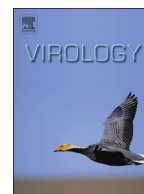




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Therapeutic effect of post-exposure treatment with antiserum on severe fever with thrombocytopenia syndrome (SFTS) in a mouse model of SFTS virus infection

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ABSTRACT

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging viral disease that is endemic in China, Korea and Japan. No effective vaccine or specific treatment for SFTS is currently available. Here, we used a mouse model to examine the effects of ribavirin, site-1 protease inhibitor PF-429242, steroids, and combination of minocycline and ciprofloxacin (MC) on SFTS infection. The antiserum from a patient who recovered from SFTS was also examined for its effect on mice. Administration of antiserum completely protected mice against lethal infection with SFTSV. It could also protect mice from showing clinical signs of the disease due to non-lethal infection. MC-treatment resulted in prolonged survival times during lethal infection. Although other agents had no significant protective effects, they did not provide detrimental effects that could lead to progression of the disease in mice. Our results suggest that antiserum treatment may be clinically useful for post-exposure prophylaxis against SFTSV infection.

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Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging disease that was first reported in rural areas of central China in 2009 (Yu et al., 2011). SFTS is currently identified in Korea and Japan (Kim et al., 2013; Takahashi et al., 2014). To date, more than 2500 cases have been confirmed in China (2047 cases during 2011–2012) with mortality ranging from 6.3% to 30% (Liu et al., 2014a, 2014b). In Japan specifically the western part, a total of 113 cases (37 deaths) had been identified until the year 2014 (Yamaguchi Prefectural Surveillance Center of Infectious Disease, 2015). The earliest case among all the reported SFTS cases worldwide was from a patient in Nagasaki who was infected in 2005 (Takahashi et al., 2014).

The causative agent, SFTS virus (SFTSV), belongs to the genus *Phlebovirus* in the family *Bunyaviridae* (Yu et al., 2011). The SFTSV is transmitted to humans through the bite of an infected tick (Zhang et al., 2012). Human-to-human-transmission occurs through contact to the blood of an infected person (Chen et al., 2013; Liu et al., 2012; Tang et al., 2013; Wang et al., 2014) and this may be through mucous membranes or skin wounds of the healthy person.

Clinical manifestations of SFTS are fever, headache, fatigue, myalgia, gastrointestinal symptom such as vomiting and diarrhea, thrombocytopenia and leukopenia, with fatality rates ranging from a few percent up to 30% (Liu et al., 2014a, 2014b; Takahashi et al., 2014; Yu et al., 2011). Although details of the mechanism of severe disease are not fully understood, it was reported that viral copy numbers in the blood or serum in the fatal cases were significantly higher than in those with survival outcomes (Gai et al., 2012; Yoshikawa et al., 2014). Thus, suppression of viral replication in patients is an important option for therapeutic treatment of the fatal form of the disease. In China, ribavirin has been prescribed to SFTS patients as an attempt to treat them (Liu et al., 2013; Oh et al., 2014). In Japan, clinicians have tried to treat severe SFTS patients with steroid such as methylprednisolone for treatment of hemophagocytic syndrome

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(IASR, 2013). However, the effects in vivo are unclear, and no effective vaccine or specific treatment for SFTS is currently available.

Injection of antiserum or blood transfusion to patients has been attempted to treat some viral diseases namely, Ebola Hemorrhagic Fever, SARS coronavirus, rabies and tick-borne encephalitis (Broker and Kollaritsch, 2008; Khawplod et al., 1996; Mair-Jenkins et al., 2015; Mupapa et al., 1999). Antiviral agents such as nucleotide analogs or protease inhibitors may also be candidates for therapeutic treatment of SFTS. Ribavirin, a guanosine analog, has been shown to be effective for treatment of some viral diseases (Keshtkar-Jahromi et al., 2011; Lange et al., 2014; McCormick et al., 1986; Sidwell and Barnard, 2006). PF-429242, the cellular protease subtilisin kexin isozyme-1 (SKI-1)/site-1 protease (S1P) inhibitor, has been shown to have antiviral activity against some RNA viruses (Olmstead et al., 2012; Pasquato et al., 2012; Urata et al., 2011). In vitro experiments suggest that PF-429242 has a suppressive effect on SFTSV propagation in cultured cells (unpublished data) and this raised the possibility of the antiviral effects of PF-429242.

In Japan, endemic areas of SFTS overlap with the region of the Japanese spotted fever, the causative agent of which is *Rickettsia japonica*. This agent is also transmitted by ticks (Mahara, 1997). In these areas, febrile patients with tick bites are suspected to have spotted fever, and they are generally treated with antibiotics such as minocycline (MINO) and ciprofloxacin (CPFX) in the early phases of the disease. However, it is not known whether these antibiotics treat or exacerbate SFTS infection.

To elucidate the pathogenesis of SFTSV infection in vivo, laboratory mouse model such as C57/BL6, BALB/c and Kunming strains have been used (Chen et al., 2012; Jin et al., 2012). However, adult mice of these strains do not exhibit apparent clinical signs and no mice have fatal outcome, indicating that these immunocompetent adult mice are not susceptible to SFTSV. On the other hand, alpha/beta interferon receptor knockout (IFNAR^{-/-}) mice are highly susceptible to SFTSV infection and adult mice have been reported to die following subcutaneous inoculation of 10⁶ focus-forming units (ffu) of SFTSV (Liu et al., 2014c). Thus, IFNAR^{-/-} mice are regarded as useful animals for SFTSV infection model in vivo.

In this study, we investigated the effects of ribavirin, PF-429242, steroids and human antiserum on SFTSV infection using an IFNAR^{-/-} mouse model. Furthermore, we also examined the effects of MINO and CPFX on the same mouse model following SFTSV infection.

Results

Lethal and non-lethal models of SFTSV infection in IFNAR KO mice

To examine the pathogenicity of the YG-1 strain of SFTSV and to observe clinical signs in mice, 129 strain background IFNAR KO (A129) mice were subcutaneously inoculated with high (10⁶ ffu) and low (10² ffu) doses of SFTSV. High dose-infected mice developed acute clinical signs including piloerection, slowness in movement, anorexia and severe weight loss after 2 days post-infection (pi). All of them died by 7 days pi (Fig. 1A and B). On the other

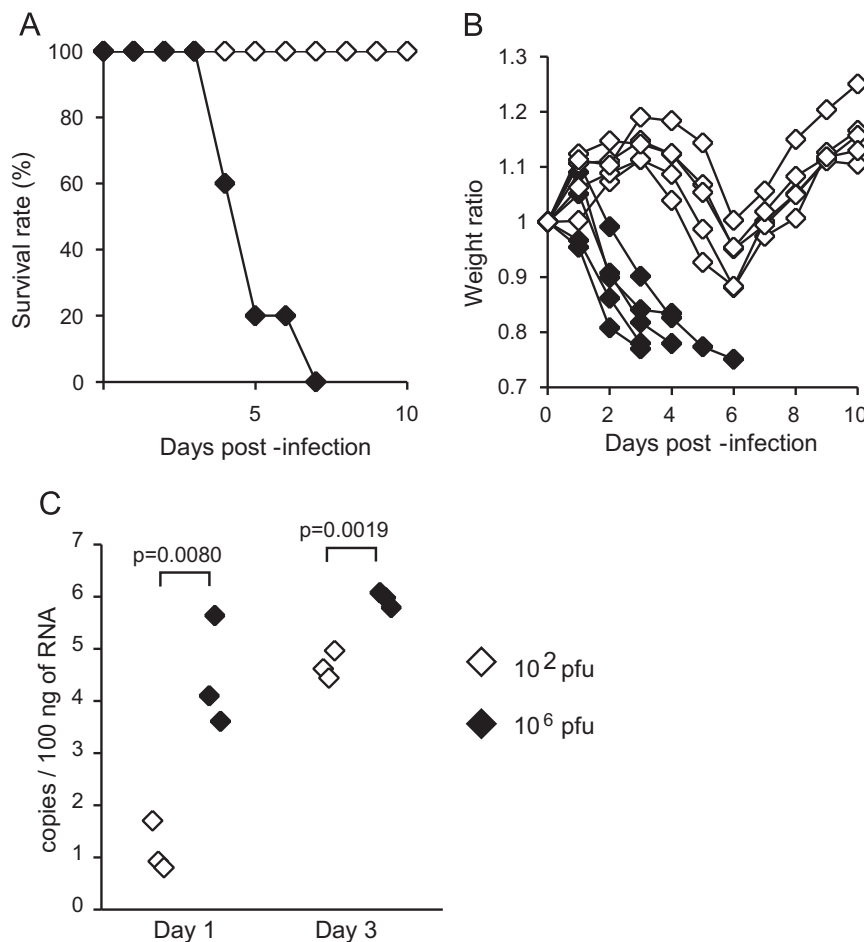


Fig. 1. Mortality, weight change and viral loads of A129 mice following subcutaneous infection with high (10⁶ ffu) and low (10² ffu) doses of SFTSV. (A) Survival curves. (B) Weight changes. The weight ratio was calculated by comparing the weight of each mouse with its weight at day 0. Mice were observed for 14 days (n=5). None of the mice died after 7 days, and the data were shown for 10 days. (C) Viral copy numbers in the spleen at 1 and 3 days (n=3). p: Student's test.

hand, low dose-infected mice exhibited weight reduction during the 3–6 days pi and recovered after 7 days pi (Fig. 1A and B). Viral loads in the spleens of high dose-infected mice were significantly higher than those of the low dose-infected mice at 1 and 3 days pi (Fig. 1C). These observations indicated that high and low dose infections in A129 mice represent lethal and non-lethal models of SFTSV infection, respectively.

Effect of treatment with ribavirin, PF-429242, steroids and antiserum on SFTSV infection in vivo

We next examined the therapeutic effects of ribavirin, PF-429242, steroids and antiserum in A129 mice infected with high

and low doses of SFTSV (Figs. 2 and 3). Antiserum was collected from a recovered SFTS human case, and this serum had a 1:2000 neutralizing antibody titer measured by 50% focus reduction neutralization.

Following lethal infection with SFTSV, all groups of mice had acute weight reduction similar to saline (mock)-treated mice (Fig. 2B). All of the PF-429242- and steroid-treated mice died, but one of five mice survived with ribavirin treatment (Fig. 2B and C). All mice survived after treatment with antiserum, although their weight reductions were similar to those of mock-treated mice during the 2–5 days pi (Fig. 2B and C).

Following non-lethal infection with SFTSV, mock-, ribavirin-, PF-429242- and steroid-treated mice showed weight reduction

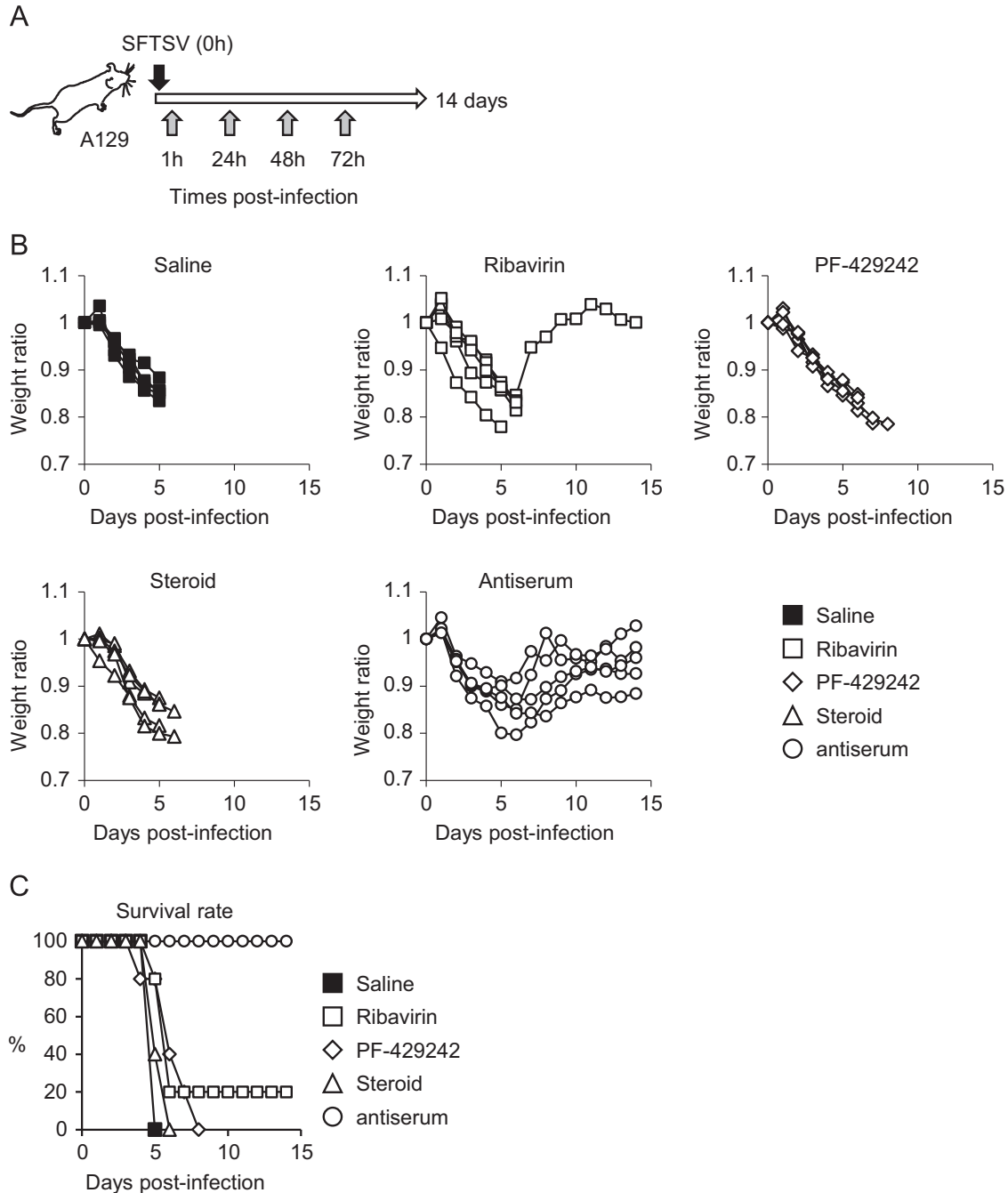


Fig. 2. Mortality and weight changes of drugs- and antiserum-treated A129 mice infected with 10^6 ffu of SFTSV. (A) Schedule of the administration of saline, ribavirin, PF-429242, steroid and antiserum following SFTSV infection. Black arrow indicates the time of SFTSV infection. Gray arrows indicate the time of drugs and antiserum administration. *h*=hour. (B) Weight ratio of individual mice in each agent-treated group. The weight ratio is indicated compared with weights at day 0. (C) Survival curves in saline-, ribavirin-, PF-429242-, steroid- and antiserum-treated groups ($n=5$).

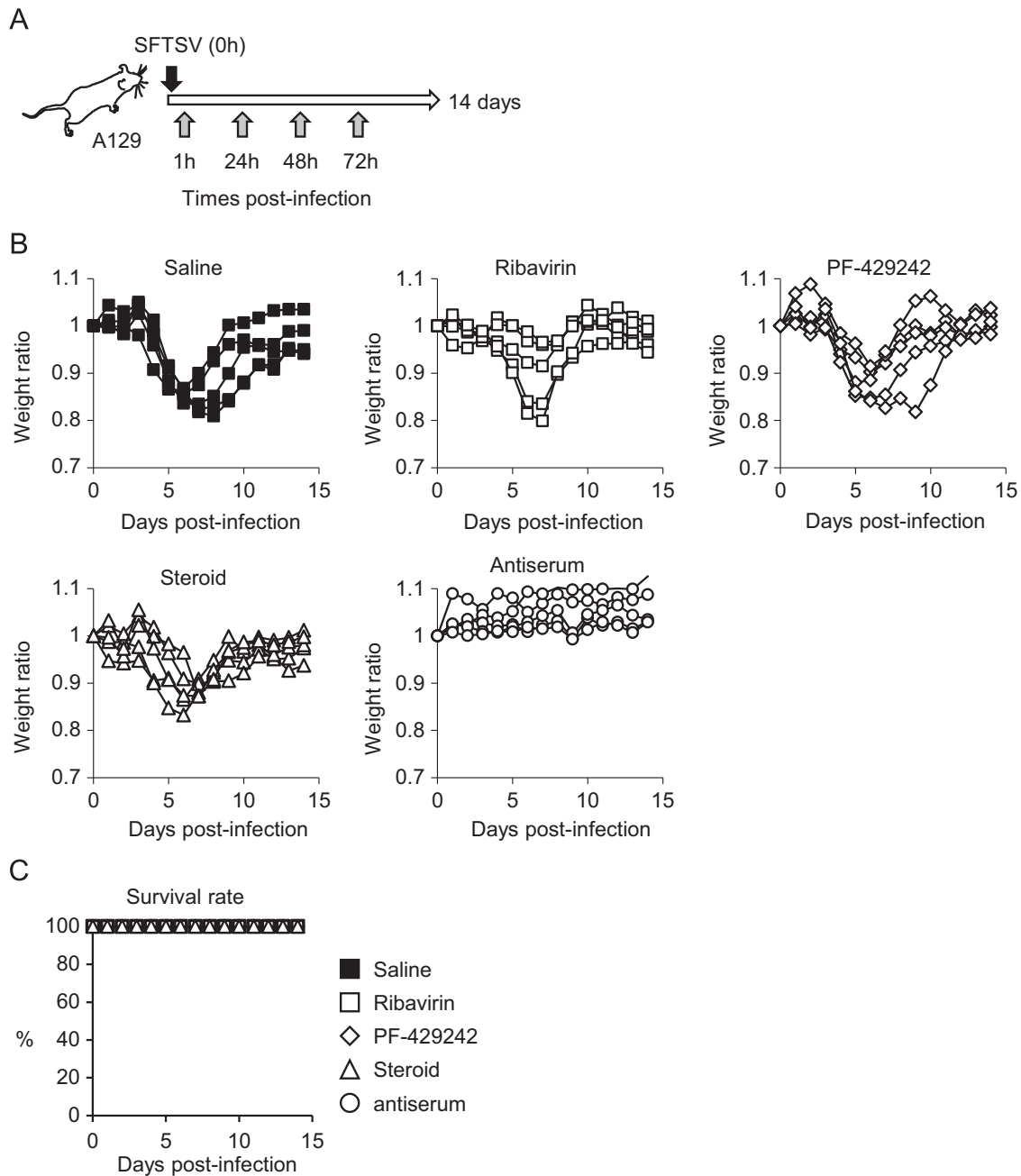


Fig. 3. Mortality and weight changes of drug- and antiserum-treated A129 mice infected with 10^2 ffu of SFTSV. (A) Schedule of the administration of saline, ribavirin, PF-429242, steroid and antiserum following SFTSV infection. Black arrow indicates the time of SFTSV infection. Gray arrows indicate the time of drugs and antiserum administration. h=hour. (B) The weight ratio of individual mice in each agent-treated group. The weight ratio is indicated compared with weights at day 0. (C) Survival curves in saline-, ribavirin-, PF-429242-, steroid- and antiserum-treated groups ($n=5$).

(Fig. 3B). Although all of the mock-, PF-429242- and steroid-treated mice had more than 10% weight reduction, three out of the five ribavirin-treated mice showed only slight (less than 10%) decreases in weight (Fig. 3B). Antiserum-treated mice, however, did not exhibit significant weight reduction or any clinical signs (Fig. 3B).

These results suggest that antiserum from SFTS patients has potential therapeutic effects against both lethal and non-lethal SFTSV infection in vivo. Although ribavirin had no statistically significant effect, it showed a slight protective effect in both lethal and non-lethal infections. PF-429242 and steroid treatments did not show apparent therapeutic benefit for SFTS infection, however, they did not seem to add detrimental effects during the progression of the disease in mice.

Suppressive effect of antiserum on viral replication in mice following lethal infection

Antiserum exhibited significant protection against fatal infection with SFTSV; thus, we compared viral loads in A129 mice infected with lethal doses of SFTSV during treatment with antiserum or mock saline.

During 1–3 days pi, copy numbers of viral RNA in the lung, spleen, liver, kidney, brain and spinal cord were not significantly different between mock- and antiserum-treated groups (Fig. 4). However, at 4 days pi, the levels in the spleen, kidney, and brain of antiserum-treated mice were significantly lower than those of the mock-treated mice (Fig. 4). Although the differences in other tissues were not significant, levels in the lung and liver tended

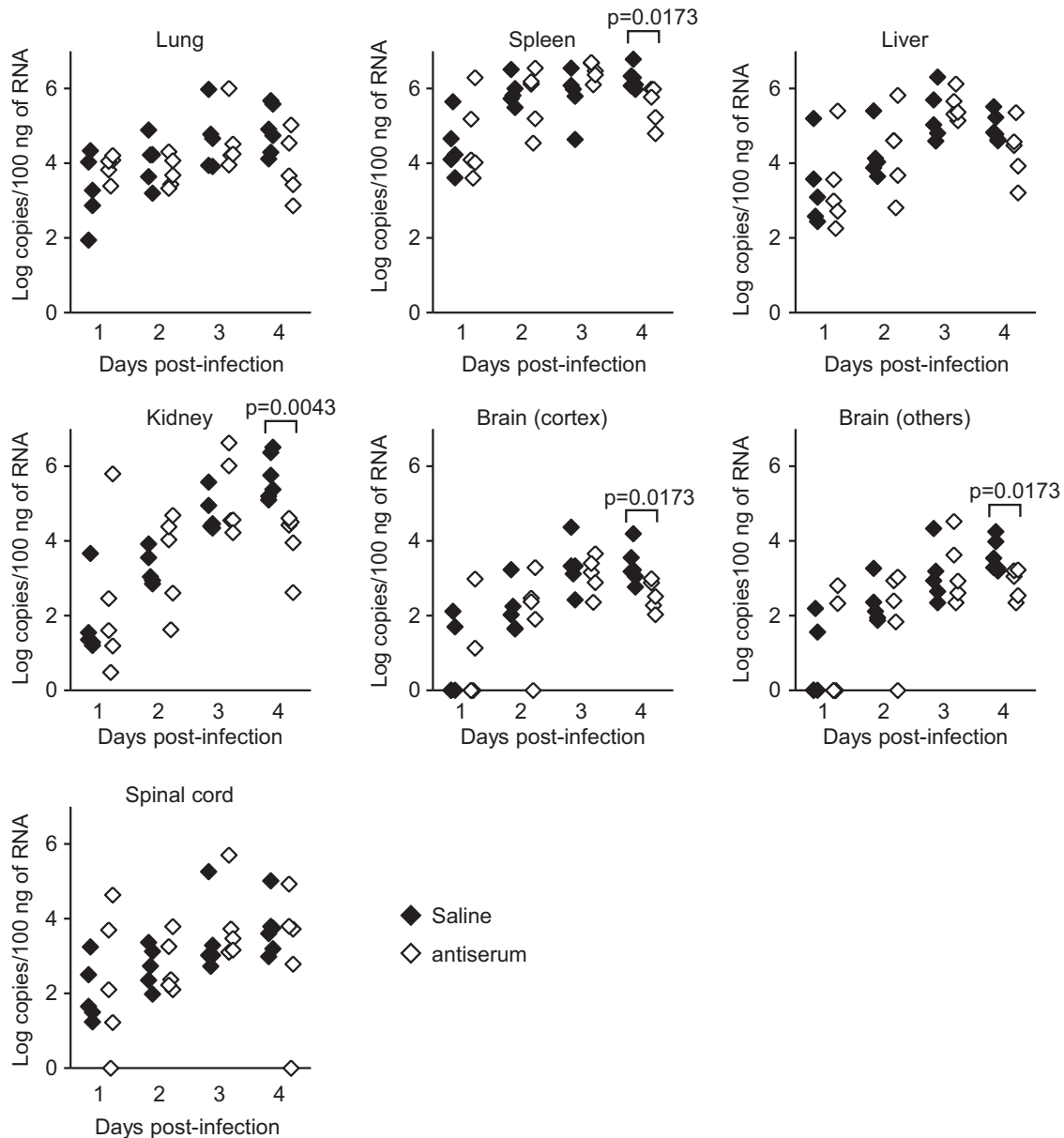


Fig. 4. Viral loads in the tissues of saline- and antiserum-treated A129 mice following infection with 10^6 ffu of SFTSV. Copy numbers in the lung, spleen, liver, kidney, brain cortex and non-cortex, and spinal cord of mice at 1, 2, 3 and 4 days were determined by RT-PCR ($n=5$). p : Mann Whitney test.

to be lower in the antiserum-treated mice than in the mock-treated mice at 4 days pi (Fig. 4).

Thus, it seems that the mice treated with antiserum had suppressed levels of viral replication. However, more evidences are required to show if the antiserum has really an effect on virus replication and help rescue mice from lethal infection. Other mechanisms may contribute to the rescue of mice from lethal infection with SFTSV.

Timing of efficient treatment with antiserum

We next examined the timing of effective treatment with antiserum on SFTSV infection in mice (Fig. 5A). When antiserum treatment was started at 24 h and 48 h, lethal dose-infected mice showed 20% and 0% survival rates, respectively (Fig. 5B). Weight reductions in these groups were similar to those of the control group (Fig. 5B). Survival rates and weight changes of non-lethal-

infected mice were not significantly different among the three groups (24 h, 48 h and control) (Fig. 5C). These observations suggest that early treatment with antiserum is important for having a significant effect on SFTSV infection.

Effects of MINO and CPEX on SFTSV infection

We next investigated the effects of MINO and CPEX on SFTSV infection in mice following a lethal dose infection (Fig. 6A). Although all mice died, survival times of minocycline and ciprofloxacin (MC)-treated mice were slightly longer than those of mock-treated mice (Fig. 6B). Their weight changes were not significantly different (Fig. 6B). Viral loads in the spleen, liver and brain cortex at 4 days pi were not significantly different between MC- and mock-treated groups (Fig. 6C). Therefore, MC-treatment may have some influences on lessening the severity of the SFTSV infection by a route other than simple suppression of viral replication in mice.

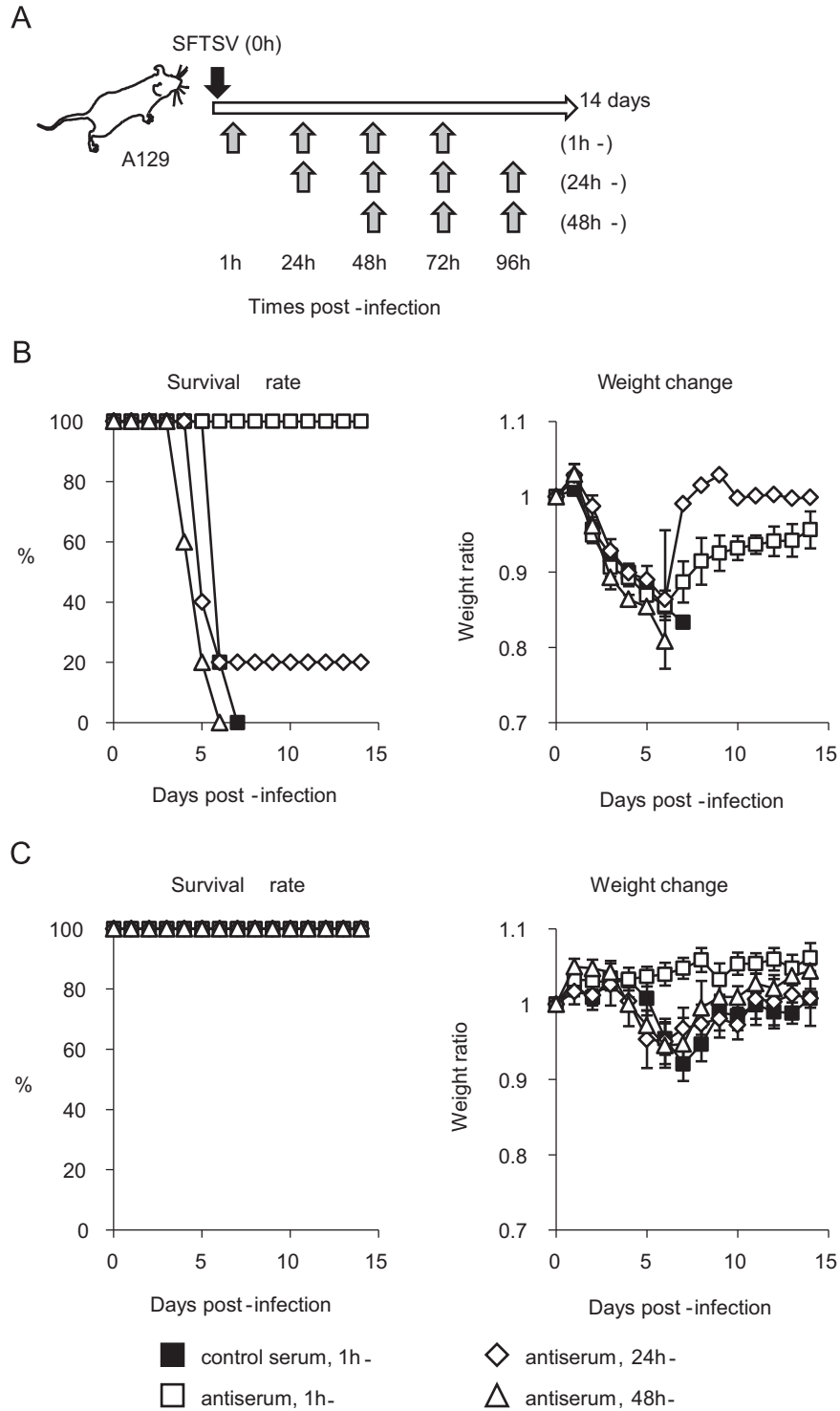


Fig. 5. Mortality and weight changes of antiserum-treated A129 mice at various times. (A) Administration schedule. Serum administration was started at 1 h (control serum or antiserum, 1 h-), at 24 h (antiserum, 24 h-) or at 48 h (antiserum, 48 h-) after infection with SFTSV. Black arrow indicates the time of SFTSV infection. Gray arrows indicate the time of antiserum administration. h = hour. (B) Mortality and weight changes of antiserum-treated mice following infection with lethal (10^6 ffu) dose of SFTSV. (C) Mortality and weight changes of antiserum-treated mice following infection with non-lethal (10^2 ffu) dose of SFTSV. Data of antiserum, 1 h are the same as in Figs. 2 and 3. $n=5$. P : Gehan–Breslow–Wilcoxon Test.

Discussion

In this study, we showed in an *in vivo* mouse model, that post-exposure prophylaxis for SFTS with human antiserum completely protected mice against lethal infection with SFTSV. This treatment also protected mice from clinical signs after infection with non-lethal doses of SFTSV. These suggest that antiserum treatment is

clinically useful for post-exposure prophylaxis against SFTSV infection.

Our results showed that early treatment with antiserum was required for efficient recovery from disease as shown in the mouse model. However, in natural infections of SFTSV after tick bites, it is difficult to do the anti-serum treatment soon after infection. On the other hand, SFTSV can infect through direct contact with

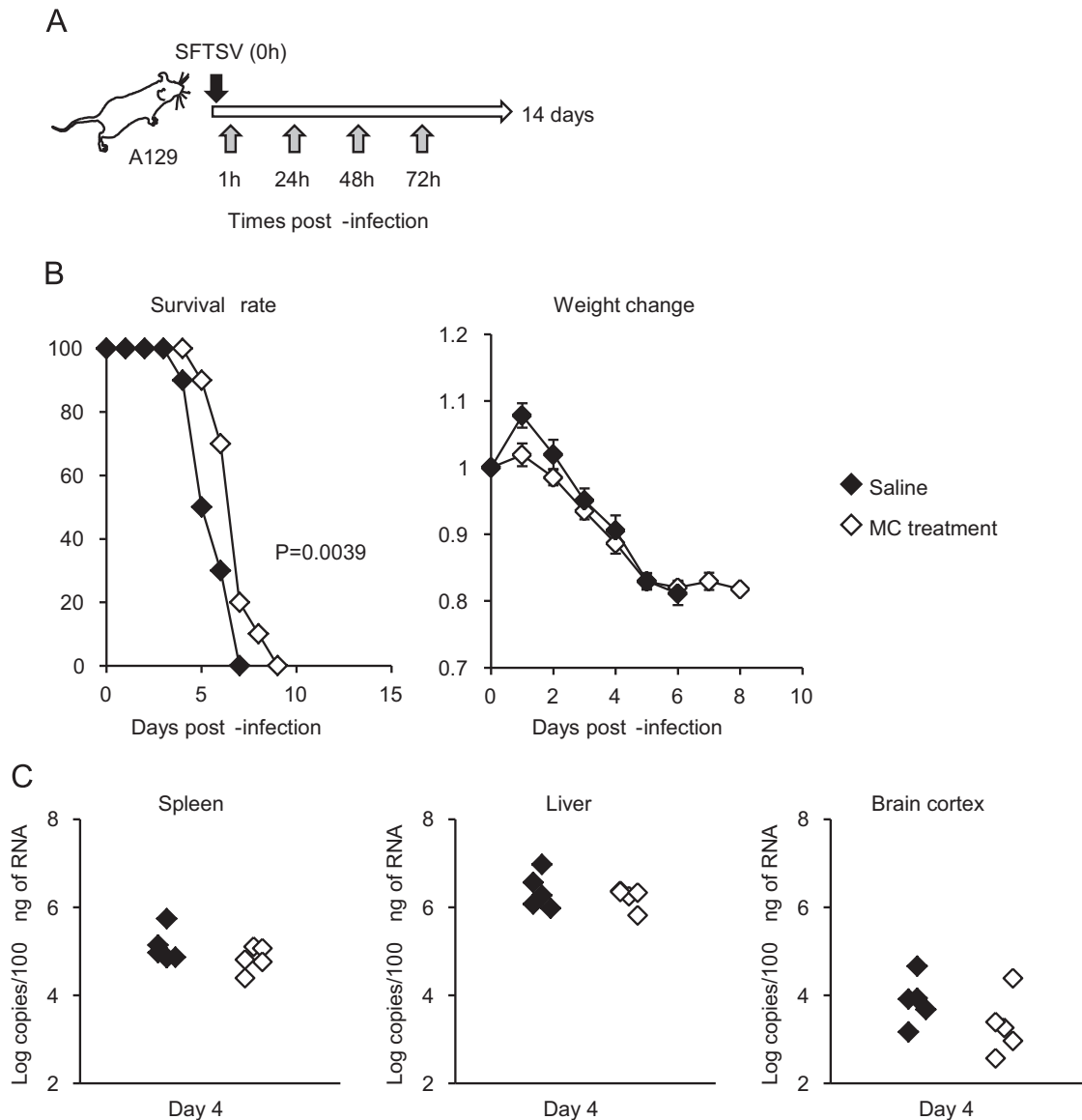


Fig. 6. Effect of MINO and CPFV (MC) on the mortality and weight changes of A129 mice infected subcutaneously with 10^6 ffu of SFTSV. (A) Schedule of the administration of MC. Black arrow indicates the time of SFTSV infection. Gray arrows indicate the time of antiserum administration. h=hour. (B) Survival curves and average weight changes in MC-treated mice (MC treatment) and mock-treated mice (Saline) ($n=10$). Error bars indicate the standard errors. (C) Viral copy numbers in the spleen, liver and brain cortex of A129 mice at 4 days ($n=5$).

infected blood or bloody secretions (Chen et al., 2013; Tang et al., 2013). Thus, antiserum treatment is likely to be more useful for individuals such as hospital health-care workers and relatives of patients who may have a hospital-acquired infection.

The antiserum used in this study showed high neutralizing antibody titers (1:2000), and treatment with this serum resulted in suppressive effects against SFTSV replication in vivo. Here, 500 μ l of inoculum (1:10 dilution of serum) per mouse was injected four times every 24 h. However, it would be difficult to prepare and inject the same scale of inoculum volume in human cases. Thus, further improvement in the production of efficient and abundant amounts of anti-viral antibodies with high neutralizing activity is needed to provide a practical strategy for treatment of SFTS. Alternatively use of pooled antisera could also provide a practical clinical source.

It has been reported that ribavirin is effective against some RNA virus infections such as hepatitis C virus (Lange et al., 2014), Lassa fever virus (McCormick et al., 1986), Crimean-Congo hemorrhagic fever virus (Keshtkar-Jahromi et al., 2011) and respiratory syncytial

virus (Sidwell and Barnard, 2006). In practice, a combination antiviral therapy of ribavirin, interferon and other agents such as sofosbuvir has been used as a standard treatment for hepatitis C (Dhingra et al., 2014). The inhibitory effects of ribavirin against SFTSV replication were recently shown in an in vitro assay (Shimajima et al., 2014). In this study, slight protective effects were observed after ribavirin treatment, although the difference was not statistically significant. Therefore, a combination of ribavirin with other antiviral agents may possibly be an effective treatment for this disease.

MC treatment resulted in prolonged survival times in mice compared with non-treated mice following lethal infection with SFTSV. However, viral loads in the tissues were not significantly different between MC- and mock-treated groups. Thus, an unknown mechanism of MINO or CPFV may contribute to the prolonged survival effect. It is noteworthy that MC treatment is unlikely to exacerbate the disease course due to SFTSV infection. Thus, MC treatment should be considered in suspected cases of spotted fever caused by *Rickettsia japonica* in the endemic area in Japan, even though diagnosis of spotted fever or SFTS is not definitive.

For animal models of SFTSV infection, immunocompetent mice and hamsters were studied, but these adult animals did not show apparent disease (Chen et al., 2012; Jin et al., 2012; Liu et al., 2014c). Liu et al. showed that IFNAR^{-/-} mice were highly susceptible to SFTSV infection (Liu et al., 2014c). In this study, we used IFNAR^{-/-} mice of the same background strain (129/Sv) reported previously (Liu et al., 2014c), but we used different virus strain (Japanese isolate YG-1 strain) for virus challenges. The previous study showed only fatal infection with 10⁶ ffu of SFTSV. Here, we also showed that 10⁶ ffu of SFTSV (YG-1 strain) caused lethal infection. Furthermore, we showed that inoculations with 10² ffu of SFTSV caused non-lethal infection. Non-lethal infection induced clinical signs including weight reduction, but the mice survived. In human cases, it has been suggested that the patient's viral load is related to disease severity (Gai et al., 2012; Yoshikawa et al., 2014). Thus, a comparison of different infective doses may provide a promising approach to elucidate the mechanism of SFTS severity. Furthermore, dose-dependent mouse models of SFTSV infection are needed for evaluation of future antiviral drugs for post-exposure prophylaxis against SFTSV infection.

Conclusion

In this study, we showed that post-exposure prophylaxis with human antiserum from an SFTS patient had significant therapeutic effects on SFTSV infection in an *in vivo* mouse model. Our results strongly suggest that antiserum treatment may be clinically useful for post-exposure prophylaxis against SFTSV infection.

Materials and methods

Virus and cells

The YG-1 strain of SFTSV was kindly provided by Ken Maeda, Yamaguchi University (Takahashi et al., 2014). Stock virus of SFTSV was prepared from cell culture medium of Vero E6 cells. Vero E6 cells were maintained in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co.) containing 10% fetal bovine serum (FBS). All experiments using live SFTSV were performed in a biosafety level 3 laboratory at Nagasaki University according to standard BSL3 guidelines.

Antiserum from a recovered SFTS case-patient

Serum was collected from the patient who contracted the disease in 2005. Collection was done after eight years from the recovery of the patient. Control serum was collected from a healthy volunteer who had not been infected with SFTSV. The experiment using human serum was performed with the approval of the ethics committee of the Institute of Tropical Medicine, Nagasaki University (Approval number: 140829129).

Focus forming assay

The SFTSV titer was determined in a focus forming assay. Confluent Vero E6 cells were inoculated with serially diluted culture supernatants of SFTSV stock and incubated in 2% FCS EMEM containing 1% methyl cellulose 4000 (Wako Pure Chemical Industries, Ltd.) for 5 days. Viral foci were detected using SFTSV antiserum, peroxidase-conjugated anti-human IgG (American Qualex) and DAB substrate (Wako Pure Chemical Industries, Ltd.). Viral titers were expressed as ffu/ml.

Neutralization test

The neutralizing antibody titers of the antiserum were determined by focus-reduction assay. Serially diluted sera were mixed with SFTSV and incubated at 37 °C for 1 h. Vero E6 cells were inoculated with the mixtures and incubated for 5 days. Focus staining was performed as described above. The neutralizing titer was determined as the reciprocal of the highest serum dilution that reduced the viral foci counts by 50%.

Mice

A129 mice were purchased from B & K Universal limited. These mice were mated in the facility at Nagasaki University. Adult (older than eight-week-old) mice were subcutaneously inoculated with 10² and 10⁶ ffu of SFTSV diluted in EMEM containing 2% FBS. Mice were weighed daily and observed for clinical signs. The experimental protocols were approved by the Animal Care and Use Committee of the Nagasaki University (approval number: 1401201115).

Treatment with drugs and antiserum in mice

Ribavirin (Wako Pure Chemical Industries, Ltd.), PF-429242 (AdooQ BioScience) and steroids (Methylprednisolone sodium succinate, Sawai Pharmaceutical Co., Ltd.) were diluted in saline, and 500 µl of each diluent was intraperitoneally administered to A129 mice at 2 mg, 1 mg and 0.4 mg per mouse, respectively, at 1, 24, 48 and 72 h following infection with SFTSV (Figs. 2A and 3A). MINO (Minomycin[®], Pfizer Japan Inc.) and CPF (Nichi-Iko Pharmaceutical Co., Ltd.) were diluted in saline, and the mixture (500 µl) was intraperitoneally injected as 1 mg of each antibiotic per mouse at 1, 24, 48 and 72 h after inoculation with SFTSV (Fig. 6A). Sera were diluted tenfold in saline, and 500 µl of diluent was intraperitoneally injected at 1, 24, 48, 72 and 92 h after inoculation with SFTSV (Figs. 2A, 3A and 4A). Saline or a diluent of the control serum at the same volume of the drugs or antiserum was used for mock treatment.

Virus titration in mouse tissues

One to four days pi, three to five mice were sacrificed, and lung, spleen, liver, kidney, brain and spinal cord were collected following perfusion with cold phosphate-buffered saline. Brains were divided into two fractions: cortex and non-cortex. The tissues were immediately submerged in RNAlater (Ambion) and were stored at -80 °C until use. Total RNA was extracted using an RNeasy Lipid Tissue Mini Kit (Qiagen). Viral copy numbers were examined by real-time RT-PCR. SFTSV-specific primers and a probe were designed based on the RdRp region of the consensus sequences of the L segment. The forward primer was SFTS_QPCR_965F: 5'-GCRAGGAGCAACAAR-CAAACATC-3', the reverse primer was SFTS_QPCR_1069R: 5'-GCCTGAGTCGGTCTTGATGTC-3' and the PrimeTime[®] qPCR probe was FAM/5'-CTCCCRCC-3'/ZEN/5'-TGGCTACCAAAGC-3'/IBFQ (Integrated DNA Technologies). Real-time RT-PCR reactions were performed using a One Step PrimeScript RT-PCR Kit (Takara Bio Inc) and a 7500 Real-time RT-PCR System (Applied Biosystems). The copy numbers were calculated as a ratio of the copy numbers of the standard control.

Statistical analyses

A Gehan-Breslow-Wilcoxon Test was performed to assess the survival curves of SFTSV-infected groups of mice. A Mann Whitney test was used to assess the significant differences in viral RNA levels.

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