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A neutralization assay with a severe fever with thrombocytopenia syndrome virus strain that makes plaques in inoculated cells

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Severe fever with thrombocytopenia syndrome (SFTS) is a recently-discovered, potentially fatal infectious disease caused by SFTS virus (SFTSV). Due to the inability of SFTSV to make clear cytopathic effects (CPE) in cell culture, titration and neutralization assays of the virus require immunostaining of inoculated cells; consequently, the assays are time-consuming and expensive. In this report, we demonstrate the use of a highly-passaged SFTSV strain, p50-2, in a neutralization assay, which made clear plaques in inoculated Vero cells under neutral red staining. Furthermore, we performed molecular analyses to determine the characteristics of the strain. The results suggested that a single amino acid mutation within the viral glycoprotein conferred the ability to make clear plaques to SFTSV.

1. Introduction

SFTSV was first discovered in China in 2009 (Yu et al., 2011). It is classified as a tick-borne Phlebovirus and is a member of the family *Bunyaviridae* (Yu et al., 2011). SFTSV infection causes SFTS in humans and is associated with a high mortality rate of 2–15% (Yu et al., 2011; Kang et al., 2012). SFTS is characterized by clinical symptoms that include high fever, gastrointestinal symptoms, myalgia, and regional lymphadenopathy (Zhang et al., 2013). The characteristic blood test findings include thrombocytopenia, leukopenia, and abnormal levels of liver markers (Zhang et al., 2013). In 2013, the discovery of SFTS and SFTSV was reported in Japan and Korea (Takahashi et al., 2014; Kim et al., 2013). While SFTS has not been reported outside these three countries, an infectious disease caused by the Heartland virus, which is phylogenetically and serologically related to SFTSV (Matsuno et al., 2015), has been reported in the United States and the clinical characteristics and laboratory findings of Heartland virus infection are similar to those of SFTSV infection (McMullan et al., 2012; Pastula et al., 2014; Muehlenbachs et al., 2014). At the present time, there are no approved vaccines or therapies for either SFTS or Heartland virus disease.

The term "cytopathic effects" (CPE) describes the morphological changes in cells that are caused by many viral infections. They are applied to some fundamental virological assays, including virus isolation, the titration of isolated viruses, and neutralization assays. A neutralization assay involves the titration of serum neutralizing activities against viruses; this is important for providing evidence of past viral infections and for evaluating the effects of vaccine candidates. SFTSV, however, does not show clear CPE (Yu et al., 2011). Thus, the neutralization assay for SFTSV requires specific reagents/procedures, such as antibodies and cell staining with antibodies to detect inoculated cells. This makes the performance of the assay time-consuming and expensive (Hayasaka et al., 2016; Le et al., 2016). We found that a highly-passaged strain of SFTSV shows clear CPE in inoculated Vero cells. Thus, in the present study, we performed an investigation to determine whether the clear CPE shown by the passaged strain was useful in the neutralization assay for SFTSV. Additionally, the mutation(s) of the genome of the strain that was responsible for the characteristic of showing clear CPE was identified.

2. Materials and methods

2.1. Cells

Vero cells (ATCC CCL81) were cultured in Dulbecco's Modified Eagle's medium (DMEM; Sigma) supplemented with 5% or 10% heat-inactivated fetal calf serum (FCS) and antibiotics (peni-

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cillin/streptomycin or kanamycin) at 37 °C. The virus-inoculated cells were maintained in DMEM containing 2% FCS and antibiotics.

2.2. Viruses

SFTSV SPL030, which was isolated from a Japanese SFTS patient (Takahashi et al., 2014), was used as the original strain in the present study. While the viral genome sequence in the patient serum (SPL030A) was deposited in GenBank with accession numbers AB817984, AB817992, and AB818000, the isolated original strain shows 2 mutations: one in the NSs-coding region; the other in the L-coding region. Both mutations led to amino acid substitutions from lysine to glycine at position 131 of the NSs and from alanine to threonine at position 531 of the L. The serial passage of SFTSV was performed at 37 °C with 3- or 4-day intervals at 1000 times dilution for the first 20 passages and 10,000–100,000 times dilution for the following 30 passages. All of the experiments involving the handling of infectious SFTSV were performed in a biosafety level 3facility at the National Institute of Infectious Diseases.

2.3. Ethics statement

All of the protocols and procedures using human sera and monkeys were approved by the research and animal committees of the National Institute of Infectious Diseases for the use of human subjects (no. 531) and for the animal experiments (no. 514008).

2.4. Virus titration

The titration of SFTSV was performed as described previously (Shimojima et al., 2014). Briefly, Vero cells were inoculated with serially diluted SFTSV and incubated for 3 days. The inoculated cells were fixed with 10% formalin and exposed to ultraviolet light to inactivate SFTSV. The cells were treated with 0.1% Triton X-100 followed by staining with rabbit anti-N antibodies (Fukuma et al., 2016) and fluorescence-labeled secondary antibodies. The SFTSV titer was determined as the 50% tissue culture infectious dose ($TCID_{50}$).

2.5. Viral growth

Confluent monolayers of Vero cells in 12-multiwell plates were infected with viruses at a multiplicity of infection (MOI) of 0.025 for 1 h. The cells were washed three times and cultured with DMEM containing 2% FCS. The supernatants were harvested soon after the start of cell culture (day 0) and at 1, 2, and 3 days post-inoculation (dpi), centrifuged at 5000 × *g* for 2 min to remove cell debris, and stored until use at −80 °C. To observe CPE, the inoculated cells were cultured for up to 7 days.

2.6. Plaque assay

Confluent monolayers of Vero cells in 6-multiwell plates were inoculated with serially diluted SFTSV for 1 h at 37 °C. After washing, the cells were cultured with DMEM containing 2% FCS and 1% agarose. Three days later, neutral red was added to the cell cultures at a final concentration of 0.0025%. The next day, the plaques were observed visually and counted or picked up for purification.

2.7. Fifty percent-focus reduction neutralization titer (FRNT50)

The $FRNT_{50}$ values of human sera were determined as follows: approximately 100 focus-forming units of the original strain of SFTSV SPL030 were mixed with serially-diluted sera and incubated for 1 h at 37 °C, then inoculated onto confluent monolayers of Vero cells in 12-multiwell plates for an additional 1 h at 37 °C. The inoculums were removed and the cells were washed once with DMEM containing 2% FCS and cultured at 37 °C in DMEM containing 2% FCS and 1% methylcellulose. Seven days later, the inoculated cells were fixed with 10% formalin and exposed to ultraviolet light to inactivate SFTSV. The cells were treated with 0.1% Triton X-100 followed by staining with rabbit anti-N antibodies (Fukuma et al., 2016) and horse radish peroxidase-labeled secondary antibodies. The foci of inoculated cells were visualized using a Peroxidase Stain DAB Kit (Brown Stain, Nacalai Tesque), then counted. The $FRNT_{50}$ values were determined as reciprocal of the highest dilution at which the number of the foci was <50% of the number obtained without serum.

2.8. Fifty percent-plaque reduction neutralization titer (PRNT50)

The $PRNT_{50}$ values of human sera were determined as follows: approximately 100 plaque-forming units of p50-2 strain of SFTSV SPL030 (see the text) were mixed with serially-diluted sera and incubated for 1 h at 37 °C, then inoculated onto confluent monolayers of Vero cells in 6-multiwell plates for an additional 1 h at 37 °C. The inoculums were removed and the cells were washed once with DMEM containing 2% FCS and cultured at 37 °C in DMEM containing 2% FCS and 1% agarose. Plaques were detected as described above. The $PRNT_{50}$ values were determined as reciprocal of the highest dilution at which the number of the plaques was <50% of the number obtained without serum.

2.9. RNA extraction and sequence analysis

Viral RNA was purified from infected cell culture supernatants using a High Pure Viral RNA Kit (Roche) according to the manufacturer's protocol. A reverse transcription reaction using SuperScript III Reverse Transcriptase (Invitrogen) was performed to synthesize cDNA. The viral genome was amplified by a conventional PCR using previously described primers (Yoshikawa et al., 2015). The sequences were determined using the Sanger sequencing method.

2.10. Cell fusion assay

Four hundred thousand Vero cells were seeded in 12-multiwell plates, and transfected with 1.5 μg of SFTSV Gn/Gc-expressing plasmids and 0.5 μg of Venus-expressing plasmids (pCAGGS) using TransIT-LT1 transfection reagent (Mirus). After 2 days of culture, the cells were washed with phosphate-buffered saline (PBS), and treated with the indicated pH conditions (pH4.6 to pH7.3) at room temperature for 2 min. The cells were further incubated with 10% FCS-supplemented DMEM for 2 h at 37 °C and observed under a fluorescence microscope.

2.11. Flow cytometry

The Vero cells that were transfected with plasmids to express SFTSV Gn/Gc were detached with 0.5 mM EDTA solution and suspended in PBS containing 2% FCS and 0.1% sodium azide. The cells were stained with the pre- and post-inoculation sera of an SFTSV-inoculated monkey (unpublished) followed by FITC-conjugated anti-human IgG. The stained cells were analyzed using a FACS Calibur instrument (BD Biosciences).

2.12. Generation of the reassortant virus

Vero cells were infected with a mixture of the original and the passaged strains at a ratio of 100:1 (MOI: ∼2.5). Three days later, the supernatant was harvested and a plaque assay was performed. Ten large plaques were picked up, purified independently once more in