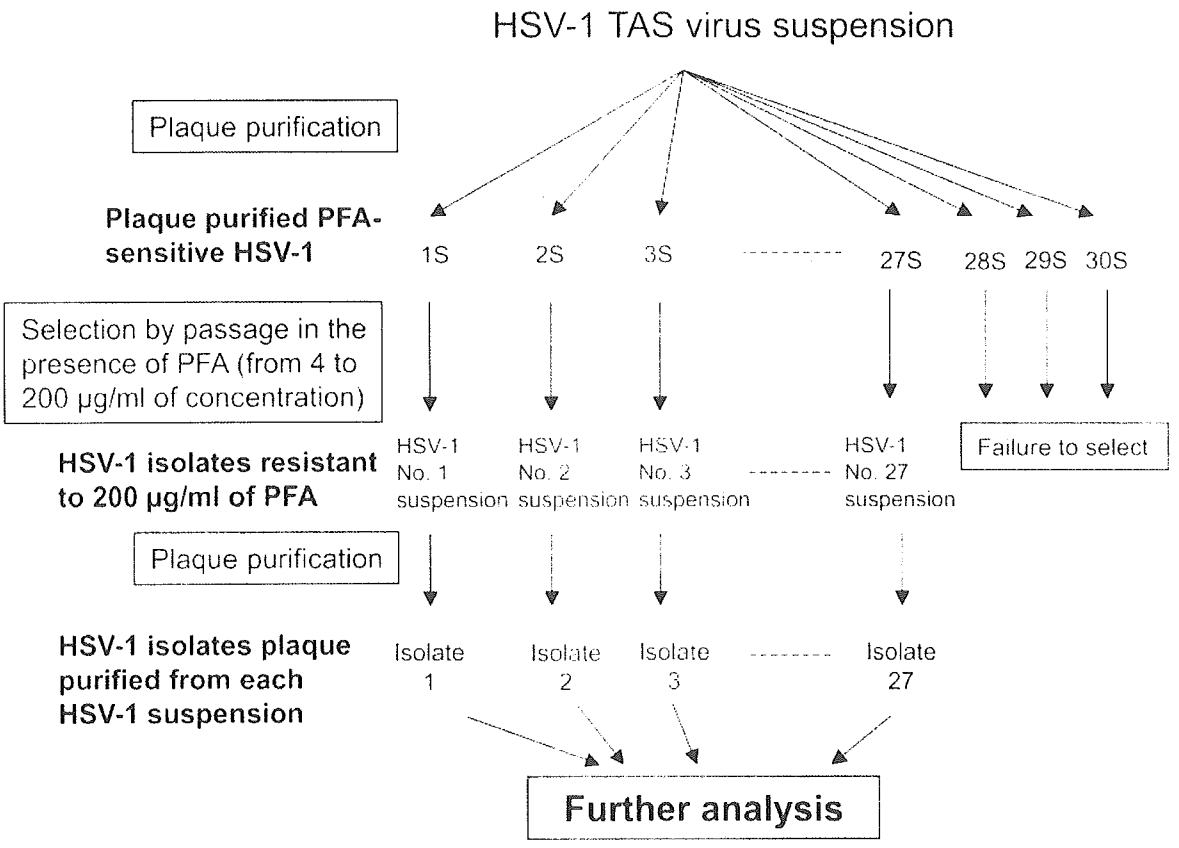

FIG. 1. Schematic representation of the procedures used for the selection of PFA^r HSV-1 isolates.

purified HSV-1 isolates were first grown in Vero cells seeded into a culture flask (25 cm^2), and the cells were then frozen and thawed once to make the working virus solution. Multiple passage of the virus was avoided. The purified HSV-1 isolates were subjected to further analyses of their sensitivities to antiviral agents and genotypic characterization.

Plaque reduction assay. HSV-1 TAS and the mutant HSV-1 isolates, which were selected from the infected cells in MEM-2FBS with PFA, were tested for their sensitivities to PFA, CDV, S2242, ACV, GCV, and PCV by plaque reduction assay, as described previously (25–28). The 50% inhibitory concentration (IC_{50}) of each antiviral agent was calculated as the concentration at which the plaque number decreased to half of that in medium without antiviral agents. Five of the purified HSV-1 TAS isolates (isolates 1S to 5S in Fig. 1 and Table 1) were chosen and tested for their sensitivities to the antiviral agents. The IC_{50}s of the antiviral agents tested for the five isolates were determined, and then the $\log_{10}$ values of the IC_{50}s were calculated. The average values and standard deviations (SDs) of the $\log_{10}$ values of the IC_{50}s for isolates 1S to 5S were then calculated. The virus isolates for which the IC_{50}s were higher than $10^{\text{average} + 3\ \text{SDs}}$ and those for which the IC_{50}s were between $10^{\text{average} + 3\ \text{SDs}}$ and $10^{\text{average} + 2\ \text{SDs}}$ were defined as highly resistant and moderately resistant, respectively. The mutants for which the IC_{50} was $10^{\text{average} + 2\ \text{SDs}}$ were defined as sensitive to the antiviral agents. Furthermore, the virus isolates for which the IC_{50} was between $10^{\text{average} + 2\ \text{SDs}}$ and $10^{\text{average} + 3\ \text{SDs}}$ and those for which the IC_{50} was less than $10^{\text{average} + 3\ \text{SDs}}$ were defined as moderately sensitive and highly sensitive, respectively.

Sequencing. The nucleotide sequences of the DNA polymerase genes of HSV-1 TAS and the mutant isolates were determined by direct sequencing methods, as described previously (28). The nucleotide sequence of HSV-1 TAS was identical to that of HSV-1 strain R98/0, which was also isolated from the same patient.

Nucleotide sequence accession number. The nucleotide sequence of HSV-1 TAS has been deposited in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/Welcome.html) under accession number AB070847 (28).

RESULTS

Selection of PFA^r HSV-1 isolates. Thirty HSV-1 isolates were first plaque purified from HSV-1 strain TAS. A plaque-purified HSV-1 isolate, which was grown in MEM-2FBS with 200 μg of PFA/ml, was successfully obtained from each of the 27 series of the 30 parent isolates. All 27 isolates were confirmed to express an intact TK polypeptide and TK activity by Western blotting and a TK activity assay, respectively, as reported previously (24) (data not shown).

Sensitivities to antiviral agents of HSV-1 isolates grown in the presence of PFA. Five plaque-purified isolates of HSV-1 TAS (isolates 1S to 5S) were tested for their sensitivities to the antiviral agents tested. The averages and SDs of the $\log_{10}$ values of the IC_{50}s of the compounds were calculated for each of the five isolates; and the reference values were calculated for determination of sensitivity or resistance to six antiviral agents: PFA, CDV, S2242, ACV, GCV, and PCV (Fig. 2).

The sensitivity profiles of the isolates selected by exposure to PFA are shown in Table 1 and Fig. 2. All 27 isolates selected after culture in MEM-2FBS with PFA were confirmed to be highly resistant to PFA. Although the virus isolates were selected in MEM-2FBS with PFA at a final concentration of 200 μg/ml, PFA IC_{50}s of less than 200 μg/ml (range, 74 to >200 μg/ml) were demonstrated for 14 isolates. On the other hand, all the PFA^r HSV-1 isolates were sensitive to both CDV and

TABLE 1. Sensitivities of PFA^r HSV-1 isolates, selected with PFA at a concentration of 200 μg/ml, to PFA, CDV, S2242, ACV, GCV, and PCV and nucleotide and amino acid sequence mutations detected in DNA polymerase gene

PFA^r isolate	Mutation(s) in DNA polymerase		IC_{50} (μg/ml [avg ± SD])[a]					
	Nucleotide	Amino acid	PFA	CDV	S2242	ACV	GCV	PCV
1	1481 A-G	494 N-S	66 ± 5	0.23 ± 0.02	2.1 ± 0.1	0.7 ± 0.2	0.14 ± 0.02	0.97 ± 0.09
2	1814 C-T	605 A-V	>200 ± ND[b]	0.70 ± 0.10	6.7 ± 0.2	5.1 ± 1.0	0.24 ± 0.03	3.2 ± 0.1
3	1814 C-T	605 A-V	>200 ± ND	0.70 ± 0.14	11 ± 2	7.9 ± 0.8	0.34 ± 0.07	3.1 ± 0.3
4	1814 C-T, 3410 G-A	605 A-V, silent	>200 ± ND	1.1 ± 0.5	7.6 ± 0.5	3.4 ± 2.1	0.20 ± 0.01	3.6 ± 0.9
5	979 C-T, 1813 G-A	327 A-T, 605 A-V	>200 ± ND	1.3 ± 0.3	9.9 ± 2.3	7.1 ± 1.1	0.31 ± 0.02	5.7 ± 0.3
6[c]	2104 C-A	702 L-I	74 ± 12	0.27 ± 0.02	1.9 ± 0.3	0.69 ± 0.05	0.15 ± 0.01	1.3 ± 0.1
7[d]	2146 T-C	716 F-L	>200 ± ND	0.30 ± 0.03	5.9 ± 0.4	6.1 ± 0.3	0.53 ± 0.07	4.2 ± 1.2
8	2156 C-T	719 A-V	130 ± 10	1.9 ± 0.4	4.8 ± 0.7	3.9 ± 1.7	0.90 ± 0.12	5.2 ± 0.4
9	2156 C-T	719 A-V	130 ± 4	1.7 ± 0.6	6.1 ± 0.2	4.6 ± 0.4	0.75 ± 0.07	5.4 ± 0.2
10	2171 G-A	724 S-N	>200 ± ND	16 ± 6	6.0 ± 0.3	7.0 ± 0.5	1.7 ± 0.6	9.6 ± 3.0
11	2171 G-A, 2747C-T	724 S-N, 916 A-V	170 ± 9	17 ± 2	9.3 ± 1.6	2.9 ± 0.3	0.72 ± 0.11	4.4 ± 0.3
12	2171 G-A	724 S-N	150 ± 5	12 ± 5	6.3 ± 0.4	8.2 ± 0.9	1.1 ± 0.2	6.2 ± 0.5
13	2171 G-A	724 S-N	>200 ± ND	16 ± 0.5	5.9 ± 0.3	9.2 ± 1.8	0.92 ± 0.12	9.0 ± 0.2
14	2171 G-A	724 S-N	150 ± 9	12 ± 3	6.6 ± 0.2	5.9 ± 0.5	0.75 ± 0.05	6.4 ± 0.4
15	2171 G-A	724 S-N	160 ± 7	15 ± 2	5.2 ± 0.4	5.3 ± 0.2	0.49 ± 0.03	2.9 ± 0.8
16	2171 G-A	724 S-N	>200 ± ND	1.4 ± 1.1	4.8 ± 0.5	3.9 ± 0.6	0.39 ± 0.14	3.6 ± 0.5
17	2171 G-A	724 S-N	>200 ± ND	10 ± 6	7.6 ± 0.1	13 ± 4	1.5 ± 0.1	6.4 ± 0.37
18	2332 C-A	778 L-M	101 ± 14	9.8 ± 1.6	3.2 ± 0.8	3.0 ± 0.7	1.1 ± 0.2	5.4 ± 0.80
19	2392 G-A	798 E-K	>200 ± ND	0.30 ± 0.02	4.6 ± 1.2	3.7 ± 0.4	0.17 ± 0.02	2.6 ± 0.05
20	2392 G-A	798 E-K	>200 ± ND	0.28 ± 0.03	4.2 ± 1.0	3.5 ± 1.2	0.20 ± 0.03	2.1 ± 0.2
21	2392 G-A	798 E-K	>200 ± ND	0.22 ± 0.02	6.2 ± 0.4	3.7 ± 0.8	0.17 ± 0.06	2.6 ± 0.3
22	2500 G-T	834 A-S	130 ± 14	0.33 ± 0.10	2.0 ± 0.2	1.3 ± 0.2	0.14 ± 0.04	2.0 ± 0.2
23	2516 C-T	839 T-I	130 ± 5	2.2 ± 0.4	2.3 ± 0.2	1.8 ± 0.1	0.12 ± 0.02	2.0 ± 0.2
24	1697 G-A, 2534C-A	565 A-G, 842 R-S	74 ± 12	0.27 ± 0.02	1.9 ± 0.3	0.69 ± 0.06	0.15 ± 0.01	1.3 ± 0.1
25	2729 C-T	910 A-V	140 ± 12	1.1 ± 0.5	2.5 ± 0.2	1.6 ± 0.2	0.52 ± 0.05	3.2 ± 0.2
26	2872 G-T	958 V-L	>200 ± ND	1.1 ± 0.3	6.7 ± 1.0	6.2 ± 0.1	0.49 ± 0.05	4.3 ± 1.0
27	2876 G-A	959 R-H	82 ± 5	0.52 ± 0.05	4.0 ± 1.0	2.9 ± 0.5	0.21 ± 0.03	2.2 ± 0.2
S1	None	None	16 ± 3	1.3 ± 0.4	2.2 ± 0.7	0.32 ± 0.18	0.60 ± 0.14	1.8 ± 0.4
S2	None	None	38 ± 17	34 ± 11	3.3 ± 1.1	1.5 ± 0.3	1.6 ± 0.3	3.4 ± 0.8
S3	None	None	22 ± 1	5.1 ± 0.6	1.7 ± 0.3	0.38 ± 0.10	0.61 ± 0.02	1.5 ± 0.2
S4	None	None	25 ± 4	12 ± 2.2	2.2 ± 0.3	0.82 ± 0.16	1.1 ± 0.2	1.8 ± 0.2
S5	None	None	21 ± 2	4.2 ± 1.1	1.8 ± 0.2	0.40 ± 0.10	0.68 ± 0.02	1.0 ± 0.1

[a] The averages and SDs of the IC_{50}s of the antiviral compounds for the 27 PFA^r HSV-1 isolates and the 5 wild-type HSV-1 isolates were determined from three independent experiments.

[b] ND, not determined.

[c] Isolate 6 possesses not only the described single amino acid substitution (2104 C-A) but also the insertion of 4 amino acid residues (EFDS) between positions 438 and 439 or between positions 442 and 443.

[d] Isolate 7 possessed not only the described single amino acid substitution (716 F-L) but also the deletion of a single amino acid (I) at position 304.

GCV. Nine and 10 of the 27 PFA^r isolates were highly sensitive to CDV and GCV, respectively (Table 1; Fig. 2). Twenty, 22, and 12 of the 27 PFA^r isolates were resistant to S2242, ACV, and PCV, respectively. The patterns of sensitivity of the PFA^r HSV-1 isolates to S2242 were similar to those to ACV (Table 1; Fig. 2).

Genotypic characterization of DNA polymerase. Amino acid changes in the DNA polymerase were demonstrated in all 27 PFA^r isolates, as summarized in Table 1; and these changes, along with those of the clinical isolates, are shown in Table 2 (10, 11, 15, 28, 30, 33). A single amino acid substitution, double amino acid substitutions, and a combination of a single amino acid substitution with an amino acid insertion or deletion were demonstrated in 22, 3, and 2 of the 27 PFA^r isolates, respectively. The single amino acid (I) deletion detected in the pre-IV region of the DNA polymerase in isolate 7 was at position 304. A unique repeat of nucleotide residues, GAATTCGAC AGC GAATTCGAGATG (identical sequences are underlined), was detected between nucleotide positions 1315 and 1338, counted from the initiation codon of the parent virus. The 12 nucleotide residues inserted into the conserved region IV gene, GAATTCGACAGC, which were detected in isolate 6, were identical to those found between nucleotide positions 1315 and 1326, resulting in the appearance of three repeats of this 12-nucleotide sequence, GAATTCGAC(G)AG(T)C(G). as GAATTCGACAGC GAATTCGACAGC GAATTCGA GATG, plus an additional insertion of four amino acid residues (EFDS). It is possible that this insertion occurred between nucleotide positions 1314 and 1315 or between positions 1326 and 1327. Of the 27 PFA^r isolates, 8 demonstrated an amino acid substitution of S for N at amino acid position 724 (724 S-N) in the DNA polymerase (Tables 1 and 2). An amino acid substitution in conserved region II was demonstrated in these 8 isolates as well as 4 other isolates (a total of 12 isolates [44%]) (Table 2). The mutations detected in the DNA polymerase were located from the pre-IV region to conserved region V (Table 2).

DISCUSSION

There have been many studies of laboratory-derived or clinically isolated PFA^r HSV-1 and HSV-2 isolates; however, there have been no studies on the characterization of PFA^r HSV-1 with a large panel of PFA^r HSV-1 isolates. In the present

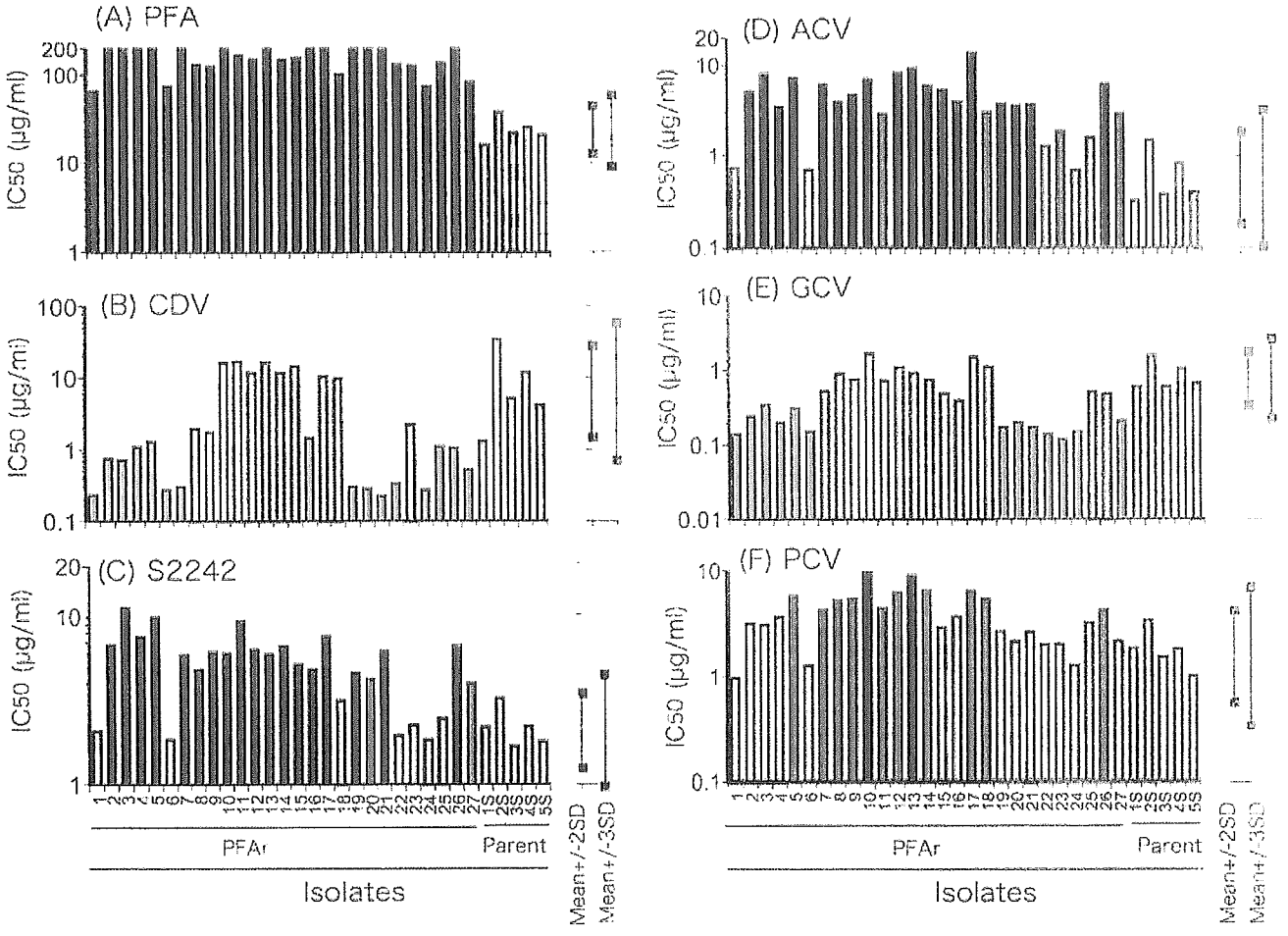

FIG. 2. IC_{50}s of PFA (A), CDV (B), S2242 (C), ACV (D), GCV (E), and PCV (F) for all 27 plaque-purified PFA^r isolates that survived in the medium with PFA and those for the 5 plaque-purified wild-type HSV-1 TAS strains (gray bars, isolates 1S to 5S). The last five plaque-purified wild-type HSV-1 TAS strains are considered wild-type HSV-1. The $10^{average \pm 2\ SD}$ and $10^{average \pm 3\ SD}$ ranges of each compound calculated from the IC_{50}s for plaque-purified isolates (isolates 1S to 5S) are shown. The former are the reference values for the determination of moderately sensitive and moderately resistant, while the latter are those for the determination of highly sensitive and highly resistant. The red, orange, yellow, green, and blue bars indicate highly resistant, moderately resistant, sensitive with normal range, moderately sensitive, and highly sensitive, respectively.

study, 27 PFA^r HSV-1 isolates were independently selected in vitro, and the sensitivities of these viruses to PFA (direct DNA polymerase inhibitor) and viral TK-unassociated antiviral drugs (CDV and S2242) or viral TK-associated antiviral drugs (ACV, GCV, and PCV) were determined. Furthermore, genotypic characterization of the DNA polymerase gene was performed. The findings of the present study are useful for the establishment of treatment strategies for and the diagnosis of PFA^r HSV-1 infections. All of the 27 PFA^r HSV-1 isolates were sensitive, moderately sensitive, or highly sensitive to both CDV and GCV, while 20 (74%), 22 (81%), and 12 (44%) of the isolates showed decreased sensitivities to S2242, ACV, and PCV, respectively. The mutations were detected from the pre-IV region to conserved region V. It is likely that conserved region II, in which the 724 S-N and 719 A-V mutations were demonstrated, is the hotspot for conferring PFA^r resistance. The reported mutations in the HSV-1 DNA polymerase of a total of 13 clinical isolates are summarized in Table 2. Six (46%) of the 13 isolates possessed the mutations responsible for resistance to PFA in conserved region II, and 3 of the 6 isolates possessed a mutation at amino acid position 724. The ratio of the appearance of mutations in conserved region II detected in the present study was similar to the ratio of mutations detected among these clinical isolates.

We recently detected a PFA^r HSV-1 infection in a patient with congenital immunodeficiency (28). The isolate was resistant to both PFA and ACV and possessed mutations in both the DNA polymerase and the TK regions that are responsible for PFA resistance and ACV resistance, respectively. Chibo et al. (10) and Stránská et al. (33) also reported on ACV^r and PFA^r HSV-1 infections in immunocompromised patients and noted that the viruses causing the infections harbored double mutations in the regions encoding DNA polymerase and TK. Infections caused by PFA^r HSV-1 isolates that possess mutations in the DNA polymerase region but not in the TK region are also common (30). The present study indicates that CDV

TABLE 2. Mutations in DNA polymerase of the 27 PFA^r HSV-1 isolates selected in the present study and the HSV-1 clinical isolates resistant to PFA

Region in DNA polymerase[a]		Amino acid substitution in DNA polymerase of PFA^r HSV-1 isolates						
		Single amino acid substitution					Double amino acid substitution	
		Isolates in this study		Clinical isolates			Isolates in this study	
Region	Amino acid positions	No.	Description (no. of isolates)	No.	Description (no. of isolates)	Reference	No.	Description (no. of isolates)
Pre-IV	1–436	0		0			1	327 A-T (isolate 5) (1)
IV	437–479	0		0			0	
IV-D-region C	480–530	1	494 N-S (1)	0			0	
D-region C	531–627	3	605 A-V (3)	0			2	565 A-G (isolate 24) (1), 605 A-V (isolate 5) (1)
D-region C-II	628–693	0		0			0	
II	694–736	11	702 L-I (1),[b] 716 F-L (1),[c] 719 A-V (2), 724 S-N (7)	6	715 V-G (1), 724 S-N (2), 724 A-T (1), 725 S-G (1), 729 S-N (1)	10, 28, 30, 33	1	724 S-N (isolate 11) (1)
II-VI	737–771	0		0			0	
VI	772–791	1	778 L-M (1)	2	783 L-M (1), 785 D-N (1)	30	0	
VI-III	792–804	3	798 E-K (3)	0			0	
III	805–845	2	834 G-T (1), 839 C-T (1)	1	841 G-S (1)	11	1	842 R-S (isolate 24) (1)
III-I	846–880	0		1	850 L-I (1)	30	0	
I	881–896	0		0			0	
I-VII	897–937	1	910 A-V (1)	2	912 D-V (1), 920 P-S (1)	30	1	916 A-V (isolate 11) (1)
VII	938–946	0		1	941 Y-H (1)	15	0	
VII-V	947–952	0		0			0	
V	953–963	2	958 V-L (1), 959 R-H (1)	0			0	
Post-V	964–1235	0		0			0	
Total no. of amino acid substitutions		24		13			6	
Total no. of isolates		24		13			3	

[a] The conserved regions in HSV-1 DNA polymerase are represented by the designations IV, D-region C, II, VI, III, I, VII, and V; and the regions between the conserved regions are represented by the designations pre-IV, IV-D-region C, D-region C-II, II-VI, VI-III, III-I, I-VII, VII-V and post-V. The amino acid positions of these conserved regions for HSV-1 shown here are based on the report by Gilbert et al (13).

[b] The amino acid substitution was that detected in isolate 6 (Table 1).

[c] The amino acid substitution was that detected in isolate 7 (Table 1).

can be used for the treatment of most PFA^r HSV-1 infections and that GCV can be used for the treatment of infections caused by PFA^r HSV-1 without mutations in TK. These results emphasize that the sensitivities of clinical isolates from high-risk patients to the antiviral agents in clinical use should be monitored and that the proper antiviral agents should be selected for the treatment of these patients. If PFA^r HSV-1 isolates are sensitive to ACV, PCV, or GCV, these drugs should also be used.

It was revealed that the pattern of sensitivity of the PFA^r isolates to ACV was similar to that to S2242. These results strongly suggest that the active forms of ACV and S2242 may inhibit DNA polymerase activity via a similar mechanism.

Double amino acid substitutions were detected in three isolates (isolates 5, 11, and 24). A 605 A-V mutation as well as a 327 A-T mutation was detected in isolate 5, and a 724 S-N mutation as well as a 916 A-V mutation was detected in isolate 11. The 605 A-V and 724 S-N mutations were also demonstrated in isolates 2 to 4 and isolates 10 and 12 to 17, respectively, indicating that these mutations alone can confer resistance to PFA and that it is unknown whether each of the mutations 327 A-T, detected in isolate 5, and 916 A-V, detected in isolate 11, alone can confer resistance to PFA. Furthermore, double amino acid substitutions of 565 A-G and 842 R-S were demonstrated in isolate 24. It is not clear whether each of the mutations detected in isolate 24 independently induce resistance to PFA or whether the combination of two mutations is necessary to confer resistance to PFA.

A combination of a single amino acid substitution and an amino acid deletion (in the pre-IV region) and a combination of a single amino acid substitution and an insertion of 4 amino acid residues (in conserved region IV) were demonstrated in one isolate each. The insertion or deletion of amino acid residues demonstrated in isolate 6 and isolate 7 should be addressed to determine whether they are responsible for PFA resistance. The amino acid substitutions detected in both isolates 6 and 7 were demonstrated in conserved region II. Although further analyses are need, it is likely that the single amino acid substitutions demonstrated in conserved region II can confer resistance to PFA.

As shown in Table 2, mutations in the DNA polymerase-encoding regions of the clinical PFA^r isolates responsible for PFA resistance were demonstrated between conserved regions II and VII. However, the mutations responsible for PFA resistance detected in the present study were located between the IV-D-region C and conserved region V (Table 2). Testing of the susceptibilities of clinical isolates to certain antiviral agents, including virus isolation and susceptibility testing, is time-consuming. Therefore, molecular techniques for determination of PFA resistance should be developed to save time while obtaining sufficient information on sensitivity to antiviral agents. For this purpose, the accumulation of data on the

nucleotide sequence of the DNA polymerase of wild-type HSV-1 strains and those of PFA^r HSV-1 strains is necessary.

In summary, 27 isolates of PFA^r HSV-1 were selected in vitro; and sensitivities to six antiviral agents, PFA (direct DNA polymerase inhibitor), CDV and S2242 (drugs independent of viral TK for activation), and ACV, PCV, and GCV (drugs dependent on viral TK for activation), were investigated. Furthermore, genotypic characterization of these PFA^r HSV-1 isolates was performed.

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Recombinant Nucleoprotein-Based Serological Diagnosis of Crimean–Congo Hemorrhagic Fever Virus Infections

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An enzyme-linked immunosorbent assay (ELISA) using recombinant nucleoprotein (rNP) was reported for the detection of immunoglobulin G (IgG) antibodies to Crimean–Congo hemorrhagic fever (CCHF) virus (CCHFV). The immunoglobulin M (IgM)-capture ELISA was developed for the diagnosis of CCHFV infections, using CCHFV rNP as an antigen. These newly developed assays were applied to a study of a CCHF-outbreak and evaluated with sera collected from patients diagnosed as having CCHF by positive reverse transcription-polymerase chain reaction (RT-PCR) and by detection of IgG response. IgM antibodies to CCHFV were detected in 10 of the 13 patients. IgM antibodies to the rNP of CCHFV were detected by the CCHFV rNP-based IgM-capture ELISA in all 6 patients in whom IgG responses were demonstrated, while it was not detected in the 10 patients in whom IgG responses were not demonstrated. Furthermore, the IgM antibodies were detected in 6 of the 61 residents living a CCHF endemic area during the endemic season, while it was not detected in any of the 48 Japanese residents that had never visited the CCHF endemic area. It is concluded that this newly developed CCHFV rNP-based IgM-capture ELISA is a useful method for the diagnosis of CCHFV infections. ***J. Med. Virol. 75: 295–299, 2005.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: Crimean–Congo hemorrhagic fever; CCHF; recombinant nucleoprotein; ELISA; RT-PCR; serological diagnosis

INTRODUCTION

Crimean–Congo hemorrhagic fever (CCHF) virus (CCHFV) is a member of the family Bunyaviridae, genus *Nairovirus* [Nichol, 2001]. CCHF is an acute viral hemorrhagic fever with a high mortality rate of up to 30% [Nichol, 2001]. Humans are infected with the virus by a tick (genus *Hyalomma*) bite or by close contact with freshly slaughtered meat or blood from viremic animals including sheep, cattle, and goats [Nichol, 2001]. CCHFV infections in humans have been reported in Africa, Eastern Europe, the Middle East, and Central and Southern Asia [Hoogstraal, 1979]. The actual number of patients with CCHF is believed to be far greater than that reported, because the disease usually occurs in remote areas. Noscomial outbreaks of CCHF are not rare [Burney et al., 1980; Suleiman et al., 1980; van Eeden et al., 1985; Fisher-Hoch et al., 1995; Papa et al., 2002].

Although there have been no reports of the efficacy of ribavirin therapy for CCHF as evaluated by a control-based study, ribavirin is known to inhibit the replication of CCHFV in vitro and in vivo [Huggins, 1989; Watts et al., 1989; Tignor and Hanham, 1993]. Furthermore, there have been several reports of patients with CCHF treated successfully with ribavirin [van de Wal et al., 1985; Fisher-Hoch et al., 1992; Papa et al., 2002; Tang et al., 2003]. Therefore, the rapid diagnosis of CCHF is important.

The study was performed under the approval of the ethical committee on medical research and human rights, National Institute of Infectious Diseases, Tokyo, Japan.

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The CCHFV recombinant nucleoprotein (rNP)-based enzyme-linked immunosorbent assay (ELISA) and immunofluorescent assay are effective for the detection of immunoglobulin G (IgG) antibodies to CCHFV [Saijo et al., 2002a; Saijo et al., 2002b]. Paired serum samples are required for diagnosis by IgG ELISA, because a significant rise in antibody titers must be demonstrated. Detection of immunoglobulin M (IgM) antibodies is another reliable procedure for the rapid diagnosis of CCHF. A patient who was diagnosed as having CCHF by detection of IgM and IgG antibodies using CCHFV rNP-based ELISAs was reported previously [Tang et al., 2003].

In the present study, a CCHFV rNP-based IgM-capture ELISA was evaluated for its efficacy in diagnosis of CCHF using sera collected from patients with acute CCHF confirmed by viral genome amplification with RT-PCR and/or by the detection of IgG responses with rNP-based IgG ELISA. The relationship of IgM and IgG responses determined by the CCHFV rNP-based ELISAs with viremia determined by RT-PCR was examined.

MATERIALS AND METHODS

Serum Samples

The western part of the Xinjiang Uygur Autonomous Region, China, is known to be the site of CCHF outbreaks [Yen et al., 1985; Saijo et al., 2002a; Saijo et al., 2002b; Qing et al., 2003; Tang et al., 2003]. Serum samples drawn from nine patients ("Panel A"), from whom the CCHFV genome was amplified by RT-PCR, in the region during the outbreak seasons in 2001 and 2002 were used. Subsequent serum samples were also collected from five of the nine patients approximately 1 week after the first samples were collected. The time of collection after onset was defined by taking the day on which fever first appeared as day 1. Although the exact days of blood sampling from the other four patients, from whom only the 1st blood samples were drawn, were unclear, these samples were collected within 10 days of onset; i.e., during the acute phase of CCHF. The samples from the two sampling times were designated as 1st and 2nd samples, respectively.

Apart from the nine patients, paired serum samples were collected from 14 individuals ("Panel B") in the same regions in the outbreak season. They were suspected as having CCHF or other viral infections based on clinical manifestations such as fever and joint pain.

There was a relatively large outbreak of CCHF in a small village in the area (data not shown). Serum samples were collected from 61 residents, "Panel C", living in the CCHF endemic area in June 2001, at which time the CCHF outbreak had nearly ended. The age of these residents ranged from 6 to 66 and the male to female ratio was 38:23.

Serum samples collected from 48 Japanese subjects, "Panel D", who had never visited the CCHF endemic area, were used as a control.

The sera used in the present study were collected under informed consent. In the case of unconscious patients and children less than 20 years of age, informed consent was obtained from their family members and parents, respectively.

Positive- and negative-control sera for IgG and IgM-capture ELISA were produced in a monkey (*Macaca fascicularis*) by immunization with the purified His-CCHFV rNP using Inject Alum™ (Pierce Biotechnology, Inc., Rockford, IL) [Saijo et al., 2002b] and were tested by each IgG ELISA and IgM-capture ELISA for verification.

CCHFV rNP-Based IgG ELISA and IgM-Capture ELISA

The CCHFV rNP was expressed as a fusion protein with a 6× His-tag on the N-terminus in the recombinant baculovirus system and was designated CCHFV rNP [Saijo et al., 2002b]. The IgG antibodies to CCHFV were detected by IgG ELISA using CCHFV rNP in the same way as described previously [Saijo et al., 2002b]. The CCHFV rNP-base IgM-capture ELISA was performed as previously reported [Tang et al., 2003].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The details of RT-PCR were also described in a previous paper [Tang et al., 2003].

RESULTS

Cut-Off Values of OD_{405} in IgG and IgM-Capture ELISA

Forty-eight serum samples collected from the "Panel D" subjects were tested as a negative control for the IgM-capture ELISA. The OD_{405}s at a dilution of 1:100 were between −0.17 and 0.15, and the average and standard deviation (SD) at that dilution were −0.008 and 0.071, respectively. The cut-off value, calculated as the average +3SD, was 0.205. Positive and negative results by the IgM-capture ELISA were judged on the basis of this cut-off value at a dilution of 1:100. This cut-off value was applied to the other dilution levels, 1:50, 1:200, and 1:400.

The average and SD values of the adjusted optical density (OD_{405}) at a dilution of 1:400 in the IgG ELISA were 0.069 and 0.039, respectively. Therefore, the cut-off value [average + 3× SD of "Panel D" sera] in the IgG ELISA format measured at a dilution of 1:400 was set at 0.186 in the study. This cut-off value was also applied to the other dilution levels, 1:100, 1:1,600, and 1:6,400.

IgM Responses Determined by IgM-Capture ELISA

The IgM antibody status to CCHFV rNP together with the results of RT-PCR and IgG ELISA are shown in Tables I and II. Positive IgM responses were demonstrated in six of the "Panel A" patients: two in the first