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Infection of Hantaan virus strain AA57 leading to pulmonary disease in laboratory mice

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ABSTRACT

Hantaan virus (HTNV) is a causative agent of hemorrhagic fever with renal syndrome (HFRS). The pathogenesis of HFRS has not been fully elucidated, mainly due to the lack of a suitable animal model. In laboratory mice, HTNV causes encephalitis. However, that symptom is dissimilar to human hantavirus infections. We found that HTNV strain AA57 (isolated from *Apodemus agrarius* in Far East Russia) caused pulmonary disease in 2-week-old ICR mice. The clinical signs of the infected mice were piloerection, trembling, hunching, labored breathing, and body-weight loss. A large volume of pleural effusion was collected from thoracic cavities of the dead mice. Overall, 45% of the mice inoculated with 3000 focus forming units (FFU) of the virus began to show clinical symptoms at 8 days post-inoculation, and 25% of the inoculated mice died within 3 days of onset of the disease. The morbidity and mortality rates of the mice inoculated with 30–30,000 FFU of HTNV strain AA57 were roughly equivalent. The highest rates of virus positivity (11/12) and the highest titers of HTNV strain AA57 were detected in the lungs of the dead mice, while lower detection rates and viral titers were found in the heart, kidneys, spleen, and brain. Interstitial pneumonia, perivascular edema, hemorrhage, inflammatory infiltration and vascular failure were observed in the lungs of the sick mice. Hantaviral antigens were detected in the lung endothelial cells of the sick mice. The symptoms and pathology of this mouse model resemble those of hantavirus pulmonary syndrome (HPS) and, to a certain extent, those of HFRS. This is the first report that, in laboratory mice, the HFRS-related hantavirus causes a HPS-like disease and shares some symptom similarities with HFRS.

1. Introduction

Hantaviruses are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) in humans (Krüger et al., 2001; Schmaljohn and Hjelle, 1997). Rodents and *Soricomorpha* species are the natural reservoirs for these viruses. Hantaviruses, which are classified in the genus *Hantavirus* within the family *Bunyaviridae*, have a genome that is composed of three negative-stranded RNA segments. The small (S), medium (M), and large (L) genome segments encode nucleocapsid protein (N), two glycoproteins (Gn, Gc), and RNA polymerase, respectively (Schmaljohn, 2001). Each rodent-borne hantavirus has a specific host, and the genus *Hantavirus* contains at present more than 50 species (Jonsson et al., 2010; Kang et al., 2010; Nichol et al., 2005). Rodent-borne hantaviruses are divided into three large groups based on the host animal: *Murinae*-borne; *Arvicolinae*-borne; and *Sigmodontinae*- or *Neotominae*-borne. Many rodent-borne hantaviruses are known to be pathogenic for humans, although these viruses do not cause any apparent symptoms in their natural hosts. Although many hantaviruses have been identified in *Soricomorpha* species, no human disease has been associated with *Soricomorpha*-borne hantaviruses to date (Song et al., 2007).

About 20,000–50,000 cases of HFRS are reported annually in Asia, Europe, and Russia. Five viruses are recognized as the major causative agents of HFRS, including Hantaan virus (HTNV), Amur virus (AMRV) and Seoul virus (SEOV) in East Asia and Far East Russia, and Puumala virus (PUUV) and Dobrava virus (DOBV) in Europe and European Russia (Garanina et al., 2009; Kariwa et al., 2007; Vapalahti et al., 2003). The hosts for HTNV, AMRV, SEOV, and PUUV are *Apodemus agrarius*, *A. peninsulae*, *Rattus norvegicus*, and *Myodes glareolus*, respectively (Schmaljohn, 2001). Recently, a detailed phylogenetic analysis revealed that DOBV forms distinct evolutionary lineages, i.e., DOBV from *A. flavicollis* (DOBV, DOBV-Af), from *A. agrarius* (Saaremaa, DOBV-Aa), and from *A. ponticus*

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(DOBV-Ap) (Avsic-Zupanc et al., 1992; Klempa et al., 2003, 2005, 2008; Nemirov et al., 1999). Regarding HFRS cases reported from East Eurasia, which includes China, Korea, and Far East Russia (Song et al., 2006; Kariwa et al., 2007; Zhang et al., 2010), HTNV is one of the main causes of the disease.

Humans become infected with hantaviruses by either inhaling rodent excreta or being bitten by infected rodents. The initial symptoms of HFRS appear suddenly and include intense headaches, back and abdominal pains, fever, chills, and nausea. Patients show flushing of the face, inflammation, and redness of the eyes, followed by severe symptoms, such as low blood pressure, acute shock, vascular leakage, and acute kidney failure, which can result in severe fluid overload (Peters et al., 1999; Schmaljohn and Hjelle, 1997). The severity of the disease largely depends on the infecting virus. HTNV and DOBV cause severe symptoms, while SEOV and PUUV infections are less severe (Antoniadis et al., 1987; Schmaljohn and Hjelle, 1997). AMRV appears to cause severe HFRS (Lokugamage et al., 2002, 2004b; Yashina et al., 2000, 2001). However, the pathogenesis of HFRS caused by AMRV has not been fully investigated.

Animal models have been developed to uncover the pathogenicity of hantaviruses. Andes virus (ANDV), which is the causative agent of HPS in South America, generates severe symptoms in Syrian hamsters (Campen et al., 2006; Hooper et al., 2001; Wahl-Jensen et al., 2007). The characteristics of ANDV infections in hamsters include rapidly progressing labored breathing, pulmonary edema, pleural effusion, and hypotension, in close resemblance to HPS in humans. In the case of HFRS-related viruses, mice have been used to examine the pathogenesis of HTNV (Ebihara et al., 2000; Kurata et al., 1983; Tamura et al., 1989; Wichmann et al., 2002). However, HTNVs cause neurovirulence in mice, unlike the symptoms associated with hantavirus infections in humans. These results emphasize that the animal models used to analyze the pathogenesis of HTNV need to be improved. Interestingly, cynomolgus macaques infected with PUUV show symptoms similar to those of HFRS (Klingström et al., 2002; Sironen et al., 2008). However, the management of infected nonhuman primates requires a larger biological containment facility and entails higher costs and labor inputs. Thus, it is desirable to establish a suitable and reasonable model with small laboratory animals to analyze the pathogenesis of HFRS.

HTNV strain AA57 (AA57) was isolated from *A. agrarius* in the Khabarovsk region of Russia. The DNA sequences of strain AA57 and of hantaviruses from HFRS patients in the Khabarovsk region were rather similar (nucleotide: 96–99%, amino acid: 98–100%; Kariwa et al., unpublished data).

In the present study, we report on the severe outcome of an infection with strain AA57 in 2-week-old ICR mice. The AA57-infected mice had severe acute pulmonary symptoms, and some of the mice died within 3 days of disease onset. We observed the clinical symptoms of the AA57-infected mice. We analyzed the distribution of the virus in various organs and the associated histopathologic changes, to determine the potential of this model to enhance our understanding of the pathogenesis of hantavirus infections in humans.

2. Materials and methods

2.1. Cells and culture medium

Vero E6 cells (ATCC No. CRL-1586; American Type Culture Collection, Manassas, VA) were maintained in Minimum Essential Medium with Eagle's salts (MEM; Invitrogen, Carlsbad, CA) that was supplemented with 10% fetal bovine serum (FBS; MP Biochemicals, Aurora, OH), 2 mM L-glutamine (Kanto Chemical, Tokyo, Japan), 100 IU/mL penicillin G (Meiji Seika, Tokyo, Japan), and 100 μg/mL streptomycin (Meiji Seika).

2.2. Hantaviruses

HTNV strain AA57 (Genebank ID: S segment; AB620031, M segment; AB620032, L segment; AB620033) and HTNV strain 76-118 cl-1 (cl-1, Genebank ID: S segment; D25530, M segment; D25529, L segment; D25528, Tamura et al., 1989) were used in this study. Virus was inoculated onto a monolayer of Vero E6 cells. The supernatant of the infected cell culture was collected on Day 5, and stored at −80 °C as a working stock. Each virus was passaged twice in Vero E6 cells.

2.2.1. Inoculation of viruses into mice

Two-week-old ICR mice (Japan SLC, Shizuoka, Japan) were used in this study. Ten or twenty mice per group were injected subcutaneously with 0.3 mL of strain AA57 (3–30,000 focus-forming units [FFU]) in MEM. MEM or 30,000 FFU of strain cl-1 were inoculated similarly into 7 or 9 mice per group. After viral challenge, the mice were observed and body weights were measured daily. A reduction in body weight, compared to the previous day, was regarded as onset of the disease. The lungs, kidneys, spleen, liver, heart, brain, and pleural effusion of each dead mouse were collected and stored at −80 °C. Some organs of the dead mice were fixed in 10% neutral-buffered formalin. At 21 days post-inoculation (d.p.i.), all the surviving mice were killed by cervical dislocation under anesthesia, and the organs, blood clots, and sera of each mouse were collected and stored at −80 °C. All animal experiments were performed in a biosafety level 3 animal facility, in accordance with the Guidelines for Animal Experimentation of the School of Veterinary Medicine, Hokkaido University.

2.2.2. Passive immunization before AA57 inoculation into mice

Mice were intraperitoneally injected with 0.3 mL of anti-HTNV strain 76-118 mouse serum or normal mouse serum. One day after injection of the mouse sera, 300 FFU of strain AA57 were inoculated into each group of mice. After viral challenge, the mice were observed and body weights were recorded until 14 d.p.i.

2.3. Virus titration

The method used for hantavirus titration has been described previously (Lokugamage et al., 2004a). The organs of the mice were homogenized in MEM using a cold pestle, mortar, and sea sand. Thereafter, 10% of each homogenate was centrifuged at 2000 × *g* for 5 min, and the supernatant was serially diluted in MEM. Diluted samples (50 μL/well) were inoculated onto monolayers of Vero E6 cells grown in 96-well plates (Nunc, Roskilde, Denmark). After adsorption for 1 h at 37 °C, the inoculum was removed, and 150 μL of MEM that contained 1.5% carboxymethyl cellulose (CMC-MEM) was layered onto the cells, which were then incubated at 37 °C for 5 days in 5% CO_2. After incubation, the monolayers were washed with PBS, fixed with methanol, and air-dried. Foci of hantaviruses were immunostained with an anti-E5/G6 monoclonal antibody (Yoshimatsu et al., 1996) and Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen). Stained foci were counted under a fluorescence microscope.

2.4. Histopathology

Heart, lung, liver, spleen, adrenal gland, kidney, and brain samples were collected from infected animals for histopathology. Tissues were fixed in 10% phosphate-buffered formalin and routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The antigens were retrieved by hydrolytic autoclaving for 10 min at 121 °C in 10 mM sodium citrate–sodium chloride buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in methanol

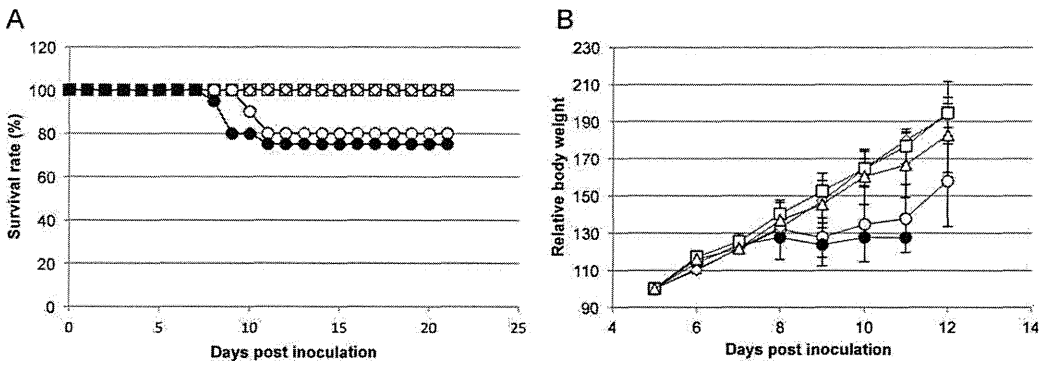

Fig. 1. (A) Survival curve of hantavirus-infected mice (ICR; age, 2 weeks). Mice were inoculated subcutaneously with Hantaan virus strain AA57 (30 or 3000 FFU), Hantaan virus strain 76-118 cl-1 (30,000 FFU) or MEM. Survival rate is expressed as the percentage. Closed circles represent AA57-inoculated mice (3000 FFU, $n=20$); open circles represent AA57-inoculated mice (30 FFU, $n=10$); diamonds represent 76–118 cl-1-inoculated mice ($n=9$); and squares represent MEM-inoculated mice ($n=7$). (B) Body-weight changes of hantavirus-infected mice (ICR; age, 2 weeks). Mice were inoculated subcutaneously with Hantaan virus strain AA57 (3000 FFU), Hantaan virus strain 76-118 cl-1 (30,000 FFU) or MEM. Relative body weight is expressed as the percentage of body weight at 5 days post-inoculation. Closed circles represent AA57-inoculated mice that died ($n=5$); open circles represent AA57-inoculated mice that recovered ($n=4$); triangles represent AA57-inoculated mice that showed no symptoms ($n=11$); diamonds represent 76-118 cl-1-inoculated mice ($n=9$); and squares represent MEM-inoculated mice ($n=7$). The error bars represent standard deviations.

for 30 min. The first antibody added was the anti-E5/G6 monoclonal antibody (1 μg/mL; incubated overnight at 4 °C). The VECTOR M.O.M. Immunodetection Kit (Vector Laboratories, Burlingame, CA) was used to visualize the HTNV antigens in the sections.

3. Results

3.1. HTNV strain AA57 is pathogenic for 2-week-old ICR mice

We had experienced that younger mice produce higher level of antibodies to hantavirus than adult mice. During course of anti AA57 immune serum preparation, we injected strain AA57 to 2-week-old ICR mice and found some of the mice died. In this study, we observed symptoms of the disease and analyzed virus loads as well as pathology in AA57 infected mice.

Two-week-old ICR mice were inoculated subcutaneously with a series of 10-fold dilutions (3–30,000 FFU) of HTNV strain AA57. Ten to twenty mice were used for each dilution of strain AA57, while nine mice were infected with 30,000 FFU of strain cl-1. Similarly, MEM was injected into seven mice as a control. Mice inoculated with strain AA57 began to show clinical symptoms from 7 d.p.i., including piloerection, trembling, hunching, labored breathing, and body-weight loss (Table 1). Some mice inoculated with 30–30,000 FFU of strain AA57 began to die from 7 to 11 d.p.i. (Table 1 and Fig. 1A). Nine of the twenty mice inoculated with 3000 FFU of strain AA57 showed reductions in body weight around 9 d.p.i. (Table 2 and Fig. 1B), and five mice died. Almost all the mouse deaths occurred within 1 day of symptom onset (Table 2). Four of nine sick mice survived and showed an increase in body weight within 1.5 ± 1.59 days of onset (Table 2 and Fig. 1B). Eleven of the twenty mice inoculated with 3000 FFU of strain AA57 showed no symptoms or had body-weight changes that were similar to those of the MEM-injected mice (Table 2 and Fig. 1B). The morbidity and mortality rates of the AA57-inoculated mice (3000 FFU) were 45% (9/20) and 25% (4/20), respectively (Table 2). The morbidity and mortality rates of the mice inoculated with 30–30,000 FFU of strain AA57 showed no significant differences (Table 2). In contrast, none of the mice inoculated with strain cl-1 showed any clinical symptoms, body-weight loss or deaths (Fig. 1A and B).

From the mice that died as a result of inoculation with AA57, 0.1–0.7 mL (average, 0.35 mL) of pleural effusion were collected from the thoracic cavity (Fig. 2). The color of the pleural effusions ranged from yellow to pink. These pleural effusions accounted for, on average, 2.8% of the total mouse body weight. The examinations of the clinical signs and at necropsy indicate that the symptoms seen in AA57-inoculated mice are similar to those see in HPS.

3.2. Viral loads in the organs of mice that died following inoculation with AA57

To determine the distribution of HTNV strain AA57 in the mice that died as a result of viral infection, the organs of these mice were subjected to virus titration (Table 3). Infectious viruses were detected in the lungs, heart, kidneys, spleen, and brain. The highest rates of virus positivity (11/12) and the highest titers of infectious virus were detected in the lungs (Table 3). The rates of virus positivity for the heart, kidneys, spleen, and brain were 4/12, 1/12, 5/12, and 3/10, respectively. The rates of virus positivity and the virus titers in these organs were lower than those in the lungs (Table 3). No virus was detected in any of the liver or pleural effusion samples (Table 3).

3.3. Histopathology

To investigate the pathogenesis of strain AA57, the HTNV AA57-infected mice were subjected to histopathologic analysis. Eight of the ten sick mice had clear histopathologic changes in their lungs at 7–11 d.p.i. (Table 4). The affected mice were shown mild perivascular edema with slight mononuclear cell infiltration around the vessels in the lungs (Fig. 3A–C). In the vessels, degenerated endothelials and adhesion neutrophils were observed and were related virus antigens (Fig. 3D and E). The slight interstitial

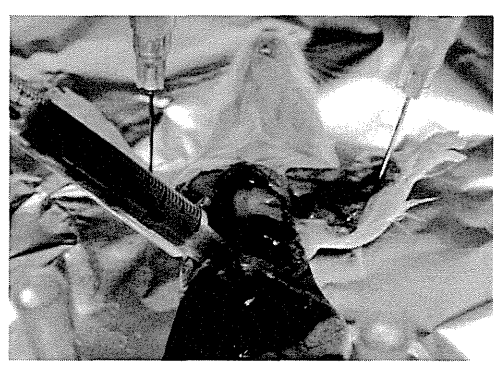

Fig. 2. Collection of the pleural effusions from an AA57-inoculated mouse that died (mouse no. AA57-96).

Table 1
Clinical signs of AA57-infected mice resulting in death.

Mice ID No.	Sex	Titer of inoculated virus (FFU[a])	Onset (d.p.i.)	Death (d.p.i.)	Survival date from onset	Clinical signs	Amount of pleural effusion (mL)
AA57-50	F	30	10	11	1	Body weight loss, labor breathing	0.4
AA57-51	M	30	10	10	0	Found dead	0.7
AA57-60	M	300	10	10	0	Body weight loss, labor breathing	0.4
AA57-64	F	300	10	10	0	Found dead	0.4
AA57-20	M	3000	9	11	3	Body weight loss, labor breathing	0.2
AA57-24	F	3000	9	9	0	Found dead	0.2
AA57-25	M	3000	8	8	0	Found dead	0.4
AA57-66	M	3000	9	9	0	Body weight loss, labor breathing	0.4
AA57-69	F	3000	9	9	0	Found dead	0.7
AA57-96	F	30,000	8	9	1	Body weight loss	0.3
AA57-98	M	30,000	9	9	0	Body weight loss, labor breathing	0.1
AA57-101	M	30,000	7	7	0	Body weight loss, labor breathing	0.2

[a] Focus forming units.

Table 2
Morbidity and mortality in AA57 infected mice.

	Infection dose (FFU[a])				
	3	30	300	3000	30,000
Morbidity	10% (1/10)	50% (5/10)	50% (5/10)	45% (9/20)	70% (7/10)
Average time of onset (d.p.i.)	11	10	10	9.11 ± 0.6[b]	8.14 ± 0.64
Mortality	0% (0/10)	20% (2/10)	20% (2/10)	25% (5/20)	30% (3/10)
No. of recovered animals	1	3	3	4	4
Disease time post onset (day)	1	2.67 ± 2.87	1	1.5 ± 1.59	1.25 ± 0.8
No. of dead animals	0	2	2	5	3
Survival time post (day)	–	0.5	0.5	0.4 ± 0.59	0.3 ± 1.43

[a] Focus forming units.
[b] 95% confidence interval.

pneumonitis with cellular infiltration was also seen in the affected mice (Fig. 3F). These affected mice showed evidence of lymphoid cell depletion in the spleen (data not shown). However, the hantavirus antigen was not detected in the spleen (Table 4). Although infiltration of mononuclear cells and hantavirus antigens were observed in the liver (Table 4), no infectious virus was detected in the liver (Table 3). These results indicate that AA57 infection does not affect liver function.

3.4. *Protection against pulmonary disease by the administration of anti-HTNV immune serum*

To confirm that, specifically, strain AA57 causes the disease seen in inoculated mice, we administrated intraperitoneally anti-HTNV strain 76-118 immune serum (cross-neutralized to AA57) to mice 1 day before inoculation of the same mice with strain AA57. The immune serum-administered mice were completely protected

Table 3
Virus titers in dead mice.

Mice ID No.	Sex	Titers of inoculated virus (FFU[a])	Virus titers of organs (FFU/g)						
			Lung	Heart	Kidney	Spleen	Brain	Liver	PE[b]
AA57-50	F	30	37,333	54,000	466	–	333	–	–[d]
AA57-51	M	30	1733	–	–	600	–	–	–
AA57-60	M	300	200	–	–	–	N.D.[c]	–	–
AA57-64	F	300	8000	200	–	–	N.D.	–	–
AA57-20	M	3000	400	–	–	–	267	–	–
AA57-24	F	3000	667	–	–	667	2400	–	–
AA57-25	M	3000	5600	–	–	533	–	–	–
AA57-66	M	3000	1200	533	–	1533	–	–	–
AA57-69	F	3000	16,000	–	–	1467	–	–	–
AA57-96	F	30,000	–	–	–	–	–	–	–
AA57-98	M	30,000	4667	867	–	–	–	–	–
AA57-101	M	30,000	400	–	–	–	–	–	–
Positive rate		11/12	4/12	1/12	5/12	3/10	0/12	0/12	

[a] Focus forming units.
[b] Pleural effusion.
[c] Not done.
[d] Virus titer is under detectable level.

Table 4
Histopathological analysis in AA57-infected mice.[a]

ID	Date of onset (d.p.i.)	Collection date (d.p.i.)	Clinical signs	Histopathological changes and virus antigen detection						
				PE[d]	Lung	Heart	Liver	Kidney	Spleen	Brain
AA57-96	8	9[b]	Body weight loss, labor breathing	+ (400 μl)	−/−[e]	−/−	+/+	−/−	+/−	−/−
AA57-98	8	9[b]	Body weight loss	+ (300 μl)	+/−	N.D.[f]	N.D.	−/−	N.D.	N.D.
AA57-101	7	7[b]	Body weight loss, labor breathing	+ (400 μl)	+/−	−/−	+/+	−/−	+/−	−/−
AA57-88	8	8	Body weight loss, labor breathing	−	+/−	−/−	+/+	−/−	+/−	−/−
AA57-89	8	8	Body weight loss, labor breathing	+ (300 μl)	+/−	−/−	−/−	−/−	+/−	−/−
AA57-90	8	8	Body weight loss, labor breathing	−	+/+	−/−	+/+	−/−	+/−	−/−
AA57-93	8	8	Body weight loss, labor breathing	−	+/+	−/−	+/+	−/−	−/−	−/−
AA57-94	8	8	Body weight loss, labor breathing	−	+/+	N.D.	+/+	−/−	N.D.	−/+
AA57-95	8	8	Body weight loss, labor breathing	−	+/−	N.D.	+/+	−/−	+/−	−/−
AA57-91	10	11[c]	Body weight loss	−	−/−	−/−	−/−	−/−	+/−	−/−
AA57-92		11	No clinical sign	−	−/−	−/−	−/−	−/−	+/−	−/−

[a] AA57 was inoculated to each mice 30,000 focus forming units subcutaneously.
[b] Collected from dead mice.
[c] Collected from a recovered mouse.
[d] Pleural effusion.
[e] Histopathological change/virus antigen.
[f] Not done.

against disease caused by strain AA57 and showed no body-weight loss, clinical symptoms, or mortality (data not shown).

4. Discussion

There have been many attempts to develop an animal model that can be used to analyze the pathogenesis of hantaviruses. However, these models have shown only limited suitability for analyzing hantavirus infections. For example, ANDV and Maporal virus cause HPS-like symptoms in Syrian hamsters (Campen et al., 2006; Hooper et al., 2001; Milazzo et al., 2002; Wahl-Jensen et al., 2007). In contrast, some strains of HTNV cause neurovirulence in mice, which is different to the symptoms of human HFRS (Ebihara et al., 2000; Kurata et al., 1983; Tamura et al., 1989; Wichmann et al., 2002). Therefore, there is an urgent need to develop an efficient animal model that will facilitate studies of the pathogenesis of HFRS-related hantaviruses.

We injected strain AA57 to 2-week-old ICR mice to obtain the immune serum and found that strain AA57 causes a severe pulmonary disease. In the present study, we examine the virus loads in organs and the pathology in AA57-infected mice. The AA57-infected mice showed pulmonary symptoms and some of the sick mice died within 1 day of disease onset. This indicates that strain AA57 is pathogenic for 2-week-old ICR mice. In contrast, mice inoculated with HTNV strain 76-118 cl-1 did not show clinical signs.

HTNV is known to cause HFRS, which is characterized by fever, internal hemorrhage, acute shock, vascular leakage, and kidney failure (Peters et al., 1999; Schmaljohn and Hjelle, 1997). However, pulmonary involvement is observed in some HFRS cases attributed to SEOV and PUUV (Caramello et al., 2002; Kikuchi et al., 1982; Linderholm et al., 1992; Rasmuson et al., 2011). In addition, pulmonary edema has been observed in HFRS cases in China, where HTNV, SEOV, and AMRV are prevalent (Hjelle et al., 1995; Zhao et al., 2009). Thus, it is possible that HTNV causes the pulmonary manifestations seen in infected humans. In the present study, large volumes of pleural effusion were collected from the AA57-inoculated mice that died as a result of the infection; this is similar to the pulmonary manifestations of HFRS (described above) and HPS patients. In addition, the pulmonary pathogenesis of AA57 in mice resembled that of ANDV-inoculated hamsters, which could be the models of human HPS (Hooper et al., 2001). The highest titers of infectious virus were detected in the lungs of the AA57-infected mice that died, and hantavirus antigens were detected in the lungs of sick mice by immunohistochemistry. Previous reports have described the detection of hantavirus antigens in the lung endothelial cells of PUUV-infected HFRS cases, HPS patients, and ANDV-infected hamsters (Hooper et al., 2001; Rasmuson et al., 2011; Zaki et al., 1995).

In previous studies, HTNV-inoculated adult or suckling mice developed neurologic diseases, and infectious viruses were detected mainly in the brain, lungs, spleen, and kidneys (Ebihara et al., 2000; Kurata et al., 1983; Tamura et al., 1989; Wichmann et al., 2002). However, the AA57-infected mice showed no neurologic signs, and they had lower rates of virus positivity and lower titers of infectious virus in the brain, heart, kidney, spleen, and liver. These results suggest that AA57 mainly replicates in the lungs, and that the pathologic changes in infected animals occur mainly in this organ. In the histopathologic analyses of AA57-infected mice at 7–11 d.p.i., acute pneumonitis, perivascular edema, hemorrhage, inflammatory infiltrates, and endothelial degeneration were observed in the lungs. Hantavirus antigen was detected in the endothelial cells. These results indicate that increased vascular permeability associated with strain AA57 leads to hemorrhage, accumulation of pleural effusion, and death in the infected mice. Therefore, our data suggest that the AA57-infected ICR mouse model may reflect the pathogenesis of human HPS and, to some extent, that of HFRS. In HFRS cases, renal failure often occurs and hantavirus antigen is detected in the kidneys. In contrast, no histopathologic changes and no infectious virus were found in the kidneys of the AA57-infected mice. These results indicate that AA57 does not replicate well in the kidneys of ICR mice, in contrast with the situation in human HFRS cases. Further investigations are needed to elucidate why strain AA57 does not affect the kidneys of ICR mice.

In the AA57-inoculated ICR mice, although pathologic changes were observed in the lungs, severe destruction of alveolar or endothelial cells was not observed. In addition, hantavirus antigens were scarcely detected in the endothelial cells. The morbidity and mortality rates did not correlate well with the dosages of inoculated virus. In addition, 2-week-old BALB/c inbred mice that were inoculated with AA57 did not show any symptoms (data not shown). These findings suggest that immunologic factors and phenotypes of the infected mice may be important for the development of the disease. Previous reports have described how the neurovirulence of HTNV varies with mouse strain and age (Kariwa et al., 1995; Wichmann et al., 2002). The human HLA haplotype has been associated with the clinical course of acute PUUV infection (Mustonen et al., 1996).

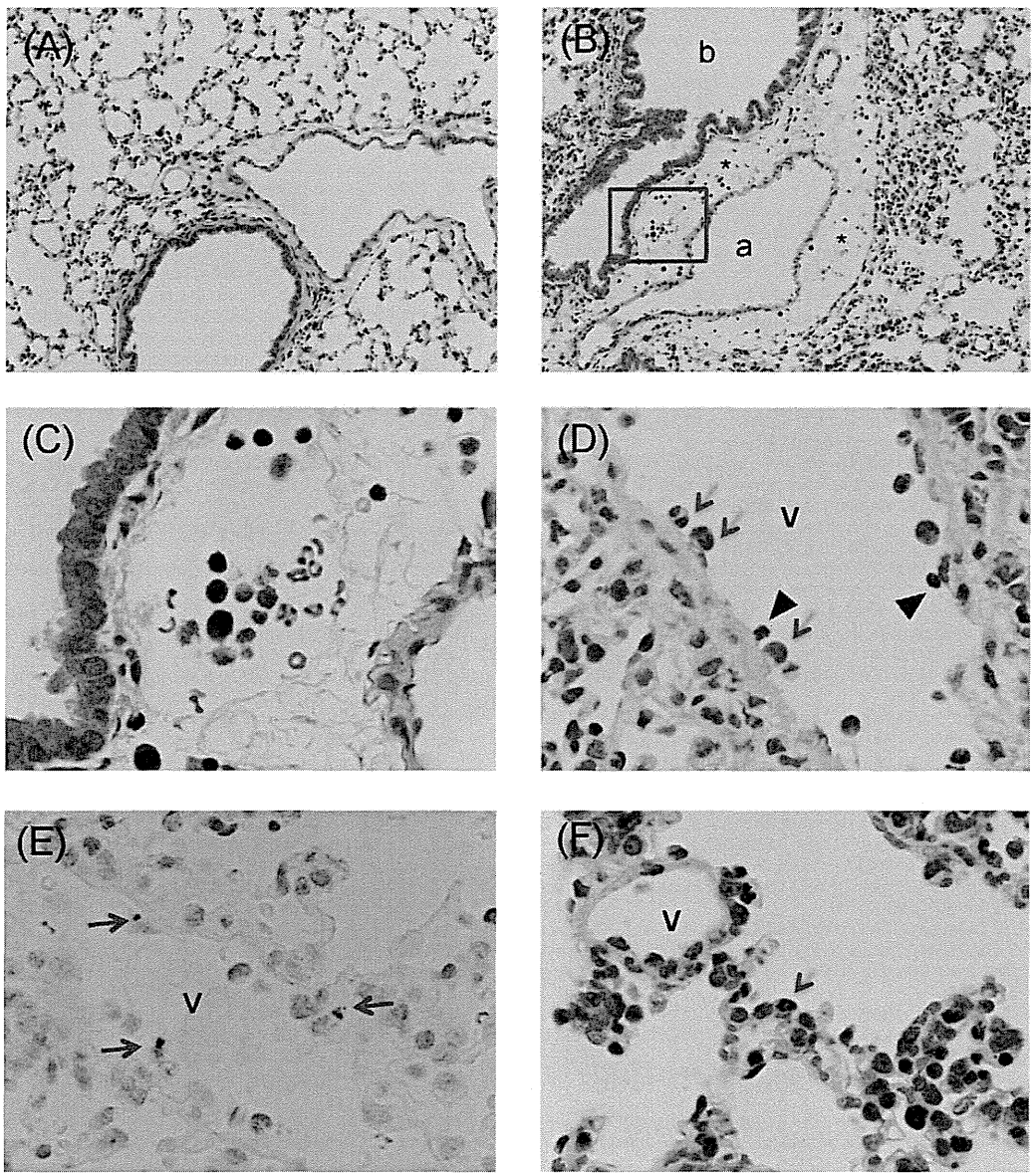

Fig. 3. Histopathological findings in the lungs of 2-week-old ICR mice after hantavirus inoculation. The lungs of mock infected mice (A) and AA57-inoculated mice (B-F) on 8 d.p.i. (A–D and F) Hematoxylin–eosin staining. and (E) immunohistochemistry for hantavirus antigen. a, Artery; b, bronchiole; v, vein. Mild perivascular edema with slight cell infiltration (asterisks) was seen around middle size artery (B). Slight mononuclear cell infiltrations, small hemorrhage, and precipitation of fibrin were found in the edema (C). Degenerated endothelial cells (arrowhead) and adhesion of neutrophils (red arrow) were observed in the middle size vein (D). Virus antigen positive cells were seen in the lesion (E). Slight neutrophils and mononuclear cells infiltration (red arrow) were presented in the alveolar wall (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Mice inoculated with strain cl-1 showed no symptoms, although strain AA57 and strain cl-1 are genetically and serologically similar (amino acid differences: nucleocapsid, 1.2%; glycoproteins, 2.1%; and RNA polymerase, 1.4%; Kariwa et al., unpublished data). This suggests that small genetic differences between strain AA57 and cl-1 (e.g., those that affect the functions of viral proteins in infected mice) confer differences in HTNV pathogenicity for mice.

In summary, we report on an animal model of old-world hantavirus infection that leads to pulmonary disease. The symptoms observed in this murine model resemble those of HPS and, in certain aspects, those of HFRS. As an experimental animal, mice have several advantages for studies of virus-host interactions. This model may be a useful model for analyzing the pathogenesis of HFRS and hantavirus infections.

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NOTE *Virology*

Application of Truncated Nucleocapsid Protein (N) for Serotyping ELISA of Murinae-Associated Hantavirus Infection in Rats

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ABSTRACT. Truncated recombinant nucleocapsid proteins (trNs) that lack N-terminally located cross-reactive epitopes of four Murinae rodent-associated hantaviruses, Seoul virus (SEOV), Thailand virus, Hantaan virus (HTNV) and Dobrava-Belgrade virus, were produced by using a baculovirus expression system. ELISA with the trNs as antigens enabled serotyping of immune sera from rats experimentally inoculated with the corresponding hantaviruses with cut-off OD values of 60% of those of whole N of HTNV. The trN-based ELISA could serotype 12 out of 13 sera obtained from wild rodents (*Rattus norvegicus*) naturally infected with SEOV using the 60% cut-off value. These results indicate that screening with whole N followed by serotyping with trNs using a cut-off OD value of 60% of that of whole N is a useful method for serological surveillance of Murinae-associated hantavirus infection among rodents.

KEY WORDS: hantavirus, serotyping ELISA.

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Hantaviruses belong to the genus *Hantavirus* in the family *Bunyaviridae* [24]. Hantaviruses are negative-stranded RNA viruses with three segmented RNAs designated small (S), medium (M) and large (L); they encode a nucleocapsid protein (N), envelope glycoproteins (Gn and Gc) and an RNA-dependent RNA polymerase (L protein), respectively [5, 23].

So far, 23 virus species have been registered within the *Hantavirus* genus [22]. Various species of small mammals, mainly rodents and soricomorphs, act as natural reservoirs, as they are infected persistently without any sign of disease [8, 14, 22]. It is generally believed that hantaviruses coevolved with their reservoir animals [7, 19]. As a consequence, hantavirus species have their own predominant reservoirs.

The members of the *Hantavirus* genus contain causative agents of two rodent-borne febrile illness in humans, hemorrhagic fever with renal syndrome (HFRS) in the Old World and hantavirus pulmonary syndrome (HPS) in the New World [22]. Among the Old World hantaviruses, Puumala virus (PUUV), Seoul virus (SEOV), Thailand virus (THAIV), Hantaan virus (HTNV) and Dobrava-Belgrade virus (DOBV) are considered to be causative agents of HFRS [22]. The predominant reservoir rodent species of PUUV is *Myodes glareolus*, which is classified into subfamily Arvicolinae. The predominant reservoir rodent species of SEOV, THAIV, HTNV and DOBV are *Rattus norvegicus*, *Bandicota indica*, *Apodemus agrarius* and *A. flavicollis*, respectively [13, 18, 20], and they are all classified into subfamily Murinae. Despite the close associations between rodent hosts and hantaviruses, exceptional host switching and spillover cases have been reported [26, 27]. Recent studies have shown that *R. tanezumi* in Indonesia and *R. rattus* in Cambodia possessed THAIV-like hantaviruses [20, 21]. Furthermore, prevalence of a novel HTNV was reported among laboratory rats (*Rattus norvegicus*) in China [30]. In our previous study, Da Bie Shan virus, an HTNV-like hantavirus, was detected in *Niviventer confucianus*, a sister genus of *Rattus* [26]. These epizootiologic findings suggest that *Rattus* species rodents act as reservoir animals of various hantaviruses. Therefore, it is important to serotype infected hantaviruses among wild rats to understand the ecology of hantavirus in nature.

Since SEOV, THAIV, HTNV and DOBV possess strong antigenic cross-reactivity in N [3, 4, 28], a neutralization test is the only serological method to define the serotypes [2, 12]. We have developed simple and rapid diagnosis for serotyping ELISA of SEOV, THAIV, HTNV and DOBV infection by using a truncated N that lacks cross-reactive epitopes within the 1–49 N-terminal aa of the N (trN) as an antigen for human sera [1, 15, 29]. In this study, we examined the applicability of the trNs for serotyping in *Rattus* sera by using immune sera from experimentally inoculated rats and naturally infected Norway rats.

SEOV strain SR-11 [9], THAIV strain thai749 [6], HTNV strain 76–118 [11] and DOBV strain Saaremaa [16] were used as representative strains of SEOV, THAIV, HTNV and DOBV serotypes, respectively. Viruses were prepared by the method previously described [1, 15]. Six-week-old WKAH/hkm female rats (SLC, Hamamatsu, Japan) were inoculated intraperitoneally with SEOV (6×10^4 FFU/animal), THAIV (4×10^2 FFU/animal), HTNV (3×10^4 FFU/animal) and DOBV (2×10^4 FFU/animal). Two

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rats were inoculated with each virus. Serum specimens were collected from the tail vein at the time of inoculation (day 0) and at 3, 6, 9, 13, 16, 19, 23, 27, 34, 40 and 49 days post inoculation (dpi) from the same rats. Animal experiments were performed after obtaining permission from the Institutional Animal Care and Use Committee of Hokkaido University. Experiments involving virus infections were performed in a BSL-3 facility.

Serotyping ELISA for IgG antibody was performed essentially by the same procedure as that reported previously [1, 10, 15, 17]. Briefly, truncated N, which lacked 49 N-terminal aa of the N (trN) of SEOV, THAIV, HTNV and DOBV and the whole N of HTNV (whole N) were expressed by a recombinant baculovirus system and used as antigens for ELISA. Serotyping ELISA for IgM was performed as described previously [10] by using polyclonal goat anti-rat IgM (KPL) as a capture antibody. TrN of SEOV, THAIV and HTNV and whole N of HTNV were used as antigens. Serum specimens were examined at 1:400 dilution.

Changes in ELISA OD values of IgG of rats after inoculation are shown in Fig. 1. The ELISA OD values for whole N of HTNV began to appear from 6 to 13 dpi in all rats inoculated with homologous and heterologous viruses and increased to a plateau level until 49 dpi. The higher cross-reactivity of whole N to immune sera from heterologous virus-infected rats indicated the applicability of the whole N of HTNV as a screening antigen to detect a Murinae-derived hantavirus antibody. In all of the eight rats, ELISA OD values of immune sera for homologous trNs were higher than those for heterologous trNs on and after 16 dpi. The OD values for heterologous trNs were <60% of those for whole Ns on or after 16 dpi. However, the type of infected virus could not be differentiated in certain periods, especially in the acute phase, according to our criteria.

The trN-based serotyping ELISA was applied to sera from Norway rats infected naturally with SEOV. A total of 13 sera were obtained from *Rattus norvegicus* captured in Hai Phong Port, Vietnam, and SEOV infection was confirmed by detecting the SEOV genome and indirect immunofluorescent assay (IFA) antibody by methods previously described [25, 27]. The rates of OD values of trNs to those of whole N of HTNV in sera were examined at serum dilutions of 1:200, 400, and 800. As shown in Fig. 2, 4 sera (#1 to #4), which possessed IFA antibody titers of 1:800, could be serotyped at a serum dilution of 1:200 according to the cut-off OD value of 60% of the OD value of whole N. The rest of the serum specimens, except serum #10, were serotyped at 1:400 or 1:800 dilutions, as higher cross-reactivity was obtained at 1:200 dilution. Particularly, serum specimens with IFA antibody titers of more than 1:6,400 required higher dilution (1:800) to be serotyped. The higher cross-reactivity might be due to the presence of high avidity antibody to common epitopes on N produced in persistently infected rats. Nevertheless, our results indicated that the trN-based ELISA was applicable for serotyping of sera from naturally infected Norway rats. Further studies with more rodent sera are required to determine reasonable serum dilutions and cut-off values for serotyping.

In our previous seroepidemiological study in Vietnam, 3 of 12 hantavirus antibody-positive human sera showed very low OD values for trNs of SEOV, THAIV and HTNV, although they reacted strongly to whole N [25]. These unserotyped cases suggested the possibility of the existence of a novel species of hantavirus. In the present study, the OD values of the trN antigens for most of the homologous sera were more than 60% of those for whole N antigen, while the OD values of trN antigens for heterologous sera were less than 60% (Fig. 1). Therefore, novel hantavirus infection would be defined if the OD values of a serum specimen for all the trN antigens are less than 60% of those of whole N antigens. Further studies with more rodent sera serotyped by a neutralization test are needed to determine more reasonable cut-off values for distinguishing a previously known hantavirus infection from a novel hantavirus infection.

We also attempted to apply the trNs for IgM serotyping ELISA. As shown in Fig. 3, ELISA IgM OD values for whole N were transiently increased in SEOV-infected rats, S1 (6 dpi) and S2 (9 and 13 dpi). However, very low reactivities were observed with trNs of SEOV, THAIV and HTNV. The lack of success using trNs for IgM serotyping ELISA is probably due to the general characteristics of IgM antibody, which is broadly reactive with low affinity.

In summary, screening with whole N followed by the trN-based serotyping ELISA is useful in epizootiological surveillance of hantavirus infection among rodents for detecting host switching and spillover as well as antigenically distinct hantavirus infections.

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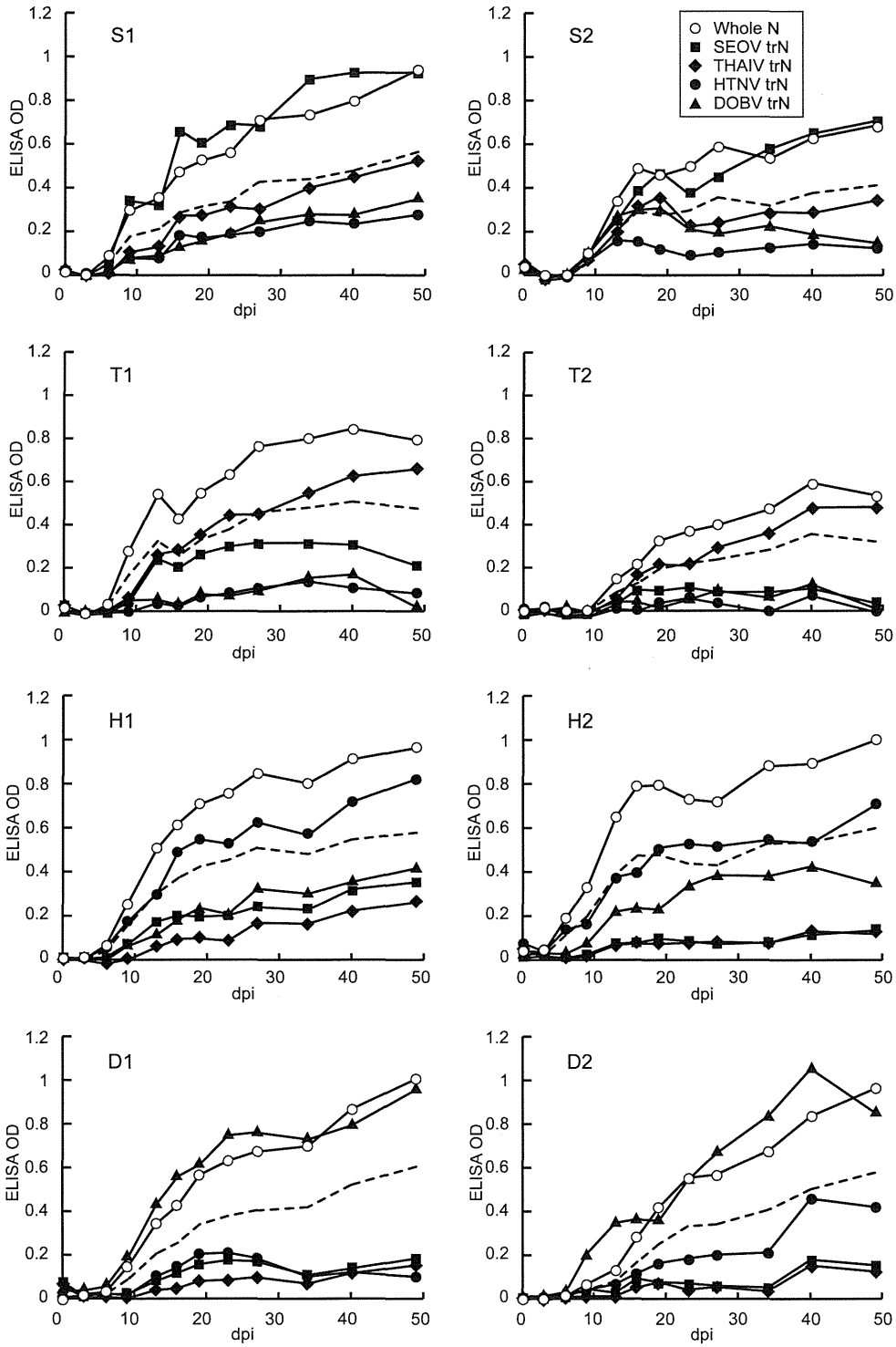

Fig. 1. Changes in ELISA OD values of IgG of rats inoculated with SEOV (S1 and S2), THAIV (T1 and T2), HTNV (H1 and H2) and DOBV (D1 and D2). Open circles show OD values for whole N of HTNV, and black symbols show OD values for trN of SEOV (square), THAIV (diamond), HTNV (circle) and DOBV (triangle). Broken lines show the 60% levels of OD values for whole N.

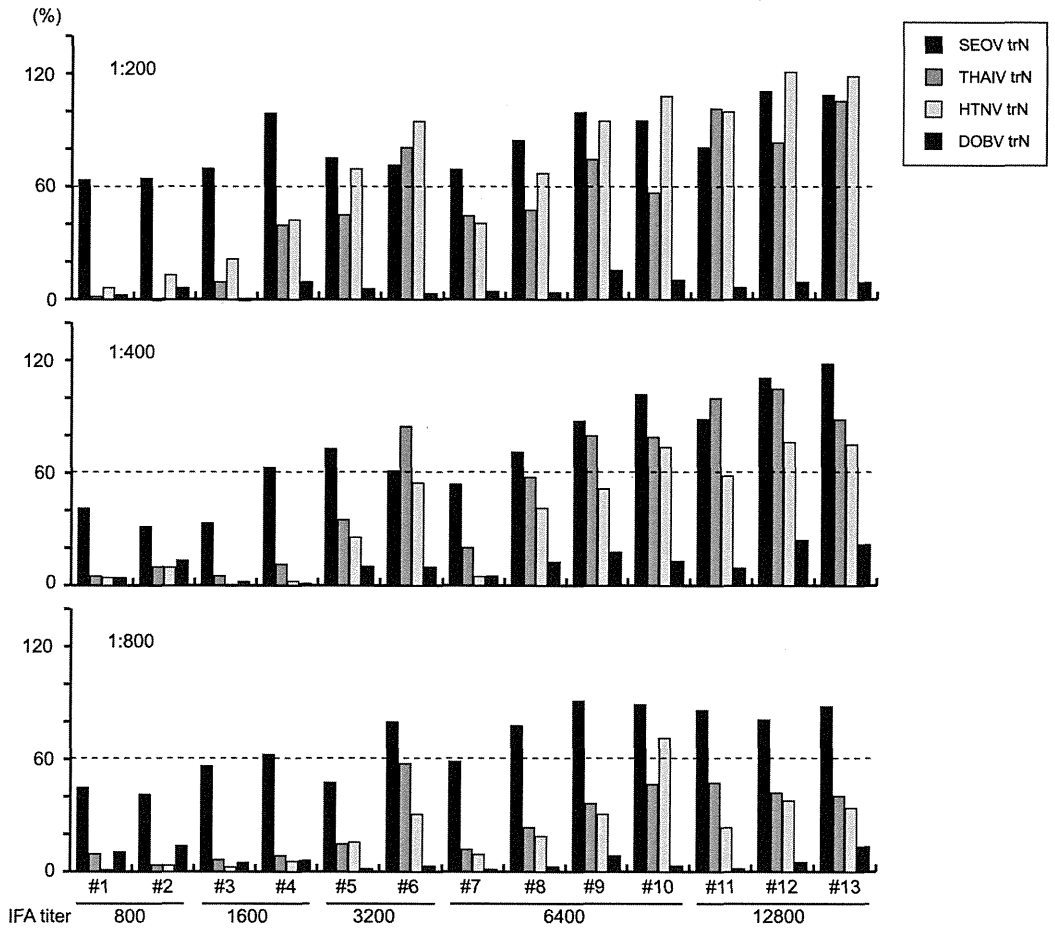

Fig. 2. Rates of OD values of trNs to those of whole N of HTNV. The thirteen sera from rats infected with SEOV were applied to the serotyping ELISA at dilutions of 1:200 (upper panel), 1:400 (middle panel) and 1:800 (lower panel).

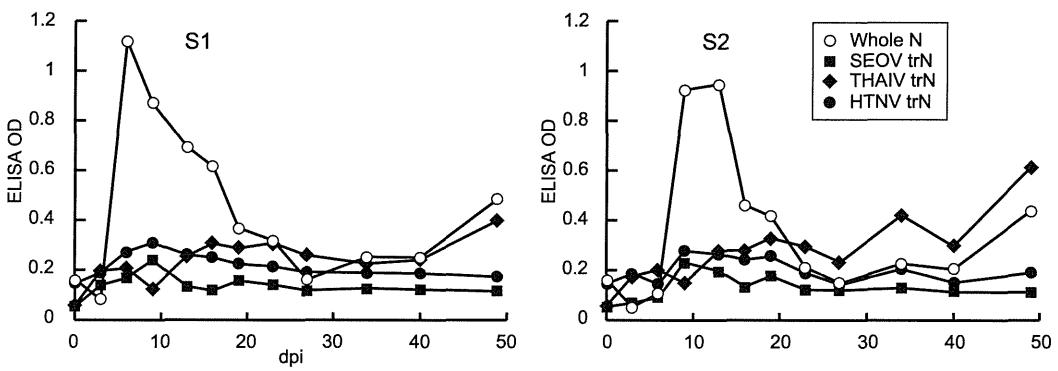

Fig. 3. Changes in ELISA OD values of IgM of rats inoculated with SEOV (S1 and S2). Open circles show OD values for whole N of HTNV, and black symbols show OD values for trN of SEOV (square), THAIV (diamond) and HTNV (circle).

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ORIGINAL ARTICLE

Novel serological tools for detection of Thottapalayam virus, a Soricomorpha-borne hantavirus

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Abstract We developed serological tools for the detection of hantavirus-specific antibodies and hantavirus antigens in shrews. The work was focussed to generate Thottapalayam virus (TPMV)-specific monoclonal antibodies (mAbs) and anti-shrew immunoglobulin G (IgG) antibodies. The mAbs against TPMV nucleocapsid (N) protein were produced after immunization of BALB/c mice with recombinant TPMV N proteins expressed in *Escherichia coli*, baculovirus and *Saccharomyces cerevisiae*-mediated expression systems. In total, six TPMV N-protein-specific mAbs were generated that showed a characteristic fluorescent pattern in indirect immunofluorescence assay (IFA) using TPMV-infected Vero cells. Out of the six mAbs tested, five showed no cross-reaction to rodent-associated hantaviruses (Hantaan, Seoul, Puumala, Tula, Dobrava-Belgrade and Sin Nombre viruses) in IFA and enzyme-linked immunosorbent assay (ELISA), although one mAb reacted to Sin Nombre virus in IFA. None of the mAbs cross-reacted with an amino-terminal segment of the shrew-borne Asama virus N protein. Anti-shrew-IgG sera were prepared after immunization of rabbits and BALB/c-mice with protein-G-purified shrew IgG. TPMV-N-protein-specific sera were raised by immunisation of Asian house shrews (*Suncus murinus*) with purified yeast-expressed TPMV N protein. Using these tools, an indirect ELISA was developed to detect TPMV-N-protein-specific antibodies in the sera of shrews. Using an established serological assay, high TPMV N protein specific antibody titres were measured in the sera of TPMV-N-protein-immunized and experimentally TPMV-infected shrews, whereas no cross-reactivity to other hantavirus N proteins was found. Therefore, the generated mAbs and the established ELISA system represent useful serological tools to detect TPMV, TPMV-related virus antigens or hantavirus-specific antibodies in hantavirus-infected shrews.

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Introduction

Hantaviruses, which are members of the family *Bunyaviridae*, genus *Hantavirus*, are negative-sense, single-stranded RNA viruses with three segments, designated large (L), medium (M), and small (S), which encode an RNA-dependent RNA polymerase, two envelope glycoproteins (G1/Gn and G2/Gc) and a nucleocapsid (N) protein [11]. Rodent-borne hantaviruses are the causative agents of two viral zoonoses: haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). In general, each hantavirus species is associated with a single predominant mammal species or a closely related species of the same genus that serves as its natural reservoir. This close relationship and the congruencies of phylogenies between hantaviruses and their hosts, could be explained by a virus-host co-evolution or, alternatively, by host-switching events followed by host adaptation processes [31, 32, 36].

Thottapalayam virus (TPMV), which was isolated from an Asian house shrew (order Soricomorpha, family Soricidae, *Suncus murinus*) that was captured during a survey for Japanese encephalitis virus in Tamil Nadu, India in 1964, for a long time represented the only exception of a non-rodent associated hantavirus [5]. Only recently was the complete genome of TPMV determined and the host association with *S. murinus* confirmed [38]. Recently, in addition to TPMV, numerous shrew- and mole-associated hantaviruses have been reported from America, Europe, Asia and Africa [1, 2, 12–14, 16, 37, 39, 40, 46]. These investigations were facilitated by the development of a broad-spectrum pan-L RT-PCR assay [15]. Previous investigations were mainly based on RT-PCR approaches. Therefore, little is known about the pathogenicity, the course of infection, or the humoral immune response in infected hosts. Moreover, the antigenic characteristics of TPMV and the new Soricomorpha-borne hantaviruses and their differences to those of rodent-borne hantaviruses have not yet been investigated. As evidenced by nucleotide (nt) and amino acid (aa) sequence analysis of the full-length S, M, and L segments and the encoded proteins, TPMV is the most genetically divergent from all other hantaviruses [38, 43]. In addition, either very low or no antigenic cross-reactivity has been observed between TPMV and other hantaviruses [6]. The current serological detection system for TPMV-specific antibodies in human and shrew sera is based on a monoclonal antibody (mAb) E5/G6 capture enzyme-linked immunosorbent assay (ELISA) using a recombinant TPMV N fusion protein with an E5/G6 epitope. Using this assay, a TPMV infection in a Laotian immigrant with a febrile illness was detected, and anti-TPMV antibodies in two out of 14 Asian house shrews captured in Indonesia were also detected [30].

For rodent-borne hantaviruses, a large panel of N- and glycoprotein-specific mAbs has been developed [3, 7, 8, 10, 17–24, 26, 34, 35, 41, 45, 49–51], but none are available for Soricomorpha-associated hantaviruses. In addition, immunological or serological investigations in shrews are also limited by the lack of commercially available species-specific secondary antibodies. For these reasons, our study was focussed on two areas: generation of TPMV-specific mAbs for detection of TPMV in infected cells and development of anti-shrew IgG antibodies applicable as species-specific secondary antibodies in immunological assays. The generation of the TPMV antigen using three different expression systems should prove to be valuable for generating TPMV-specific mAbs that are able to recognize the native antigen in virus-infected cells.

Materials and methods

Generation of recombinant full-length and truncated N proteins in *Escherichia coli* and insect cells

The full-length recombinant N protein of TPMV (accession no. AY526097) was expressed previously in both *E. coli* and insect cells using a baculovirus vector [30]. To construct plasmids expressing truncated N proteins spanning aa 1-80, 1-177, and 1-311 in *E. coli*, the corresponding coding regions were amplified by PCR using the following primers (restriction sites are given in capital letters): forward primer TPMVNATG#637, 5′-ttc aGA ATT Cga tga ctc aag gga aaa tga ctc ccg aag a; reverse primers TPMV290, 5′-ggC TCG Aga gca agc ata gct tgc ccg g, TPMV580, 5′-atC TCG AGg tcc tcc atg cat gag tca t; TPMV980, 5′-aaC TCG AGt ggg gtg gct tct gac tca a. To express a truncated N protein of shrew-borne Asama virus (ASAV; accession number EU929070), a region of the S segment encoding the amino-terminal 103 aa of the N protein was amplified by PCR using the primer pair ASAVs-ATG-EcoR I (5′-ata GAA TTC atg gac aac att gag gac atcc) and ASAVs-tr-NP-103-Xho I-TAG (5′-ata CTC GAG cta gat tgt att acc ata ccg c). The TPMV- and ASAV-derived PCR products that were obtained were ligated to the plasmid vector pET43.1b (Novagen, Merck, Darmstadt, Germany). The recombinant expression plasmids were used to transform competent BL21 cells (Invitrogen, Life Technologies, New York, USA). Synthesis of recombinant proteins was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG: Wako, Osaka, Japan). Truncated N proteins fused to Nus-tag (491 amino acids) were extracted from *E. coli* and purified using a His-trap column according to the instructions of the manufacturer (Amersham, GE Healthcare Bio-Sciences, New York,

USA). An attempt to express the entire ASAV N protein in *E. coli* failed (data not shown).

Construction of a yeast expression plasmid and yeast expression of TPMV and other hantavirus N proteins in *Saccharomyces cerevisiae*

The entire open reading frame of the TPMV N protein was amplified by PCR using plasmid pFastBac–TPMV N [30] as a template with primers TPMV-5′ (5′-gat TCT AGA act caa ggg aaa atg act ccc gaa gag-3′) and TPMV 3′ (5′-gat TCT AGA tta cag ttt aat agg ctc ctg act tga-3′), which resulted in the addition of XbaI restriction sites to facilitate subsequent cloning. The XbaI-digested PCR product was cloned into XbaI-linearized yeast expression plasmid pFX7-His6 [33]. DNA sequence determination revealed a few nt exchanges compared to the published sequence (accession no. AY526097), which led to four aa exchanges (Q20K, A102T, G346S, G357A). For expression, *S. cerevisiae* strain gcn2 was transformed with a pFX7-derived expression plasmid encoding the TPMV N protein (pFX7-His6-TPMV N). Cultivation of yeast cells and expression and purification of TPMV, Tula virus (TULV), Puumala virus (PUUV) and Dobrava-Belgrade virus (DOBV) N proteins were performed as described previously [27, 28, 33].

Preparation of TPMV-N-protein specific monoclonal antibodies

Female BALB/c mice (Japan SLC, Inc, Hamamatsu, Japan and Taconic Europe, Ry, Denmark) were immunized four times with different recombinant TPMV N protein antigens (Table 1). Three days prior to fusion, the mice received a final immunization, and spleen cells from immunized mice were fused with myeloma P3X63Ag8U1 or SP2/0 cells using PEG1500 (Sigma-Aldrich, St. Louis, USA). Hybridoma supernatants were screened for TPMV-N-protein-specific antibodies in IFA and ELISA. Positive clones were re-cloned twice and TPMV-specific mAbs were further characterized as described below. The mAbs EB5, ED5, 1A3 and B5H9 were selected for subtyping in cell culture supernatant by ELISA using peroxidase-conjugated rabbit anti-mouse IgG-1, IgG-2a, IgG-2b, IgG-3, IgM, IgG (Zymed, Vienna, Austria), Lambda and Kappa (Caltag, Buckingham, UK) (1:5000). The mAbs 2H6 and 1F1 were subtyped using a Mouse Monoclonal Subtyping Kit (AbD Serotec, Kidlington, UK).

Table 1 Characteristics of monoclonal antibodies produced in this study

Clone ID	Expression system used for generation of immunogen	Class/ subclass	ELISA detection with screening antigen
EB5	Baculovirus	IgM	*E. coli*-expressed TPMV N protein
ED5	Baculovirus	IgM	*E. coli*-expressed TPMV N protein
1A3	*Escherichia coli*	IgG1/kappa	*E. coli*-expressed TPMV N protein
B5H9	*Escherichia coli*	IgG*	*E. coli*-expressed TPMV N protein
2H6	*Saccharomyces cerevisiae*	IgG1/kappa	Yeast-expressed TPMV N protein
1F1	*Saccharomyces cerevisiae*	IgG1/kappa	Yeast-expressed TPMV N protein

Enzyme-linked immunosorbent assay, ELISA; Ig, immunoglobulin; N, nucleocapsid; TPMV, Thottapalayam virus

* The subclass and light chain of B5H9 were not defined

Indirect immunofluorescence assay (IFA)

In-house IFAs were performed essentially as described previously [47]. Acetone-fixed, TPMV, HTNV, SEOV, PUUV, TULV, or DOBV-infected Vero cells were used as antigens. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Zymed), was used as a secondary antibody. Commercial IFAs (Euroimmun, Lübeck, Germany) were performed according to the manufacturer's instructions using FITC-conjugated anti-mouse Ig (DakoCytomation, Glostrup, Denmark) as a secondary antibody.

Enzyme-linked immunosorbent assay (ELISA)

Flat-bottomed 96-well plates were coated with purified *E. coli* or insect-cell-expressed TPMV and ASAV N proteins in phosphate-buffered saline (PBS), or with *S. cerevisiae* expressed TPMV, TULV, PUUV and DOBV N proteins in 0.05 M carbonate buffer and incubated overnight at 4 °C. PBS with 0.05 % Tween 20 containing 3 or 1 % bovine serum albumin (Sigma-Aldrich, St. Louis, USA) was added to block unsaturated binding sites, and the plates were incubated for 1 h at 37 °C or room temperature. After washing three times with 0.05 % PBS-Tween, hybridoma supernatants were added, and the plates were incubated for 1 h at 37 °C. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000) and *o*-phenylendiammonium dichloride substrate (Sigma-Aldrich, St. Louis, USA) or 3,3′,5,5′-tetramethylbenzidine (Bio-Rad, Hercules, California, USA). After incubating for 15 min at room temperature, the absorbance at 450 nm was measured.

Western blot test

The reactivity of the mAbs with full-length or truncated recombinant N proteins of TPMV and ASAV was tested in

a western blot test (WB) as described previously [48]. The purified yeast-expressed TPMV N protein was tested in the WB with mAbs raised against PUUV (2C6, 7A5, 5E11, 5C5, [52]; A1C5, [51]; 4C3, 2E12, 5A3, 1C12, [25]), SNV/ANDV (7G2, 4H3, [18]) and HTNV (E5/G6, ECO2, [49]; B5D9, [51]). The synthesis of the Nus fusion proteins was confirmed in the WB by staining with anti-NUS-tag mAb (Novagen).

Purification and biotinylation of the immunoglobulin fraction

The mAbs ED5 (IgM) and 1A3 (IgG) were purified using an IgM purification kit (Pierce, Thermo Scientific, Rockford, Illinois, USA) using protein-A Sepharose column chromatography and a MAPS II kit (Bio-Rad), respectively. The purified mAbs were biotinylated using a Biotin-AC5-OSu kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Competitive binding to *E. coli*-and insect-cell-expressed TPMV N proteins in ELISA

The relative binding avidity of mAbs to solid-phase recombinant antigen was evaluated by indirect ELISA. Flat-bottomed 96-well microtitre plates were coated with serial twofold dilutions of purified truncated N protein in PBS and then incubated overnight at 4 °C. Nonspecific binding was blocked with PBS containing 3 % bovine serum albumin for 1 h at room temperature. The plates were then washed three times with 0.05 % PBS-Tween. Dilutions of each biotin-labeled mAb were added and incubated for 1 h at room temperature. After additional washing, HRP conjugate (1:2000; Prozyme, Hayward, Canada) was added to the wells and incubated for 1 h at room temperature. Thereafter, *o*-phenylendiammonium dichloride substrate was added, and the plates were incubated for 30 min at room temperature. The relative binding avidity was defined as the amount of antibody required to yield an A450 value ranging from 0.4 to 1.4. Absorbance values at 405 nm were compared with those in the absence of a competitive antibody. For use in competitive binding assays, the serial fivefold dilutions of each unlabeled antibody were added to 96-well plates coated with antigen as described above. After a one-hour incubation at room temperature, the plates were washed, and binding of biotinylated mAbs was detected with avidin-peroxidase as described earlier.

Establishment of an ELISA to detect TPMV-specific antibodies in shrew sera

To establish this diagnostic tool, anti-shrew-IgG antisera were first prepared. Briefly, two BALB/c mice and two rabbits were immunized five times at four-week intervals with 200 μg protein-G-purified shrew IgG. To obtain shrew anti-TPMV-N sera, three Asian house shrews were immunized four times at four-week intervals with 50 μg of dialyzed TPMV N protein, and blood was taken after each immunization. In addition, two Asian house shrews were inoculated intraperitonially with 10^4 FFU of TPMV (Arikawa et al. unpublished data).

To test the shrew anti-TPMV-N-protein antisera, the anti-shrew-IgG, and the sera from experimentally TPMV-infected shrews, an indirect cross-titration ELISA was performed. Briefly, plates were coated as described earlier. The different shrew sera were tested on plates coated with TPMV, TULV, PUUV or DOBV N protein. As secondary antibodies, the anti-shrew-IgG antisera were cross-titrated. To determine the amount of antibody bound, an anti-mouse-IgG HRP-conjugate was used as a third antibody. All incubations were done for 1 h at 37 °C, and between the single incubation steps, the plates were washed three times as described above.

Results

For the generation of TPMV-N-protein-specific mAbs, the entire N protein of 435 aa residues was expressed using *E. coli* and baculovirus-mediated insect cell systems [30] and a yeast expression system. The high-level yeast expression of the entire His-tagged TPMV N protein resulted in a protein of the expected molecular weight (data not shown). This purified protein did not react in the immunoblot with any of the PUUV-specific mAbs (2C6, 7A5, 5E11, 5C5, A1C5, 4C3, 2E12, 5A3, 1C12), the SNV/ANDV-specific mAbs (7G2, 4H3) or the HTNV-specific mAbs E5/G6, ECO2, B5D9 (data not shown).

Six different mAbs were generated that recognized the TPMV N protein expressed in *E. coli*, insect cells and *S. cerevisiae*, and these were characterized by ELISA, IFA and WB (Tables 1, 2). The subtyping of the generated mAbs showed that the clones EB5 and ED5 were of the IgM class, whereas the remaining clones belong to the IgG class (Table 1). All six mAbs showed a characteristic cytoplasmic fluorescent pattern in IFA when using an acetone-fixed smear of TPMV-infected Vero cells and recombinant TPMV N protein expressed in insect High Five cells (Table 2). Their specificity was confirmed in ELISA and IFA against N proteins from rodent-associated hantaviruses (Hantaan virus, HTNV; Seoul virus, SEOV; PUUV; TULV; DOBV; Sin Nombre virus, SNV). Only the mAb ED5 was found to have cross-reactivity to SNV in IFA (Table 2). None of the TPMV-specific mAbs cross-reacted in the ELISA and WB with an amino-terminal segment of the shrew-borne ASAV N protein (Table 3).

Table 2 Cross-reactivities of monoclonal antibodies with different hantaviruses in indirect immunofluorescence assay (IFA) using hantavirus-infected Vero cells and insect cells expressing hantavirus nucleocapsid (N) protein

Clone ID	TPMV		HTNV	SEOV	PUUV	TULV	SNV*	DOBV
	Infected Vero cells	N protein expressed in High Five cells	Infected Vero cells					
EB5	+	+	−	−	−	−	−	−
ED5	+	+	−	−	−	−	+	−
1A3	+	+	−	−	−	−	−	−
B5H9	+	+	−	−	−	−	−	−
2H6	+	+	−	−	−	n.d.	−	−
1F1	+	+	−	−	−	n.d.	−	−

+ positive, − negative, n.d. not done, TPMV Thottapalayam virus, HTNV Hantaan virus, SEOV Seoul virus, PUUV Puumala virus, TULV Tula virus, SNV Sin Nombre virus, DOBV Dobrava-Belgrade virus

* Recombinant N protein of SNV was expressed by baculovirus vector in High Five insect cells

Table 3 Reactivities of monoclonal antibodies against *E. coli*-expressed full-length and truncated Thottapalayam virus (TPMV) and Asama virus (ASAV) nucleocapsid (N) proteins in ELISA and western blot test

Clone ID	ELISA					Western blot test				
	TPMV N (aa)				ASAV N (aa)	TPMV N (aa)				ASAV N (aa)
	1-80	1-177	1-311	1-435	1-103	1-80	1-177	1-311	1-435	1-103
EB5	−	−	−	+	−	−	n.d.	n.d.	−	−
ED5	+	+	+	+	−	+	+	+	+	−
1A3	+	+	+	+	−	+	+	+	+	−
B5H9	−	−	−	+	−	−	n.d.	n.d.	−	−
2H6	+	+	+	+	−	+	+	+	+	−
1F1	+	+	+	+	−	−	n.d.	n.d.	−	−

aa amino acid position, + positive, − negative, n.d., not done

Four of the six mAbs that were generated were reactive in ELISA with *E. coli*-derived truncated TPMV N proteins including the amino-terminal 80 aa (Table 3). Three of these four mAbs (1A3, ED5 and 2H6) showed an identical pattern of reactivity in ELISA and WB (see Fig. 1; Table 3), whereas mAb 1F1 failed to detect the full-length and truncated N proteins in the WB. The two remaining mAbs, EB5 and B5H9, reacted with the full-length TPMV N protein in the ELISA but not in the WB. The relative binding avidity of the biotin-labeled mAbs (1A3, ED5), tested by avidin-biotin indirect ELISA, showed different competitive effects when tested with unlabeled mAbs to recombinant TPMV N protein (Fig. 2). Based on the different levels of competition, two groups were defined, one comprising mAbs 1A3 and ED5, and the other comprising EB5 and B5H9.

Purified shrew IgG from *S. murinus* was used to produce anti-shrew-IgG sera in two BALB/c mice and two rabbits. TPMV-N-protein-specific shrew control sera were produced in *S. murinus* by immunization with the yeast-expressed TPMV N protein. The specific titres of these

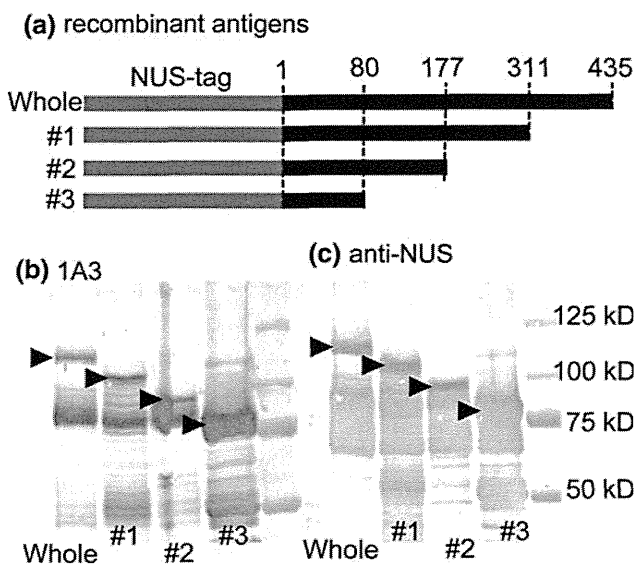

Fig. 1 Schematic representation of the structure (**a**) and western blot reactivity of full-length and truncated Thottapalayam virus nucleocapsid protein derivatives with TPMV-specific monoclonal antibody (mAb) 1A3 (**b**) and anti-NUS mAb (**c**)

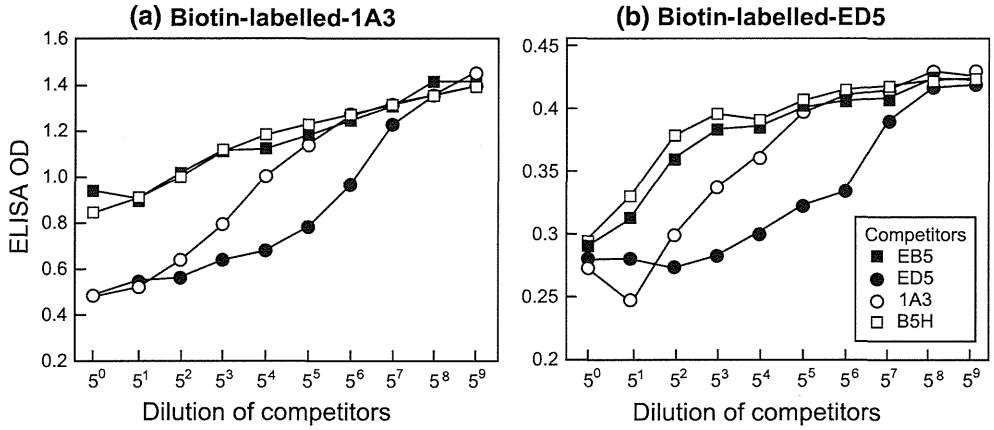

Fig. 2 Competitive binding assay with biotin-labeled mAbs 1A3 (**a**) and ED5 (**b**). Absorbance values (OD) at 405 nm are shown on the y-axis and are compared with those in the absence of competitive antibody. Dilutions of the competitive antibodies are shown on the x-axis. The relative binding avidity was defined as the amount of antibody required to yield an A450 of 0.4–1.4. Purified mAbs (1 mg/ml) were diluted from 1:5 to 1:510 and added as competitors

antisera and of the sera of experimentally TPMV-infected shrews were determined by ELISA. Using an initial cross-titration ELISA, the optimal dilution of the anti-shrew IgG for detection of TPMV-N-protein-specific antibodies in an immunized shrew was selected to be 1:32,000. By using the mouse anti-shrew-IgG at this selected dilution, strong TPMV-specific ELISA reactivities were detected in sera from all three immunized shrews and the two infected shrews (Fig. 3 and data not shown). In addition, in all three shrews, TPMV-N-protein-specific antibodies were already detected after the second immunization (data not shown). The TPMV-N-protein specific antibody titre in one of the immunized animals reached a level of 1:128,000 12 days after the final, fourth immunization. In general, no cross-reactivity to other hantavirus N proteins was found in any of the three TPMV-immunized shrews (data not shown). Only in one of the infected animals (shrew 1), 40 days postinfection, during the high IgG antibody response against TPMV, was a low cross-reactivity against DOBV detected (Fig. 3).

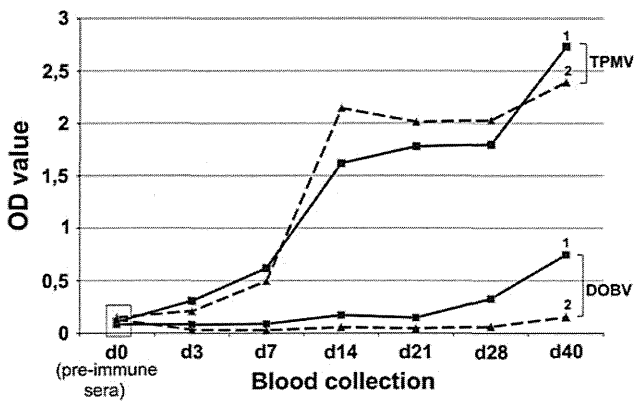

Fig. 3 ELISA reactivity of the sera from two experimentally TPMV-infected Asian house shrews against yeast-expressed TPMV and DOBV N proteins. Absorbance values (OD) of the ELISAs measured at 405 nm are shown on the y-axis. Time points after the experimental infection are given on the x-axis. Reactivities of non-immunizied shrew sera (pre-immune sera) are highlighted by a box. d, days postinfection

Discussion

Hantavirus-specific mAbs have been generated by various approaches [3, 10, 17, 19–24, 26, 34, 35, 41, 45, 49, 51]. Previously, yeast-expressed polyomavirus-derived virus-like particles harbouring 120 aa residues of the PUUV N protein and his-tagged N protein derivatives of SNV and ANDV were used for the generation of mAbs [18, 52]. These mAbs were found to react to the N antigens used for their generation, but also to a different extent to N proteins of other hantaviruses. Most importantly, these mAbs can be used to detect native viral antigen in virus-infected cell lines by IFA and in immunohistochemistry analysis of rodent and human tissue samples [18]. The TPMV-specific mAbs described here were also obtained by immunization with a His-tagged yeast-expressed TPMV N protein, confirming the value of this procedure. The generation of mAbs with similar properties using TPMV N protein from *E. coli* or baculovirus-mediated insect cell expression systems indicates the usefulness of all three different expression systems generating antigens for the production of mAbs. Moreover, the mAbs that were obtained, regardless of the expression system used for the generation of the antigen, were all found to detect native viral antigen in cell cultures.

The hantavirus N protein possesses immunodominant, linear and conformational cross-reactive epitopes within the first 100 aa of its N-terminus [9, 44, 49].

Mapping of the epitopes of N-protein-specific mAbs resulted in the identification of antigenic epitopes between aa 1-45 and 1-120 of PUUV, 166-175 of HTNV, 226-293 of TULV and 244-286 of ANDV and SNV [18, 24, 25, 42, 49]. In line with these observations, some of the TPMV-N-protein-specific mAbs described here are directed against the amino-terminal 80-aa region. The lack of reactivity of mAbs EB5 and B5H9 with the full-length N protein in the WB and with truncated N proteins in the WB and ELISA may suggest a conformation-dependent or discontinous epitope. Similarly, mAb 1F1 did not detect any TPMV N protein derivatives in the WB, suggesting that its binding site is at a conformational or discontinous epitope within the 80-aa amino-terminal region of the N protein. Future epitope mapping studies using linear synthetic peptides may allow localization of the linear epitopes of the mAbs ED5, 1A3 and 2H6 within the aa 1-80 region.

The lack of cross-reactivity of our mAbs to rodent-borne hantavirus N proteins (HTNV, SEOV, PUUV, TULV and DOBV) and ASAV N protein confirmed that TPMV is genetically and antigenetically highly divergent from all other known hantaviruses. In line with this observation, the yeast-expressed N protein of TPMV failed to react with a panel of mAbs raised against N proteins of PUUV, SNV/ANDV and HTNV. This particular feature of TPMV can be explained by the low aa sequence similarity of its N protein to those of other hantaviruses, which is also reflected in the isolated position of TPMV in phylogenetic trees [38, 43]. This lack of cross-reactivity is in contrast to the broad-spectrum cross-reactivity observed for rodent-borne hantavirus N-protein-specific mAbs with N proteins of these hantaviruses.

The mouse and rabbit anti-shrew-IgGs generated in this study are applicable in serological assays for the detection of IgG antibodies in sera of immunized and infected *S. murinus*, as demonstrated. Based on molecular data [29] and chromosome homology [4], *Suncus* represents a separated genus within the family Soricidae, which is closely associated with the genus *Crocidura*. The close relationship of these two genera, in spite of the detection of different new hantaviruses in members of the genus *Crocidura* [16, 40], may suggest that the application of the anti-shrew-IgG generated here is not limited to immunological approaches in *Suncus*. Indeed, an initial WB experiment demonstrated cross-reactivity of the rabbit anti-*Suncus* IgG with purified IgG of *Suncus*, *Crocidura* and *Sorex*, but a lack of reactivity with laboratory mouse IgG (data not shown). Additional efforts are needed to establish and validate serological assays to detect hantavirus-specific antibodies in these shrews by using defined negative and positive control sera. The efficiency of such serological assays could be improved by labelling the anti-shrew-IgGs with HRP, FITC or other fluorescent dyes.

In conclusion, the reactivity of the mAbs generated here in IFA using TPMV-infected cells indicates that these mAbs represent useful tools for detection of TPMV (and antigenically related hantaviruses) in cell culture and tissue of potentially infected animals or humans, where the shrew anti-TPMV-antisera could serve as a positive control. These serological tools will be helpful for discovering novel insectivore-associated hantaviruses and/or characterizing the humoral immune response and antigen expression in hantavirus-infected insectivores.

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Ethical statement Shrews, laboratory mice and rabbits were handled according to the Laboratory Animal Control Guidelines of the Hokkaido University Animal Research Committee in Japan and EU Council Directive 86/609/EEC for the protection of animals used for experiments in Germany.

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