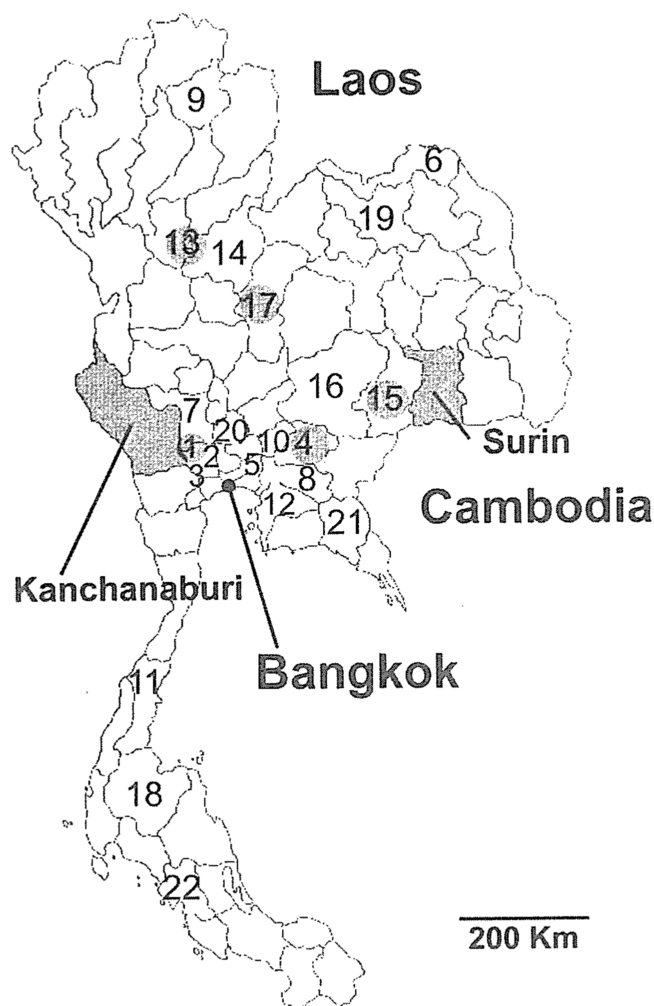


FIGURE 2. Map of Thailand showing the localization of rodent trapping sites. The numbers of the trapping sites correspond to those given in Table 1. The locations of the trapping sites where anti-hantavirus–positive rodents were captured are shown as gray circles. The geographical origin of patient #277 in Surin Province is shown as a gray area. The locality of collection of the *Bandicota indica* used for isolation of THAIV strain Thai749, in the western province Kanchanaburi is given as gray area.

TABLE 2
Trapping sites, collected rodent species and seropositivity for hantavirus

Site no.	Trapping site	Rodent species	No. of seropositives/ No. of animals tested
1	Nakhon Pathom/Bang Len	*Rattus rattus*	0/9
		Bandicota indica	**1/83**
2	Nakhon Pathom/Nakhon ChaiSi	*R. rattus*	0/1
3	Nakhon Pathom/Sam Phran	*R. rattus*	0/1
4	Prachin Buri	*R. losea*	0/7
		B. indica	**2/18**
5	Bangkok Metropolis	*R. norvegicus*	0/16
		R. rattus	0/9
6	Nong Khai	*R. rattus*	0/1
7	Suphan Buri	*R. argentiventer*	0/11
8	Chanchoengsao/Bang Nam Prieo	*R. norvegicus*	0/34
		R. rattus	0/6
		B. indica	0/34
9	Phayao	*R. rattus*	0/1
10	Nakhon Nayok	*R. rattus*	0/1
11	Chumphon/Bang Son (Pathiu)	*R. tiomanicus*	0/97
12	Chon Buri	*B. indica*	0/2
		B. savilei	0/1
		R. rattus	0/2
		R. exulans	0/1
13	Phitsanulok/Phrom Piram	*R. argentiventer*	0/19
		R. losea	0/4
		B. savilei	**1/3**
14	Phitsanulok	*B. indica*	0/7
		R. rattus	0/6
15	Buri Ram	***B. indica***	**2/3**
		R. rattus	0/2
16	Nakhon Ratchasima	*B. indica*	0/2
		R. rattus	0/1
17	Phetchabun	*B. indica*	0/3
		R. rattus	**1/1**
18	Surat Thani	*R. rattus*	0/2
19	Udon Thani	*R. rattus*	0/1
		R. exulans	0/2
20	Ayutthaya	*B. indica*	0/1
		R. rattus	0/1
		R. exulans	0/2
21	Chanthaburi	*R. rattus*	0/1
		R. norvegicus	0/2
22	Trang	*R. rattus*	0/2
		R. exulans	0/2
	All	***B. indica***	5/152 (3.3%)
		B. savilei	1/5 (20.0%)
		R. rattus	1/48 (2.1%)
		R. exulans	0/7
		R. norvegicus	0/52
		R. losea	0/11
		R. argentiventer	0/30
		R. tiomanicus	0/97
	Total		7/402 (1.7%)

The groups in which the positive rodents were detected are shown by the bold-faced type.

other Murinae-associated hantaviruses. These results were corresponding to the previous report from Dr. Chu et al. [3]

Serological survey of rodent sera. In total, serum samples from 402 rodents captured at 22 different sites (Figure 2) were examined for IgG reactive against hantavirus antigens. Serological screening was carried out by ELISA or with an agglutination kit (HANTADIA). As shown in Table 2, 7 of 402 (1.7%) serum samples were antibody-positive. Of the 7 seropositive specimens, 5 were derived from *B. indica*, one from *B. savilei*, and one from *Rattus rattus*. The 5 provinces in which the seropositive rodents were located (i.e., Nakhon Pathom, Prachin Buri, Phitsanulok, Buri Ram, and Phetchabun) are distributed close to Bangkok in the eastern and northern parts of Thailand (Table 2, Figure 2).

To characterize the apparent homologous virus, 5 positive *Bandicota* sera were selected, and FRNT investigations were performed (Table 3). All of the rodent sera showed the highest FRNT titers to THAIV, which indicates that THAIV or THAI-like viruses exist among rodents in Thailand. Two other positive sera, one from *B. indica* and one from *R. rattus*, were not available for the FRNT due to an insufficient amount of serum.

Serological survey of human sera. A total of 260 paired sera were obtained from 260 patients who were clinically diagnosed with leptospirosis but were serologically negative for *Leptospira* antigens. Two paired sera (#53 and #54, #277 and #277/2004) showed positive reactions against the HTNV antigen but negative or very low reactivity against the PUUV antigen (Figure 3A). The ELISA OD values of anti-hantavirus IgG in serum #53 and #54 were 0.309 and 0.398,

TABLE 3

Analysis of human and rodent serum samples in focus reduction neutralization test (FRNT) using *Hantaan* virus (HTNV), Seoul virus (SEOV), and *Thailand* virus (THAIV)

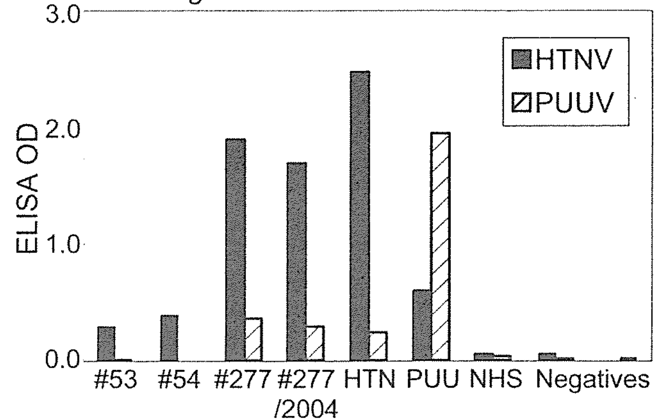
Serum specimen/antiserum	Reciprocal end-point titer† against HTNV	SEOV	THAIV
A172 *(Bandicota indica)*	< 40	< 40	**80**
Bi65 *(B. indica)*	< 40	< 40	**≥ 1280**
Bi74 *(B. indica)*	< 40	< 40	**≥ 1280**
Bi324 *(B. indica)*	< 40	< 40	**80**
Bs355 *(B. savilei)*	< 40	< 40	**160**
Anti-HTNV/mice	**≥ 1280**	< 40	< 40
Anti-SEOV/rat	< 40	**≥ 1280**	80
Negative sample of *B. indica*	< 40	< 40	< 40
#277	40	< 40	**160**
Anti-HTNV	**640**	< 40	< 40
Anti-SEOV	80	**640**	160
NHS‡	< 40	< 40	< 40

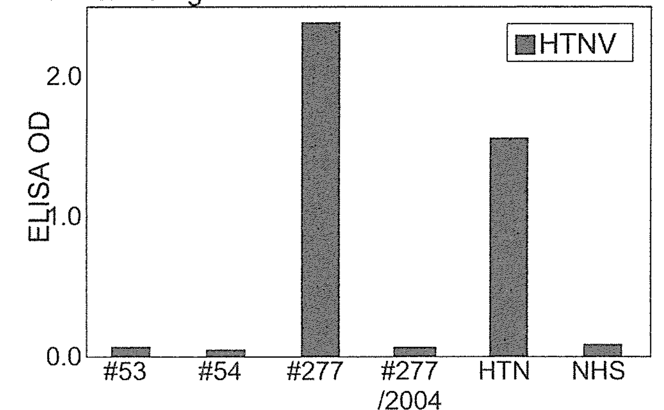
† The highest neutralizing antibody titer for each serum is given in bold.
‡ Serum sample from a healthy human individual.

respectively. The virus-specific IgM was not detected (Figure 3A, 3B). Therefore, this patient may have been infected with a hantavirus many years ago and was suffering from an illness that was unrelated to recent hantavirus infection. Serum #277 contained high concentrations of HTNV-reactive IgM and IgG (Figure 3A, 3B). The #277/2004 serum, which was collected 12 months after the onset of disease, showed high IgG concentration but quite lower IgM concentration. The presence of anti-hantavirus antibodies in serum #277 and #277/2004 was also confirmed by IFA testing using SEOV-infected Vero E6 cells and by Western blotting using recombinant HTNV N protein antigen (data not shown). The detection of HTNV-reactive IgM in patient serum #277 in acute phase but not in convalescent phase may represent an indication of an hantavirus infection.

The serotyping of serum #277 by ELISA revealed reactivities to the truncated N proteins of HTNV, SEOV, and DOBV. However, unlike the positive control sera, serum #277 was equally reactive against the 3 test antigens (Figure 3C). This may indicate that the patient with serum #277 was probably infected with a hantavirus other than HTNV, SEOV, and DOBV. To further characterize the antibody response of serum #277, neutralizing capacity against HTNV, SEOV, and THAIV was tested using FRNT (Table 3). The results indicated that the patient with serum #277 was infected with either THAIV or a THAI-like virus, since the neutralizing antibody titer against THAIV was at least 4-fold higher than that against HTNV or SEOV.

The clinical profile of the patient with serum #277 was consistent with HFRS. The male patient was a 26-year-old farmer from Surin province in northeastern Thailand who was admitted to a mobile "fever unit" with a 40°C fever that had developed over the previous days. The physical examination on admission showed a well orientated patient who suffered from headache, abdominal pain, and conjunctival suffusion. Urine analysis displayed a proteinuria, glucosuria, erythrocyturia, and leukocyturia. The serum level of the alanine aminotransferase was 110 IU/l, the aspartate aminotransferase level was 240 IU/l, and the alkaline phosphatase level was 480 IU/l. The patient showed neither hemorrhages nor oliguria. The serological tests performed for leptospirosis, dengue fever, influenza, and scrub typhus were negative.





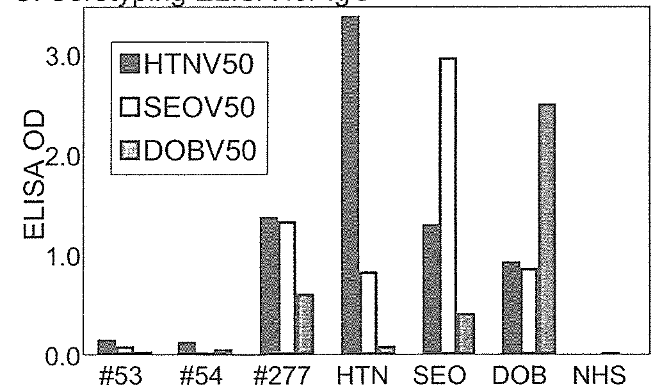


FIGURE 3. Serological screening of patient sera obtained in Thailand. (**A**) Detection of anti-hantavirus IgG in patient sera #53, #54, #277, and #277/2004 by ELISA using the recombinant N protein antigens of HTNV (closed bar) and PUUV (hatched bar). HTN and PUU are positive control sera from patients who were previously confirmed to be infected with HTNV in China and with PUUV in Sweden, respectively. NHS represents a human serum sample that was confirmed as negative for hantavirus-specific antibodies. Typical negative reactions in the initial screening assay are shown as negative. (**B**) Detection of anti-hantavirus IgM by μ-capture ELISA using the recombinant N protein antigen of HTNV. HTN is a positive control serum sample of the acute phase from a patient previously confirmed as being infected with HTNV. (**C**) Serotyping ELISA for human sera #53, #54, and #277 using truncated N antigens (amino acids 50-429) of HTNV (HTNV50), SEOV (SEOV50), and DOBV (DOBV50). Human sera HTN, SEO, and DOB were used as positive controls in the assay and have been characterized previously.

All the methods used showed basically the same tree topology. Therefore, only the ML trees with the Tamura-Nei evolutionary model are shown. We obtained a total of 260 paired sera (acute phase and convalescent phase) from different patients who had a fever of unknown etiology and were found to be seronegative for leptospirosis, dengue fever, influenza, and scrub typhus. The sera were collected in Surin Province of Thailand (Figure 2) in 2002 (454 sera), 2003 (65 sera), and 2004 (1 serum).

DISCUSSION

To further characterize the genetic and antigenic relatedness of THAIV to other Asian hantavirus species, first we cloned and sequenced the almost entire S genome segment of THAIV strain Thai749. Our sequence and phylogenetic analysis based on the nucleotide sequence of the N-protein-encoding ORF on the S segment revealed the same conclusions as previously drawn from complete M segment analyses; THAIV is most closely related to the SEOV species but different enough to appear as a distinct branch on the phylogenetic tree.[5,11] The different aa sequence similarities are reflected also in the reactivity of N-, G1-, and G2-specific MAbs with the corresponding proteins of THAIV and other hantaviruses. In general, our IFA reactivities of all anti-G1 and anti-G2 MAbs with Thai749 are in line with data of ELISA investigations published previously. In contrast, the reactivity of these MAbs in hemagglutination inhibition assay and especially plaque reduction neutralization test (PRNT) differed markedly to our IFA data, most likely due to the differences of the test formats.[3] The definition of THAIV as a distinct species was based on its association to a unique rodent species (i.e., *B. indica*).[4] Recently, THAIV genome was amplified by RT-PCR from *B. indica* captured in central Thailand (personal communication from Alexander Plyushin). In addition, the 2-way cross-neutralization test with sera from a patient and naturally infected bandicoot rats showed more than a 4-fold difference. This is in line with data of PRNT investigations of a rat anti-Thai749 immune serum with a large panel of strains of different hantavirus species.[3] Therefore, this report provides additional support for defining THAIV as a distinct species among the hantaviruses.

Schmaljohn et al.[34] reported that the N proteins of HTNV, SEOV, and PUUV have an overall amino acid sequence identity of 50%. However, certain regions of the N protein, such as that spanning amino acid residues 240–310 display only a low level of sequence identity (about 11%) to each other. Therefore, the corresponding N protein-encoding sequence between nt 760–970 is considered as variable region among hantaviruses. By phylogenetic analysis based on nucleotide sequences between positions 375–959 of S genome segment, which contains the variable region, we found a close genetic association of THAIV with the *R. rattus*-associated Cambodian virus strains. Therefore, it is suggested that THAIV and closely related viruses occur throughout Indochina.

The present study extends our knowledge of the geographical distribution and natural host relationships of hantaviruses indigenous to Thailand. A serological survey of rodent samples originating from 22 provinces of Thailand resulted in the identification of hantavirus-reactive samples of *B. indica* from 3 different provinces located in the central plains and northeastern parts (Khorat plateau) of the country. Determination of the endpoint titers of these sera in neutralization assays using HTNV, SEOV, and THAIV revealed infections with THAIV or a THAI-like virus. Similarly, a serum sample originating from *B. savilei* confirmed the occurrence of THAIV or a THAI-like virus in an additional province in the north of Thailand. However, as no viral genetic material is available from *B. savilei* we can not exclude that the detection of THAIV-reactive antibodies is the result of a spill over infection that might have occurred in this region due to a high infectious pressure of this virus. Our findings on the geographical distribution of THAIV overlap with the observations of Nitatpattana et al.,[35,36] who found hantavirus-infected giant bandicoot rats in the central plains as well as in 3 northeastern provinces of Thailand (Khon Kaen, Buri Ram, Surin). A majority of the hantavirus-positive rodents were collected from rice field habitats.[36] In the latter study the highest seroprevalence was observed in giant bandicoot rats from Khon Kaen, an area that lies at the center of the Khorat plateau, whereas comparative quantities of animals collected further east, from Nakhon Phanom and Kalasin, were all hantavirus negative. Unfortunately, we were not able to collect serum samples of bandicoot rats from southern Thailand. Interestingly, a recent serological study conducted in neighboring Cambodia employing HTNV as antigen (660 rodents) found roof rats, Norway rats, and unidentified *Rattus* species infected with hantaviruses closely related to SEOV, but none of 75 bandicoot rats and 183 Polynesian rats (*Rattus exulans*).[32] Therefore, search for THAIV or THAI-like viruses should be extended to southern provinces as well as neighboring Cambodia.[32] THAIV is antigenically cross reactive to HTNV and SEOV. Therefore, previous seroepidemiological studies with the heterologous viruses would detect the prevalence of THAIV infection with the same sensitivity as with THAIV antigen. For further epidemiologic studies, serological typing would certainly profit to elucidate the situation of THAIV infection. Virus isolation and genome amplification from *B. indica* originating from different provinces in Thailand have not been attempted so far, but would be very important to extend our knowledge on the distribution and variability of THAIV and THAI-like viruses in Indochina.

Serological detection of THAIV-reactive antibodies in patients with fever of unknown origin from Surin province confirmed the circulation of THAIV or THAI-like viruses in Thailand. Particularly, results of patient #277 suggest that THAIV or THAIV-like virus causes HFRS. We interpret the close geographical proximity of this THAIV-reactive human sample to the seropositive samples from bandicoot rats as a first indication of a potential epidemiologic relationship. Elwell et al.[4] reported that people living in an area where seropositive giant bandicoot rats were trapped showed a higher seroprevalence than those living in a low prevalence area. Nitatpattana et al.[36] observed that *B. indica* was the species with the highest prevalence of anti-hantavirus antibodies in a study on rodents from northeastern Thailand. Similarly, our study revealed the greater bandicoot rat as the species with the highest seroprevalence against hantavirus in general, and THAIV in particular (3.3% in *B. indica*), and identified the lesser bandicoot rat (*B. savilei*) as a potential new host for THAIV. In combination, this suggests that a higher prevalence of infection of bandicoot rats as such already poses a higher risk for humans to become infected with THAIV than with other hantaviruses from other rodent species. This especially applies to rural areas, where both commensal (e.g., ro-

dent species inhabiting houses) as well as field rodents like bandicoot rats live in close association with humans.[37] In the case of THAIV this is possibly aggravated by the fact that 50–80% of residents in some rural areas trap, cook, and eat *B. indica*.[4,37]

Although a recent publication attributed a first clinical case to hantavirus infection in Thailand,[8] the causative hantavirus species was not further characterized. In our study we identified a patient who developed a clinical profile similar to that of HFRS with high concentrations of IgM and IgG to HTNV by an initial screening of various human sera. Because this serum showed lower titers to HTNV, SEOV, and DOBV antigens compared with virus-specific human positive control sera, and, importantly, contained significant concentrations of virus-neutralizing antibodies against THAIV, these observations suggest that THAIV or a THAI-like virus caused this infection. Furthermore, our FRNT results show close similarities between the particular patient serum and sera from rodents of the genus *Bandicota,* especially *B. indica* representing a host of THAIV.[4] In most hantaviral disease cases, both IgM and IgG to hantavirus are positive at the onset of clinical disease.[38] The reduction of the titer of HTNV-reactive IgM in a follow-up serum sample from convalescent phase of the patient may indicate that THAIV or a related virus is a causative agent of HFRS. However, since hantavirus-reactive IgM might be detected up to 6 months after onset of disease,[39] the possibility that the febrile illness might be caused by infection with other pathogen could not be excluded. Therefore, further epidemiologic study is needed to find out similar patients with hantavirus antibody. Nevertheless, the results of Suputthamongkol et al.[8] and our study indicate that human disease caused by hantaviruses may be more prevalent in Thailand than anticipated earlier, because clinical cases may have been confused with leptospirosis, a rodent-transmitted disease that causes similar symptoms in humans like conjunctival suffusion, hemorrhagic manifestation, renal failure, and hepatic dysfunction.[40] Further epidemiologic studies, including virus isolation, are needed to elucidate the relationship between fevers of unknown origin, presence of THAIV or THAI-like viruses in rodents, and potential transmission from rodents to humans.

In conclusion, we have demonstrated that distribution of *Bandicota*-associated THAIV or THAI-like viruses extends from the central plains of Thailand to the north and northeast. Our genetic and serological studies confirmed the definition of THAIV as a distinct hantavirus species. Moreover, our data suggest that THAIV, besides HTNV and SEOV, may represent an additional causative agent of HFRS in Asia. Recently, we found anti-hantavirus antibody-positive sera both in humans and rodents in Vietnam.[41] Molecular, epidemiologic, and serological studies on hantaviruses in rodents and humans have also been reported from Cambodia and Indonesia.[32,42.] Taken together, this indicates a wide distribution and potentially high diversity of hantaviruses in Southeast Asia calling for further studies on human hantavirus infections, its rodent reservoirs, and possible transmission routes.

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Development of Serological Assays for Thottapalayam Virus, an Insectivore-Borne Hantavirus[∇]

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Thottapalayam virus (TPMV), a member of the genus *Hantavirus* in the family *Bunyaviridae*, was isolated from an insectivore, *Suncus murinus* (musk shrew), captured in southern India in 1964. While the isolation of TPMV predates the discovery of the prototype Hantaan virus, little is known about its genetics and biology. To date, preliminary evidence suggests that TPMV differs significantly, both antigenically and genetically, from all known rodent-borne hantaviruses. However, since detailed epizootiological studies have not been conducted, it is unclear if TPMV is naturally harbored by an insectivore host or if TPMV represents a "spillover" from its natural rodent reservoir host. Moreover, to what extent TPMV causes infection and/or disease in humans is not known. To address these issues, we first studied the antigenic profile of TPMV using monoclonal antibodies against Hantaan and Seoul viruses and polyclonal immune sera against Puumala virus and TPMV. Armed with this newfound information, we developed an enzyme-linked immunosorbent assay system for the diagnosis of TPMV infections in shrews and humans, using a recombinant TPMV N antigen manipulated to have an E5/G6 epitope to be captured by monoclonal antibody clone E5/G6. Using this assay, we found anti-TPMV antibodies in sera from a patient with high fever of unknown etiology in Thailand and from two shrews captured in Indonesia. Seropositivity was verified by the indirect immunofluorescence antibody test, Western blotting analysis, and focus reduction neutralization test. Collectively, our data indicate that TPMV is harbored by *Suncus murinus* as its host in nature and is capable of infecting humans.

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Like other viruses in the family *Bunyaviridae*, members of the genus *Hantavirus* are enveloped viruses with a tripartite, negative-stranded RNA genome, consisting of large (L), medium (M), and small (S) segments. The L segment encodes an RNA-dependent RNA polymerase; the M segment encodes a glycoprotein precursor, which is cleaved into surface glycoproteins, Gn and Gc; and the S segment encodes a nucleocapsid protein (N) (15). Some hantaviruses cause zoonotic diseases in humans, known as hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome (HPS) (14). Presently, 22 species are classified within the genus *Hantavirus* based on antigenic and genetic differences (9). In the Old World, four antigenically related and genetically distinct hantaviruses are known to cause hemorrhagic fever with renal syndrome: Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), and Dobrava virus (DOBV). Several sigmodontine rodent-borne hantaviruses in the New World, including Sin Nombre virus (SNV) and Andes virus, cause HPS. For both diseases, virus transmission to humans occurs via aerosolization of infectious rodent excreta (6).

Each hantavirus appears to have coevolved with a specific rodent species, in which it maintains an enzootic cycle. As the only known presumed exception, Thottapalayam virus (TPMV) was isolated from an insectivore, *Suncus murinus*, captured in southern India in 1964 (3). Either very low or no antigenic cross-reactivity has been observed between TPMV and other hantaviruses (4, 5). And as evidenced by nucleotide and amino acid sequence analyses of the full-length S segment, TPMV is the most genetically divergent of all other hantaviruses (6, 17). Analyses of the recently acquired full-length M and L segments of TPMV are congruent (J.-W. Song and R. Yanagihara, unpublished observations). However, since detailed epizootiological and epidemiological surveys of TPMV infection have not been conducted, the fundamental biology of TPMV, including its true natural host and pathogenicity to humans, is unclear.

Previously we developed enzyme immunoassays using baculovirus-expressed recombinant N (rN) antigens of various hantaviruses (including HTNV, SEOV, PUUV, and DOBV) for the serological diagnosis of hantavirus infections (1, 7, 8, 18). With this method, the monoclonal antibody (MAb) clone

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TABLE 1. Reactivities of MAbs to TPMV and rodent-borne hantaviruses

Specificity	Epitope	MAb	Reactivity of MAb to[a,b]:								
			TPMV	HTNV	SEOV	THAIV	DOBV	SaaV	PUUV	PHV	SNV
N (HTNV)		FDO3	±	+	+	+	+	+	+	+	NT
		KAO6	–	+	+	+	+	+	–	–	NT
		ECO2	–	+	+	+	+	+	–	–	–
		ECO1	–	+	+	+	+	+	+	–	–
		GBO4	–	+	+	+	+	+	+	+	NT
		E5G6	–	+	+	+	+	+	+	+	+
		C16D11	–	+	+	+	+	+	+	+	–
		F23A1	–	+	+	+	+	+	–	+	+
		C24B4	–	+	–	–	±	±	–	–	–
		BDO1	–	+	–	–	–	–	–	–	–
		G5	–	+	–	–	–	–	–	–	+
N (SEOV)		DCO3	–	–	+	–	–	–	–	–	–
Gn/Gc (HTNV)	Gn-a	8B6	–	+	±	+	±	+	±	–	NT
		6D4	–	+	+	+	+	+	–	–	NT
		10F11	–	+	+	+	+	+	+	–	NT
	Gn-b	2D5	–	+	–	–	–	–	–	–	NT
		3D5	–	+	–	–	–	–	–	–	NT
		16D2	–	+	–	–	–	–	–	–	NT
	Gc-a	HCO2	–	+	+	+	–	–	–	–	NT
		16E6	±	+	+	+	+	+	±	–	NT
	Gc-b	EBO6	–	+	+	–	±	±	–	–	NT
	Gc-c	11E10	–	+	±	–	+	–	+	+	NT
	Gc-e	17G6	+	+	±	+	+	+	+	±	NT
		3D7	+	+	+	+	+	+	+	±	NT
		5B7	+	+	+	+	+	+	+	±	NT
	Gc-c	20D3	+	+	±	+	+	+	–	–	NT
	Gc-f1	8E10	+	+	+	+	+	+	+	±	NT
		1C6	+	+	+	+	+	+	+	±	NT
		1G8	–	+	+	+	+	+	+	±	NT
		23G10-2	+	+	+	+	+	+	+	±	NT
		3B6	±	+	+	+	+	+	+	±	NT
	Gc-f2	23G10-1	+	+	+	+	+	+	–	–	NT
		7G6	–	+	+	+	+	+	–	–	NT
		18F5	–	+	±	+	+	+	–	–	NT

[a] TPMV, Thottapalayam virus; HTNV, Hantaan virus; SEOV, Seoul virus; THAIV, Thailand virus; DOBV, Dobrava-Belgrade virus; SaaV, Saaremaa strain of DOBV; PUUV, Puumala virus; PHV, Prospect Hill virus; SNV, Sin Nombre virus; NT, not tested.
[b] Results with HTNV, SEOV, PUUV and PHV (2), THAIV, DOBV, and SaaV (12), and SNV (7) are available in previous reports.

E5/G6 is utilized as an effective capture antibody, since it binds to a linear epitope of the N protein among all hantaviruses (11, 18). Thus, after determining the antigenic profile of TPMV, we developed a robust serological assay to diagnose TPMV infections in animals and humans, using the TPMV rN antigen manipulated to contain specific amino acid substitutions to allow binding with MAb E5/G6. Using this assay, we detected anti-TPMV antibodies in a human with febrile illness and in two musk shrews. These results indicate that TPMV is carried by musk shrews in nature and is capable of causing infections in humans.

MATERIALS AND METHODS

Viruses and cells. The prototype VRC-66412 strain of TPMV, originally isolated in suckling mice (3) and subsequently adapted to growth in the E6 clone of Vero cells (CRL 1586; American Type Culture Collection), was used. HTNV strain 76-118, SEOV strain SR-11, and PUUV strain CG1820 were used as representative rodent-borne hantaviruses. Viruses were propagated in Vero E6 cells maintained in Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% nonessential amino acids (Gibco). High Five cells (Invitrogen, Carlsbad, CA) were maintained in Grace's insect growth medium (Gibco) supplemented with 10% fetal bovine serum. Recombinant baculoviruses of HTNV, PUUV, and SNV were prepared as described previously (1).

MAbs and immune sera. Monoclonal antibodies (MAbs) and immune rabbit sera for N of HTNV and SEOV and MAbs to Gn and Gc of HTNV, as described previously, were used (2, 18). Immune rabbit serum for PUUV N was kindly provided by Hiroaki Kariwa of the Graduate School of Veterinary Medicine, Hokkaido University. Immune rabbit serum to TPMV N was prepared by intradermal injections of an 11-week-old Std:JW/CSK rabbit (specific-pathogen-free rabbit; SLC, Shizuoka, Japan) with 350 μg of TPMV rN expressed in *Escherichia coli* and 500 μg of Freund's complete adjuvant. A booster immunization of the same antigen with Freund's incomplete adjuvant was administered at 24 days, and blood was collected at 58 days. Immune mouse sera to TPMV were obtained 4 weeks following intraperitoneal inoculation of BALB/c mice (CLEA Japan, Osaka, Japan) with 2.0×10^3 focus-forming units of native TPMV (indirect immunofluorescence antibody [IFA] titer against TPMV was 1:12,800). Finally, sera were obtained from shrews (CLEA Japan) inoculated subcutaneously with 5.2×10^4 focus-forming units of native TPMV at 40 days postinoculation.

Human patient and wild shrew sera. Of the 478 human sera available for testing, 284 were collected between 2003 and 2004 from patients in Surin Province who had leptospirosis-like symptoms but who were serologically negative for both *Leptospira* and dengue virus. The other 194 sera were collected from patients with febrile illnesses of unknown etiology as part of the Emerging Infectious Diseases collaborative project, conducted by the Thai National Institute of Health and the Japan International Cooperation Agency in Nongkhai Province in 2005. In addition, sera were collected from 14 wild shrews

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(*Suncus murinus*) captured in Thousand Islands, Indonesia, in July and October 2005.

Preparation of recombinant TPMV N antigen. Culture supernatant of TPMV-infected Vero E6 cells was ultracentrifuged (265,000 × *g*, 4 h, 4°C), and RNA was isolated from the viral pellet and dissolved with Isogen (Invitrogen) following the manufacturer's instructions. Reverse transcription-PCR was performed using the KOD-plus system (Toyobo, Tokyo, Japan) to amplify the entire TPMV N-coding S segment with primers 5'-TTCAG *AATTC* GATGA CTCAA GGGAA AATGA CTCCC GAAGA-3' and 5'-TATCC *TCGAG* TTACA GTTTA ATAGG CTCCT GACTT GAAAT C-3' (the EcoRI and XhoI sites are shown in italics). After amplification, the DNA fractions were subcloned into the pET-43b(+) vector using restriction enzymes that recognized the restriction sites added by PCR and transformed into *E. coli* strain Origami (Invitrogen). A single colony was inoculated into Circle growth medium (BIO101 systems, Carlsbad, CA) containing tetracycline, kanamycin, and ampicillin for small-scale culture incubation at 37°C overnight. The culture fluid was then centrifuged, the collected cells were inoculated into 100 ml of fresh medium, and isopropyl-β-D-thiogalactopyranoside induction was performed according to the procedure for pET system expression. The cultured cells were collected by centrifugation, resuspended in 5 ml of 0.5 M NaCl binding buffer (0.5 M NaCl, 20 mM imidazole, 20 mM potassium phosphate), and sonicated four times for 15 s each on ice. Thereafter, the fusion protein was purified using a His trap column (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

IFA test. An IFA test was performed using previously described procedures (18). Acetone-fixed smears of Vero E6 cells infected with hantavirus or High Five cells infected with recombinant baculovirus were used as antigens. Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) (heavy plus light chains) antibody (1:2,000; Molecular Probes, Eugene, OR) was used as a secondary antibody to MAbs. For rabbit and human sera, fluorescein isothiocyanate-conjugated protein A (1:2,000; Sigma, St. Louis, MO) was used. IFA titers were expressed as the reciprocal of the highest serum dilution that produced characteristic intracytoplasmic fluorescence.

Peptide synthesis and antigenic analysis. Peptides were synthesized and analyzed by previously published methods (11). Briefly, using an Autospot ASP222 peptide synthesizer (ABiMED, Langenfeld, Germany), a variety of 10-mer peptides were spotted on a membrane. The spotting membrane was blocked in Block Ace (Yukijirushi Co., Tokyo, Japan) for 30 min at room temperature, stained with an E5/G6 hybridoma culture supernatant for 60 min, and detected using horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:500; Zymed, South San Francisco, CA) and 3-amino-9-ethylcarbazole (Sigma).

Construction of recombinant baculovirus expressing TPMV N with an E5/G6 epitope. The subcloned DNA fragment was excised from pET-43(+), described above, by digestion with the same enzyme and inserted in the donor plasmid pFAST-BAC1 (Gibco). Based on the results of the E5/G6 epitope analysis of TPMV N, amino acid-altering nucleotide mutations required for E5/G6 binding were added, using the GeneTailor site-directed mutagenesis system (Invitrogen). TPMV wild-type rN (rN/wt) and TPMV rN with the E5/G6 epitope (rN/E5G6) were expressed using the Bac-to-Bac baculovirus expression system according to the manufacturer's instructions (Gibco). These baculoviruses were inoculated into High Five cells to acquire the rN antigen, using previously described methods (1).

Western blotting analysis. Western blotting was performed using previously published methods (19). The infected High Five and Vero E6 cells were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (ATTO, Tokyo, Japan). Immune rabbit serum to hantavirus N was used to detect antigen on the membrane. Binding antibodies were detected using HRP-conjugated protein A (Prozyme, San Leandro, CA), and 4-chloro-1-naphthol (Sigma) was used as the peroxidase substrate.

ELISA. Enzyme-linked immunosorbent assays (ELISAs) using whole HTNV, PUUV, SNV, and TPMV rN were performed according to previously described methods (1, 7, 8, 18). MAb E5/G6 (2 μg/ml) was used as a capture antibody to coat 96-well plates for 60 min at 37°C. Nonspecific binding was blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS). After a 60-min incubation, the plates were washed three times with PBS containing 0.05% Tween 20. Each antigen was added and incubated for 60 min at 37°C, followed by three washings. For detection of rabbit IgG, HRP-conjugated goat anti-rabbit IgG antibody (1:5,000; Jackson, Bar Harbor, ME) was used as the secondary antibody, and *o*-phenylenediamine (Sigma) was added as the peroxidase substrate. Absorbance at 450 nm was measured using a SpectraMax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). For detection of human IgG, alkaline phosphatase-conjugated goat anti-human IgG (γ-chain specific) antibody (1:2,000; Sigma) was used as the secondary antibody and *p*-nitrophenyl phosphate (Sigma) was added as the substrate before measuring the absorbance at 405 nm. For detection of shrew IgG, HRP-conjugated protein A (1:5,000; Prozyme) was used as the secondary antibody and *o*-phenylenediamine was added as the peroxidase substrate.

FRNT. Endpoint titers of neutralizing antibodies were determined by the focus reduction neutralization test (FRNT), as described elsewhere (1). Foci of virus-infected cells were detected by staining the cells with Alexa Fluor 488-labeled MAb 5B7, which recognizes the Gc of hantaviruses (10). FRNT titers were expressed as the reciprocal of the highest serum dilution leading to a greater than 80% reduction in the number of infected cell foci.

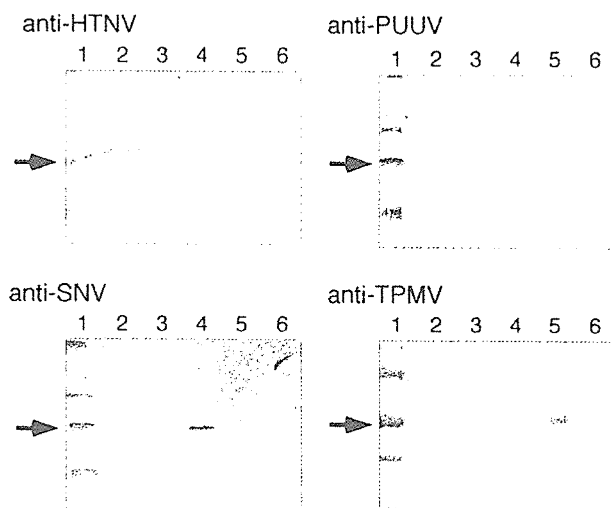

FIG. 1. Western blot analysis of hantavirus antigens using polyclonal rabbit immune sera. We tested the cross-reactivity of each hantavirus, including TPMV, using sera from rabbits immunized with rN antigens expressed in *E. coli*. For HTNV, PUUV, and TPMV antigens, viruses were inoculated on Vero E6 cells, harvested, dissolved, and used. For SNV antigen, High Five cells expressing SNV rN by use of recombinant baculovirus were used (7). Lanes 1, molecular weight marker; lanes 2, HTNV; lanes 3, PUUV; lanes 4, SNV; lanes 5, TPMV; lanes 6, uninfected Vero E6 cells. Arrows indicate the band at 50 kDa.

RESULTS

Antigenic profiling of TPMV using MAbs and polyclonal antibodies. To characterize the TPMV antigenic profile, we performed the IFA test using a panel of MAbs against HTNV N, Gc, and Gn and SEOV N (Table 1). None of the MAbs against HTNV N and Gn cross-reacted with TPMV, while 8 of 16 MAbs against HTNV Gc did. By contrast, all other hantaviruses exhibited various degrees of cross-reactivity to MAbs against N and Gn, except for Prospect Hill virus, which did not react with MAbs against HTNV Gn. TPMV seemed to share partly common epitopes in the Gc region but not in the N or Gn regions, although all other serotyped viruses had some common epitopes in each region.

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We next immunized a rabbit with TPMV rN expressed in *E. coli* and obtained a polyclonal immune serum with an IFA titer of 1:6,400, which strongly reacted also to reduced-TPMV antigens by Western blot analysis (Fig. 1). Using this immune serum, however, TPMV did not cross-react with other hantaviruses (Fig. 1), suggesting that TPMV was the most antigenically divergent of all hantaviruses isolated to date.

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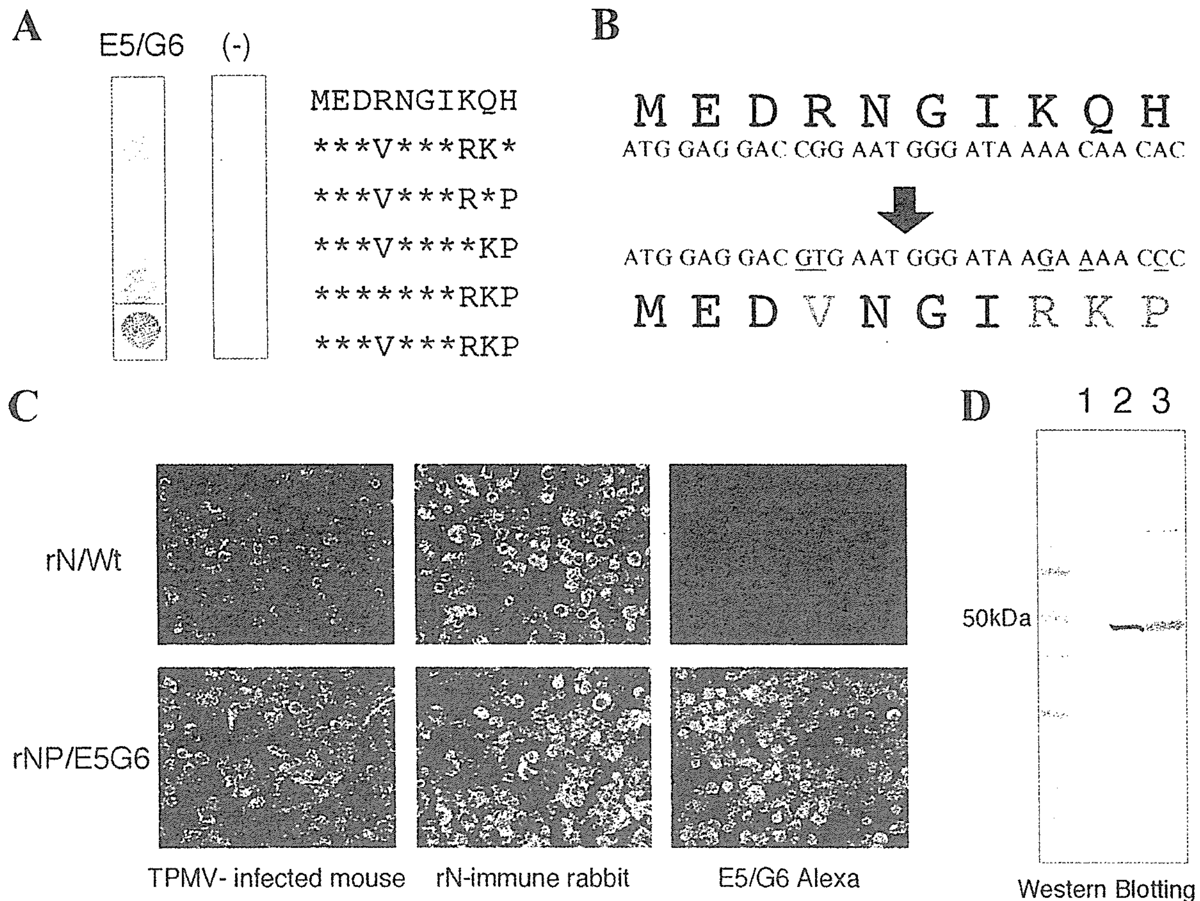

FIG. 2. A. E5/G6 epitope analysis of TPMV N. Using a variety of synthesized 10-mer peptides, we confirmed E5/G6 reactivity against TPMV sequence. Further, we determined which amino acid changes in this region were essential for E5/G6 binding. The peptide changes at positions 178 (R→V), 182 (K→R), 183 (Q→K), and 184 (H→P) were sufficient for MAb E5/G6 binding. B. Insertion of several amino acid mutations changing E5/G6 binding. C. Confirmation of the antigenicitiy of each baculovirus-infected High Five cell antigens expressed by recombinant baculoviruses. The rN antigen having the original sequence (rN/wt) reacted with immune serum but not with MAb E5/G6. On the other hand, the rN with the E5/G6 epitope (rN/E5G6) reacted with immune serum, as well as MAb E5/G6. D. Western blot analysis using sera from rabbits immunized with *E. coli*-expressed rN antigens. Both rN antigens (rN/wt and rN/E5G6) were detected by a band of about 50 kDa, which corresponded to authentic TPMV N. Lane 1, rN/wt; lane 2, rN/E5G6; lane 3, TPMV-infected Vero E6 cells.

Epitope analysis and construction of TPMV rN possessing the E5/G6 epitope. We have developed an ELISA system for diagnosing hantavirus infections with excellent specificity and sensitivity (1, 7, 8, 18). In this assay, baculovirus-expressed recombinant hantavirus antigen is captured on 96-well plates coated with MAb E5/G6 (18). Although there are several amino acid variations, the N protein of all 21 rodent-borne hantavirus species reacted with MAb E5/G6 (11). Therefore, MAb E5/G6 is an effective tool for capturing hantavirus N, with the only exception being TPMV (Table 1). Accordingly, to use this assay for TPMV, we manipulated the E5/G6 epitope region of TPMV rN to allow binding with MAb E5/G6. The MAb E5/G6 made by immunizing mice with HTNV rN reacted effectively with the sequence YEDVNGIRKP at 165 to 174 amino acids (11, 18). However, TPMV has the sequence ME DRNGIKQH for the corresponding E5/G6 epitope region and did not react with MAb E5/G6 (Table 1). Using a peptide synthesizer, we synthesized this 10-mer peptide and confirmed the effect of some amino acid mutations on MAb E5/G6. As a result, the peptide MEDVNGIRKP with four changes (R to V, K to R, Q to K, and H to P) reacted with MAb E5/G6 (Fig. 2A). Based on the E5/G6 epitope analysis, we inserted five nucleotide mutations in the TPMV S segment to produce the four amino acid changes (Fig. 2B) and prepared recombinant baculoviruses expressing TPMV rN with the E5/G6 epitope.

High Five cells inoculated with the recombinant baculoviruses were harvested, and the antigenicities of TPMV rN/wt and TPMV rN/E5G6 was confirmed by the IFA test (Fig. 2C). Both rNs reacted with TPMV-infected mouse sera and rN-immune rabbit sera. But only rN/E5G6 reacted with MAb E5/G6, as expected. In addition, we confirmed the antigenicity of rN/wt and rN/E5G6 by Western blotting analysis using rN-immune rabbit sera (Fig. 2D). These data show that both TPMV rNs have the same band of approximately 50 kDa, which is the size of TPMV N, as well as TPMV-infected Vero

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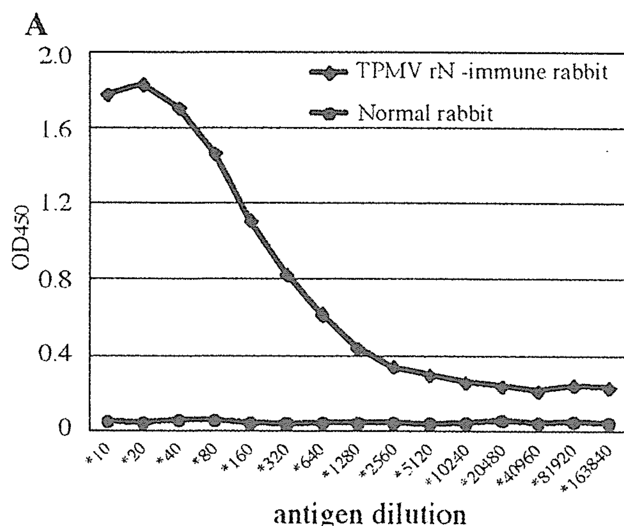

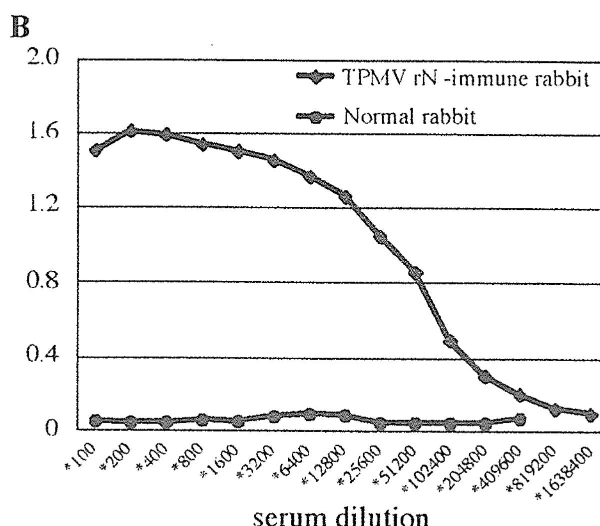

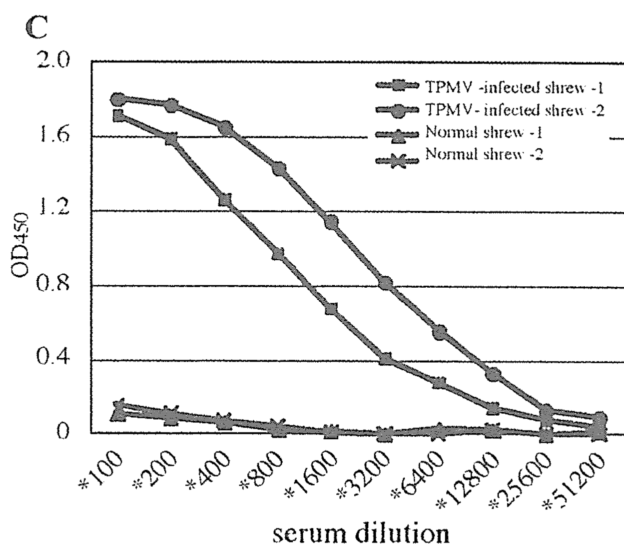

FIG. 3. A. To determine a suitable dilution of the antigen for the ELISA, we tested the reactivities of serial twofold dilutions of rN/E5G6 to a constant amount (1:200 dilution) of antibodies from TPMV rN-immune rabbit. The 1:20 to 1:40 dilution seemed to be appropriate. B. The results of ELISA using a constant amount of rN/E5G6 antigen (1:40) to twofold dilutions of the immune rabbit serum. TPMV antibodies could be detected at serum dilutions at or exceeding 1:200,000. C. Detection of antibodies against TPMV in sera from shrews experimentally infected with TPMV.

E6 cell antigen. So, we succeeded in producing TPMV rN with the E5/G6 epitope, which has the antigenicity of TPMV as well as reactivity with MAb E5/G6.

Developing the E5/G6 capture ELISA system for TPMV. Using the TPMV rN/E5G6 antigen, we developed an IgG antibody-detecting capture ELISA system, according to methods described previously (1, 7, 8, 18). Figure 3A shows the results of an ELISA with twofold dilutions of TPMV rN/E5G6 to a constant dilution of rN-immune rabbit sera (1:200 dilution), and Fig. 3B shows the results of an ELISA with a constant amount of TPMV rN/E5G6 antigen (1:40 dilution) to serial twofold dilutions of rN-immune rabbit sera. The ELISA assay system using the TPMV rN/E5G6 antigen detected anti-TPMV antibodies with high sensitivity. Results with sera from shrews experimentally infected with TPMV also supported the sensitivity of this assay system (Fig. 3C).

In addition, we compared the antigenic cross-reactivities of TPMV and other hantaviruses using this ELISA system (Table 2). In the reactions with each homologous combination, the optical density value was remarkably high. Although the heterologous combinations showed a variety of reactivities, according to the antigenic similarity between viruses, TPMV antigen did not cross-react with other antihantavirus antibodies. This result indicated that rN/E5G6 is a useful tool for the specific detection of anti-TPMV antibodies.

Serological survey of TPMV infection among febrile patients in Thailand. Employing the newly developed capture ELISA

TABLE 2. Cross-reactivities in capture ELISA among TPMV and representative disease-causing hantaviruses

Source of antigen	Cross-reactivity of immune rabbit serum to:			
	HTNV	PUUV	SNV	TPMV
HTNV	0.781	0.453	0.037	0.015
PUUV	0.671	1.487	0.669	0.000
SNV	0.614	1.362	1.672	0.036
TPMV	0.011	0.007	0.002	1.578

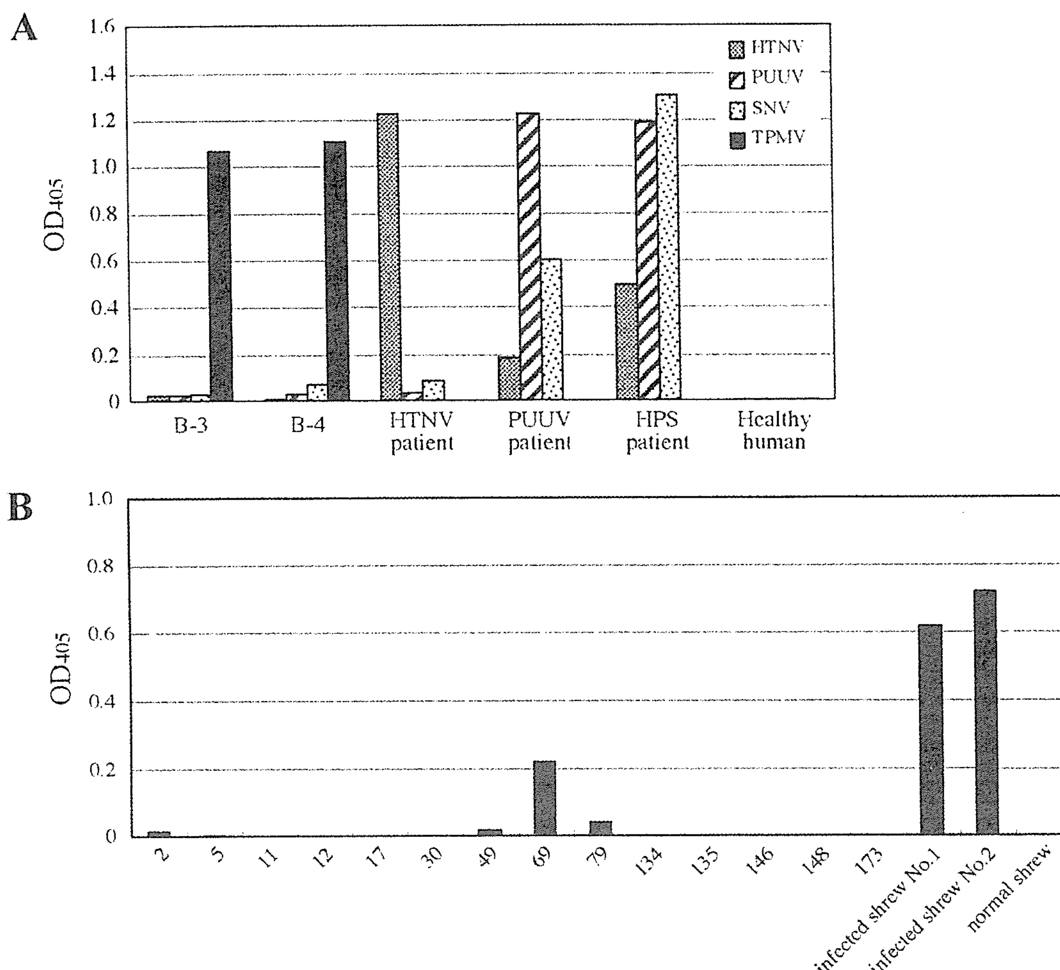

FIG. 4. Serological surveys for TPMV infection in Southeast Asia. We examined 478 sera from patients with fever in Thailand and found two sera (B-3 and B-4 from the same patient at different phases of illness) with anti-TPMV IgG antibodies. Results of the ELISA are shown in panel A. Sera B-3 and B-4 reacted with TPMV antigen. HTNV, PUUV, and HPS patient immune sera served as positive controls. In addition, we tested sera from 14 wild shrews (*Suncus murinus*) captured in Indonesia in 2005 (B). Serum no. 69 was positive for anti-TPMV IgG antibodies, while sera no. 2, 49, and 79 were weakly positive.

system, we tested 478 serum samples from patients with fever in Thailand who were serologically negative for leptospirosis and dengue fever. Each serum was tested with whole HTNV, PUUV, SNV, and TPMV rN for serotyping (Fig. 4A). Serum samples no. B-3 and no. B-4, which were from the same patient during different phases of illness, were positive for anti-TPMV IgG antibodies. Sera from seven other cases were weakly positive to HTNV (data not shown). Sera no. B-3 and no. B-4 also reacted with TPMV-infected Vero E6 cells by the IFA test and Western blot analysis (Fig. 5A and B) and by FRNT using native TPMV (Table 3). However, virus-specific IgM was not detected (data not shown). Therefore, this patient may have been infected with TPMV previously, although it is not clear if he had shown symptoms.

This anti-TPMV-antibody-positive patient was a 58-year-old Laotian male who fell ill in Laos and came to a hospital in Nongkhai Province, along the border of Thailand and Laos, in April 2005. He presented with high fever, chills, headache, cough, sore throat, vomiting, diarrhea, abdominal pain, and exhaustion. The patient recovered fully after being hospitalized for several weeks. However, these symptoms were not necessarily related to TPMV infection directly, because he lacked IgM against TPMV. Unfortunately, no information is available about his occupation or his exposure to shrews or wildlife.

Serological survey of TPMV infection in wild shrews captured in Indonesia. Of sera collected from 14 shrews captured in Indonesia in 2005, one (no. 69) was positive for anti-TPMV IgG antibodies by ELISA. Sera from three other shrews (no. 2, 49, and 79) were weakly positive by ELISA (Fig. 4B). By contrast, in the IFA test using TPMV-infected Vero E6 cells as antigen, sera no. 49 and 69 were positive (Fig. 5A), whereas sera no. 2 and 79 were negative. Sera no. 49 and 69 were also positive by Western blotting analysis using TPMV-infected Vero E6 cell antigens (Fig. 5B). Only no. 49 was positive by FRNT (Table 3).

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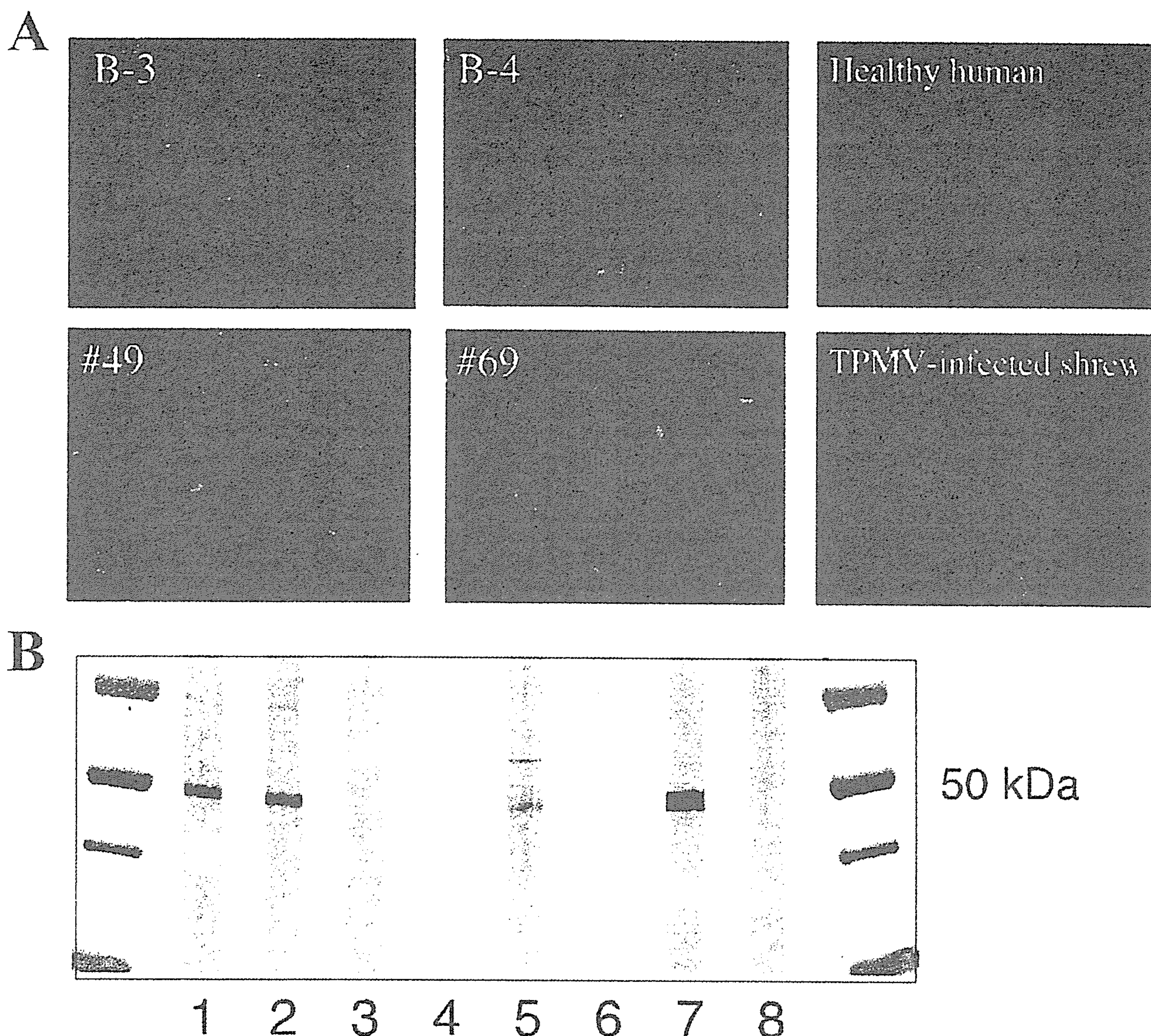

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FIG. 5. A. Results of IFA test using TPMV-infected Vero E6 cell antigens. As a positive shrew serum control, serum from a shrew experimentally infected with TPMV was used. Sera no. 49 and 69 were positive against TPMV antigen. But sera no. 2 and 79 were negative by the IFA test (data not shown). B. Western blot analysis of TPMV-positive sera with TPMV antigen. The human positive sera B-3 and B-4 in ELISAs also reacted with TPMV-infected Vero E6 antigen by Western blot analysis. On the other hand, for shrews, only no. 49 and 69 showed a band at 50 kDa with TPMV antigen, and sera no. 2 and 79 did not. Lanes 1 (B-3) and 2 (B-4) are human positive samples in ELISA; lane 3 (no. 2), lane 4 (no. 49), lane 5 (no. 69), and lane 6 (no. 79) are shrew positive samples in ELISA; lane 7 (serum from a shrew experimentally infected with TPMV) is a positive control. Lane 6 (normal shrew serum) is a negative control.

TABLE 3. FRNT with native TPMV in human and shrew sera[a]

Serum no.	FRNT titer
Human	
B-3	40
B-4	80
(−)	<40
Shrew	
49	80
69	<40
(+)	320
(−)	<40

[a] Human (−) is a serum from a healthy individual as a negative control. Shrew (+) is a serum from a shrew experimentally infected with TPMV as a positive control, and (−) is a serum from a normal uninfected shrew.

DISCUSSION

Long unclassified, TPMV is now known to be a member of the genus *Hantavirus*. Surprisingly little is known about TPMV, however, despite the fact that its isolation predates that of Hantaan virus. For example, until very recently sequences of the full-length S-, M- and L-genomic segments of TPMV were not known. Also, although TPMV was isolated from tissues of a musk shrew, the identity of its natural reservoir host has remained shrouded in some uncertainty, with some believing that TPMV must represent spillover from a rodent host. The dearth of information about TPMV can largely be attributed to

the lack of systematic, well-designed studies focusing on its epizootiology and pathogenicity. One of the barriers to conducting such studies has been the unavailability of highly sensitive and specific serological assays.

To address this limitation, we first compared the antigenic profile of TPMV with those of representative hantaviruses, which segregate into three groups according to the subfamilies of their rodent reservoir hosts: that is, Murinae-, Arvicolinae- and Sigmodontinae-associated hantaviruses (13, 16). Viruses in each group have antigenic properties similar to each other's (5, 7). As determined by the IFA test using MAb and polyclonal immune sera, TPMV had the most divergent antigenic profile among hantaviruses, which conforms to data from an earlier report using the plaque reduction neutralization test (5). Moreover, immune serum, prepared by inoculating BALB/c mice with TPMV, had a high IFA titer against TPMV of 1:12,800. However, in Western blot analysis, the mouse immune serum did not detect TPMV antigen in TPMV-infected Vero E6 cell lysates or in TPMV rN antigen prepared with *E. coli*, whereas other hantavirus N proteins were detected by mouse serum immunized with the respective hantavirus (data not shown). These data suggest that TPMV induces either no or very low levels of linear epitope-recognizing antibodies in mice. The antigenic difference of TPMV N from that of other hantaviruses indicated a requirement for a new ELISA system for the serological diagnosis of TPMV infection.

We have developed an E5/G6 capture ELISA system which has excellent specificity and sensitivity profiles for the diagnosis of hantavirus infection (1, 7, 8, 18). In this ELISA system, each rN antigen is captured in wells coated with MAb E5/G6. Since TPMV seemed to have no affinity to MAb E5/G6, we inserted several amino acid mutations into the region corresponding to the E5/G6 epitope of TPMV N. Because antibodies against the E5/G6 epitope are not induced in hantavirus-infected patient sera and E5/G6 does not compete with other antibodies induced by hantavirus infections (18), we expected that inserting amino acid-altering point mutations within this region would not change its antigenicity. Finally, we succeeded in developing an E5/G6 capture ELISA which can identify TPMV rN-immune rabbit sera and sera of shrews experimentally infected with TPMV with high specificity.

We previously proposed that three kinds of whole-length rN antigens of HTNV, PUUV, and SNV were required for the serological diagnosis of rodent-borne hantavirus infections (7). Now, by adding TPMV rN/E5G6, it is possible to diagnose both rodent- and insectivore-borne hantavirus infections. Using these four rN antigens, we examined 478 serum samples from patients with high fever in Thailand who were serologically negative for leptospirosis and dengue virus and found two anti-TPMV IgG antibody-positive sera from a single individual. Anti-TPMV IgG antibodies in these sera were confirmed by IFA, Western blotting, and FRNT. Because the patient came to the hospital after his condition had worsened, the relationship between his illness and TPMV infection could not be accurately determined. Thus, while this case suggests the infectivity of TPMV for humans, its pathogenicity for humans remains uncertain.

In testing sera from 14 wild shrews captured in Indonesia in 2005, one sample (no. 69) reacted strongly against TPMV, and three other samples (no. 2, 49, and 79) reacted weakly by ELISA. In the IFA test, using TPMV-infected Vero E6 cells as the antigen, two of these sera (no. 49 and 69) were positive, and this was confirmed by Western blot analysis. However, only serum no. 49 neutralized TPMV by FRNT, suggesting the possible existence of TPMV variants or other antigenically distinct insectivore-borne hantaviruses in nature. To fully demonstrate that shrews are the natural reservoir of TPMV, it is necessary to survey additional species and detect the viral genome using RT-PCR assays in the future.

This is the first report of TPMV infection serologically confirmed with both humans and shrews. Our data indicate that TPMV can infect humans and is maintained in musk shrews as its natural host. The availability of newly developed serological assays for TPMV will facilitate future studies aimed at further elucidating the epizootiology and molecular phylogeny of insectivore-borne hantaviruses. Moreover, such studies will provide important insights about the role of TPMV and TPMV-like hantaviruses in the pathogenesis of febrile illnesses.

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The kinetics of proinflammatory cytokines in murine peritoneal macrophages infected with envelope protein-glycosylated or non-glycosylated West Nile virus

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Abstract

The envelope (E) protein glycosylation status of the New York strain of West Nile (WN) virus is an important determinant of virus neuroinvasiveness. To elucidate the determinant of the difference between E protein-glycosylated and non-glycosylated WN virus infections, the cytokine expression of murine peritoneal macrophages infected with each virus was examined. Tumor necrosis factor (TNF) α and interleukin (IL)-1β were up-regulated with replication of the E protein-glycosylated virus. Interferon (IFN) β and IL-6 were up-regulated with the clearance of both viruses. These results suggest that TNFα and IL-1β expression are related to the virulence of E protein-glycosylated WN virus.

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Keywords: Envelope protein glycosylation; Peritoneal macrophage; Tumor necrosis factor alpha (TNFα); West Nile virus

1. Introduction

West Nile (WN) virus is an arthropod-borne virus of the genus *Flavivirus* in the family *Flaviviridae*. It is a member of the Japanese encephalitis virus (JEV) serocomplex, which includes JEV, St. Louis encephalitis virus, and Murray Valley encephalitis (MVE) virus. Since the outbreak of WN encephalitis in humans and horses in New York City in late August 1999, WN virus has spread throughout North and Central America (Beasley et al., 2001; Garmendia et al., 2001). Currently, no effective therapies or vaccines against human WN virus infection exist.

The WN virus strain isolated in New York City (NY strain) causes large-scale mortality in wild birds; however, this phenomenon was not observed with previously isolated WN virus strains (Garmendia et al., 2001). Therefore, the pathogenicity of the NY strain appears to differ from that of previously isolated strains, and the study of the pathogenesis of this strain may be useful for developing new vaccines and therapies.

Recently, variants with different amino acid sequences at an N-linked glycosylation site in the envelope (E) protein region were isolated from NY strain stocks (Beasley et al., 2004, 2005; Shirato et al., 2004b). When 6-week-old BALB/c mice were infected subcutaneously with these variants, those infected with viruses carrying the glycosylated E protein developed lethal infections, whereas those infected with viruses carrying non-glycosylated E protein showed low mortality. In contrast, intracerebral infection was lethal, with no difference between E protein-glycosylated and non-glycosylated viruses. Therefore, the glycosylation of the E protein is a molecular determinant of the neuroinvasiveness of the NY strain of WN virus.

The initial target of infection for dengue virus, a mosquito-borne flavivirus, is dendritic cells (Wu et al., 2000); these cells migrate to the lymph nodes after arbovirus infection (Johnston et al., 2000). However, subsequent events leading to viral invasion of the central nervous system (CNS) remain unclear.

Abbreviations: CNS, central nervous system; E, envelope; NY strain, West Nile virus strain isolated in New York City in 1999; TNFα, tumor necrosis factor alpha; WN virus, West Nile virus

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It has been reported that macrophage-depleted mice exhibited more severe WN virus-induced disease (Ben-Nathan et al., 1996), and maximum antigen-presenting activity was exhibited by activated macrophages in WN virus-infected mouse lymph nodes (Pisarev et al., 2003). Therefore, we focused on the interaction between WN virus and macrophages. In this study, we investigated virus replication and cytokine induction in macrophages infected with E protein-glycosylated and non-glycosylated WN viruses.

2. Materials and methods

2.1. Viruses

Two variants, 6-LP (AB185914) and 6-SP (AB185915), of West Nile virus strain NY99-6922 were used in this study. These viruses were isolated from the NY99-6922 strain by plaque purification (Shirato et al., 2004b) and have a single point mutation at an N-linked glycosylation site in the envelope (E) protein region sequence. The 6-LP virus has N-linked glycosylated E protein; the 6-SP virus does not. Viruses were propagated once in the brains of suckling mice as previously described (Shirato et al., 2003), and the working stocks of the viruses were propagated once in C6/36 cell cultures.

2.2. Cell culture and virus infection

Murine peritoneal macrophages were obtained from 6- to 12-week-old BALB/c mice (Japan SLC Inc., Sizuoka, Japan). The mice were injected with 3 ml of phosphate-buffered saline (PBS) containing 4% thioglycollate medium, Brewer modified. At 3 days post-injection, the mice were perfused with cold PBS, and induced macrophages were collected. The cells were washed twice with cold PBS, seeded in a 24-well plate, and incubated in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal calf serum and antibiotics. After a 24-h incubation, the cells were washed twice with DMEM and infected with viruses at the indicated multiplicity of infection (m.o.i.). Viruses were also infected into a monolayer of VeroE6 cells formed in 24-well plates. The supernatants were collected at the indicated times post-infection (p.i.) and stored at −80 °C until used. The virus titer of the supernatants was determined by plaque assay using a BHK cell monolayer, as previously described (Shirato et al., 2004b).

2.3. RNA extraction and reverse transcription

Total RNA was extracted from cells using TRIzol (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol, and the extracted RNA was re-extracted with a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). First strand cDNA was synthesized from total RNA using reverse transcriptase M-MLV (Takara Bio Inc., Siga, Japan) and oligo-dT primer, according to the manual. After synthesis, the cDNA was diluted with distilled water.

Table 1
Primer sets used in the quantitative SYBR Green-based real-time PCR

Primer		Sequence
TNFα	Sense	5′-GGCAAGGATGAGCCTTTTAGG-3′
	Anti-sense	5′-TTGGTTTGGGAGGAAAGGG-3′
IL1β	Sense	5′-CCTTCCAGGATGAGGACATGA-3′
	Anti-sense	5′-CAGCACGAGGCTTTTTTGTTG-3′
IFNβ	Sense	5′-GGCGGACTTCAAGATCCCTAT-3′
	Anti-sense	5′-GGATGGCAAAGGCAGTGTAAC-3′
IL6	Sense	5′-GGGACTGATGCTGGTGACAA-3′
	Anti-sense	5′-TCCACGATTTCCCAGAGAACA-3′
IP-10	Sense	5′-GGACGGTCCGCTGCAA-3′
	Anti-sense	5′-GCTTCCCTATGGCCCTCATT-3′
RANTES	Sense	5′-GCAAGTGCTCCAATCTTGCA-3′
	Anti-sense	5′-ATCCCCAAGCTGGCTAGGA-3′

2.4. Quantitative SYBR Green-based real-time PCR

To quantify the expression of cytokine mRNA, quantitative SYBR Green-based real-time PCR was performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Table 1 lists the primer sets used in this study. The amplifications were performed using an ABI/PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR was performed using SYBR-Green PCR Master Mix (Applied Biosystems). The PCR conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Each amplification was performed in duplicate. The amplification was confirmed by dissociation curve analysis. The number of copies for each cytokine was obtained from the standard curve. The cytokine data were normalized to the GAPDH data for the same sample. The data are presented as the relative number of copies corresponding to the number of GAPDH copies in 5×10^5 cells.

2.5. Virus detection by real-time PCR

To detect the virus genome within cells, we performed real-time PCR as previously described (Shirato et al., 2005). After virus infection, the cells were treated with pronase (0.1 mg/ml, EMD Biosciences Inc., San Diego, CA, USA) and incubated for 40 min at 4 °C. After incubation, the cells were washed with PBS and subjected to RNA extraction.

2.6. TNFα ELISA

The level of TNFα in culture supernatants was determined using an Endogen Mouse TNFα ELISA kit (Pierce Biotechnology Inc., Rockford, IL, USA), according to the manufacturer's protocol. Each sample was tested in duplicate.

2.7. Statistics

Statistical significance was determined using the unpaired *t*-test or Mann-Whitney test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Virus replication in macrophages

WN virus replication in murine peritoneal macrophages was determined (Fig. 1). The E protein-glycosylated WN virus, 6-LP, and non-glycosylated virus, 6-SP, were infected into macrophages at an m.o.i. of 1 or 0.01. Previous data (Shirato et al., 2004b) and Fig. 1C both show that the E-protein-glycosylated virus replicated at a higher titer than did the non-glycosylated virus in tissue culture and murine organs. In macrophages, the two viruses exhibited similar kinetics at both m.o.i., with a peak at 18 h p.i., after which the virus titer decreased. Replication in macrophages did not differ between the two viruses (Fig. 1A and B). The virus titer was determined at 72 h p.i.; however, the virus titer remained low, near the detection limit (10 pfu/ml). In addition, intracellular viral RNA was quantitated by real-time PCR as previously described (Shirato et al., 2005). However, there was no difference in intracellular virus copy numbers (data not shown).

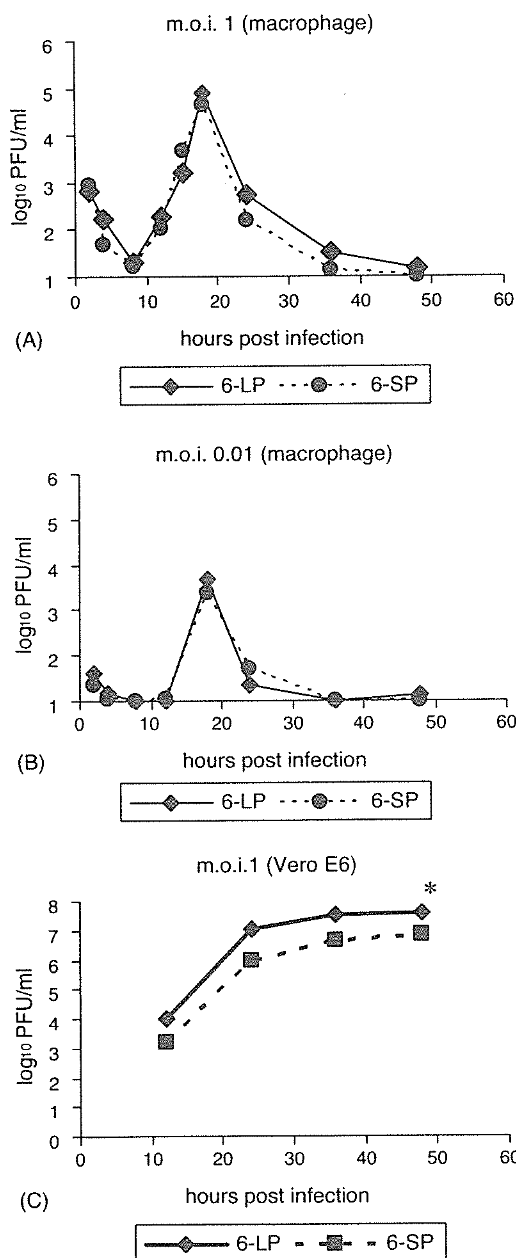

Fig. 1. West Nile virus replication in murine peritoneal macrophages (A and B) and Vero E6 cells (C). Cells were infected with virus, and supernatants were collected at the indicated times p.i. The virus titer was determined by plaque assay using BHK cell monolayers. (A) Macrophages infected with virus at an m.o.i. of 1. (B) Macrophages infected with virus at an m.o.i of 0.01. (C) Vero E6 cells infected with virus at an m.o.i. of 1. Six determinations were made per time point. (*) Statistically significant difference between E protein-glycosylated and non-glycosylated virus infections.

3.2. Cytokine quantitation in E protein-glycosylated and non-glycosylated WN virus-infected macrophages

The proinflammatory cytokine and chemokine mRNA levels in E protein-glycosylated and non-glycosylated WN virus-infected macrophages were evaluated by quantitative real-time PCR (Fig. 2). The TNFα and IL-1β mRNA were highly up-regulated until 18 h p.i. in macrophages infected with E protein-glycosylated WN virus as compared with non-glycosylated virus- or mock-infected macrophages (Fig. 2A and B). During E protein-glycosylated WN virus infection, TNFα and IL-1β mRNA were up-regulated with virus replication and down-regulated with virus clearance. On the other hand, IFNβ and IL-6 mRNA were up-regulated from 18 h p.i. in virus-infected macrophages as compared to mock infection (Fig. 2C and D). IFNβ and IL-6 mRNA were up-regulated with virus clearance in WN virus-infected macrophages; however, the difference between E protein-glycosylated and non-glycosylated virus infections was not statistically significant. The IP-10 and RANTES mRNA levels in virus-infected macrophages were also determined (Fig. 2E and F). IP-10 mRNA was up-regulated during the time course of infection, and the up-regulation was higher than that of the mock infection. Up-regulation of the RANTES mRNA was also seen after virus infection in WN virus-infected macrophages, and RANTES mRNA increased with virus clearance. However, no significant difference between E protein-glycosylated and non-glycosylated virus infections was observed.

3.3. TNFα production in virus-infected macrophages

The quantity of extracellular TNFα production of WN virus-infected macrophages was determined by ELISA (Fig. 3). In agreement with the increased TNFα mRNA levels, TNFα protein was increased until 18 h p.i. in macrophages infected with E protein-glycosylated virus as compared with non-glycosylated virus ($p < 0.05$). TNFα protein production was up-regulated with E protein-glycosylated virus replication and down-regulated with virus clearance. TNFα did not increase in macrophages infected with non-glycosylated virus.

4. Discussion

The aim of this study was to examine the relationship between E protein glycosylation and macrophage infection

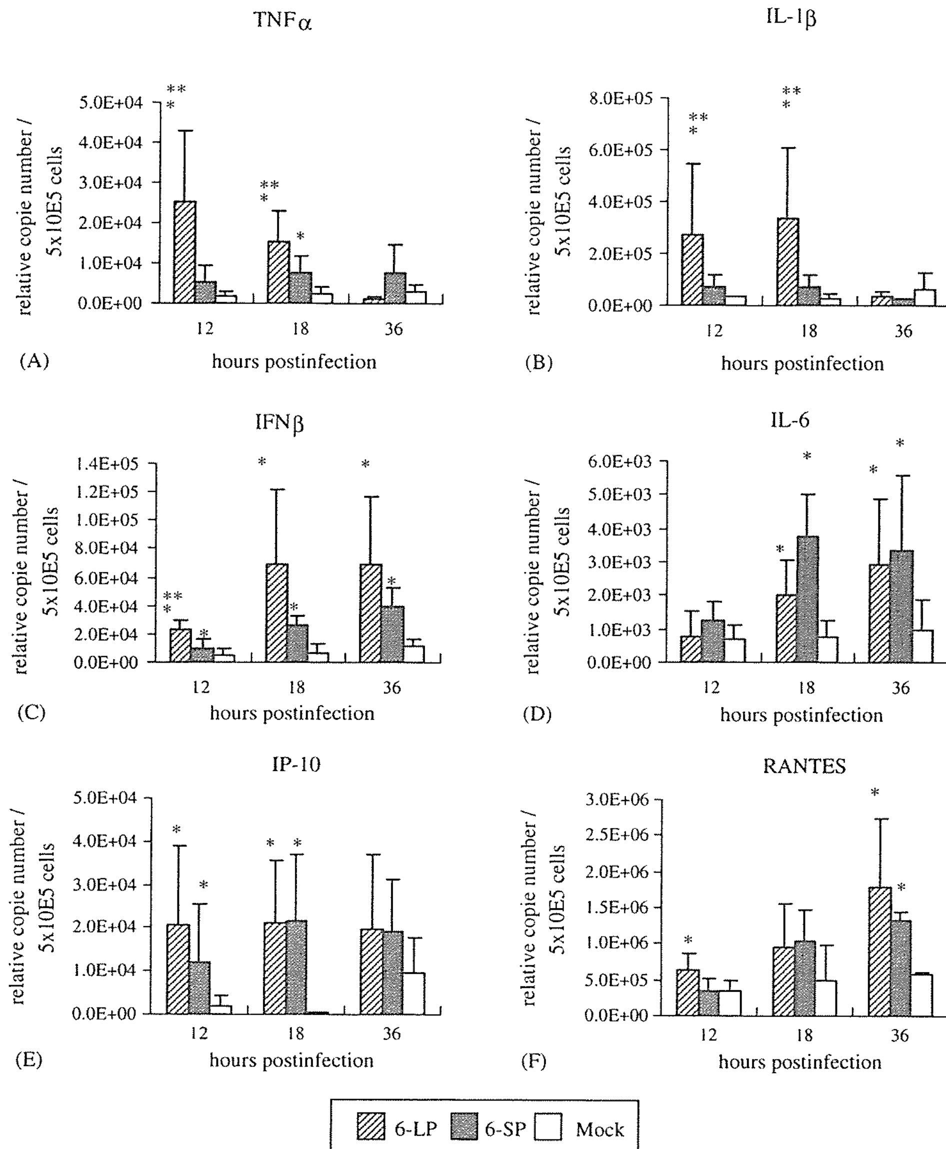

Fig. 2. Cytokine mRNA expression in WN virus-infected murine peritoneal macrophages. The cells were infected with WN virus at an m.o.i. of 1, total RNA was extracted at the indicated times p.i., and cDNA was synthesized. SYBR Green-based quantitative real-time PCR was performed using synthesized cDNA. The numbers of copies of cytokines were obtained from the standard curve. The cytokine data were normalized to the GAPDH data for the same sample. The data are presented as the number of copies relative to the number of copies of GAPDH in 5×10^5 cells. (A) TNFα, (B) IL-1β, (C) IFNβ, (D) IL-6, (E) IP-10, and (F) RANTES. Four determinations were made per time point. (*) Statistically significant difference compared with mock-infected macrophages; (**) statistically significant difference between E protein-glycosylated and non-glycosylated virus infections.

with WN virus. The results showed that virus replication in macrophages did not differ between E protein-glycosylated and non-glycosylated viruses. Previous data in BHK cells and the results of this study in Vero E6 cells both show that the final concentration of E protein-glycosylated WN virus is 10 times that of non-glycosylated virus in cell culture (Shirato et al., 2004b). In addition, Scherret et al. (2001) reported that a glycosylated clone of Kunjin virus produced 10- to 100-fold the virus concentration produced by a non-glycosylated clone. In the current report, the peak virus titer in murine macrophages was seen at 18 h p.i., after which the virus titer decreased to around the detection limit. This finding suggests that the virus was eliminated from murine peritoneal macrophages before it replicated efficiently. Therefore, we hypothesized that the virus was eliminated by some protective mechanism, such as cytokines, before the difference in replication rates between E protein-glycosylated WN virus and non-glycosylated virus became apparent.

Cytokine production differed between E protein-glycosylated and non-glycosylated WN virus infections in murine peritoneal macrophages. TNFα and IL-1β mRNA were up-regulated with virus replication in macrophages infected with E protein-glycosylated WN virus. IFNβ and IL-6 mRNA were up-regulated with WN virus clearance in macrophages infected with either E protein-glycosylated or non-glycosylated WN virus. In addition, the up-regulation of TNFα protein production in macrophages infected with E protein-glycosylated WN virus coincided with the mRNA kinetics. This result suggests that TNFα and IL-1β expression

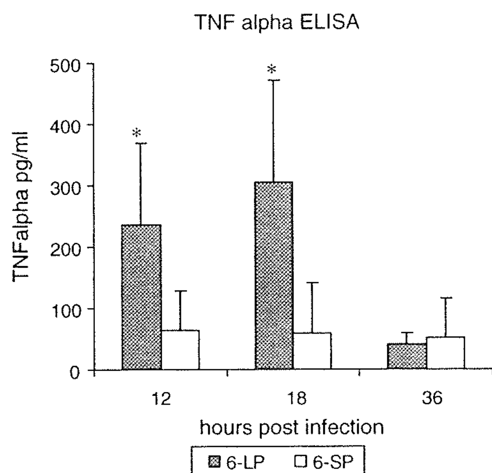

Fig. 3. TNFα production as determined by ELISA in WN virus-infected murine peritoneal macrophages. The cells were infected with virus at an m.o.i. of 1. The supernatants were collected at the indicated times. The TNFα concentration was determined by ELISA. Three determinations were made per time point. (*) Statistically significant difference between E protein-glycosylated and non-glycosylated virus infections.

is related to the replication of virulent E protein-glycosylated WN virus and that IFNβ and IL-6 expression is related to the clearance of WN virus. Thus, the kinetics of cytokine expression was different between E protein-glycosylated and non-glycosylated WN virus infections in macrophage. However, the kinetics of virus replication was not different between them. This result suggests that the different cytokine expression between E protein-glycosylated and non-glycosylated WN virus infected macrophage was caused by not virus replication but direct stimulation of cell with E protein, although the difference of cytokine expression kinetics also may be caused by the structural difference by an amino acid substitution. Davis et al. (2006) reported that WN virus bound to lectin DC-SIGNR (CD209L) and loss of N-linked glycosylation of E protein reduced the DC-SIGNR-mediated cell attachment. The difference of cytokine expression between the E protein-glycosylated and non-glycosylated WN virus infection in macrophage may be caused by the difference in an interaction of cell membrane lectin and E protein by its glycosylation status.

Recently, Wang et al. (2004) reported that Toll-like receptor (TLR) 3-dependent inflammatory responses, especially TNFα, were involved in the increasing blood-brain barrier (BBB) permeability and brain penetration of the WN virus. In a previous report, high mortality was observed when mice were infected with E protein-glycosylated WN virus via the subcutaneous route, but those infected with non-glycosylated virus showed very low mortality (Shirato et al., 2004b). In contrast, mice infected with E protein-glycosylated and non-glycosylated virus via the intracerebral route exhibited lethal infection (Shirato et al., 2004b). Therefore, viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the WN virus, rather than its neurovirulence. Our results and previous data suggest that TNFα induced by E protein-glycosylated WN virus infection leads to BBB leakage and allows virus penetration into the CNS and that the difference in TNFα production is related to the difference in neuroinvasiveness between E protein-glycosylated and non-glycosylated WN virus.

Recently, Lee et al. (2005) reported that TNFα was produced from monocyte-derived macrophages by stimulation with human immunodeficiency virus type 1 glycoprotein (gp) 120 and that TNFα production was mediated by phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways after gp120 interacted with chemokine receptor 5. Recently, putative receptor molecule of mosquito-borne flavivirus was reported (Chu et al., 2005), however, the downstream signal pathway remain unclear. Therefore, further studies are needed to elucidate the mechanism of the TNFα induction pathway stimulated by the E glycoprotein of WN virus.

IL-1β was also up-regulated in E-protein-glycosylated WN virus-infected macrophages. Byrne et al. (2001) reported that Langerhans cell migration into lymph nodes is related to TNFα rather than IL-1β although they used WNV Sarafend strain, which had biological differences as compared to NY strains (Li et al., 2005, 2006). In peripheral inflammation against WN virus infection, TNFα may be more important than IL-1β.

The IFNβ and IL-6 mRNA were up-regulated with virus clearance in WN virus-infected macrophages. It has been reported that pretreatment with several IFN inducers, such as poly (I-C), was effective against WN virus infection in vitro and in vivo (Morrey et al., 2004; Pantelic et al., 2005). Our result suggests that IFNβ production relates virus clearance in WN virus-infected macrophages. It was reported that IL-6 increased BBB permeability and was related to neuronal injury in CNS virus infection (Brett et al., 1995; Hariri et al., 1994). However, when IL-6-deficient mice were infected with WN virus, the mice exhibited lethal infection just as wild type mice did (Wang et al., 2004). Therefore, IL-6 did not aggravate the illness caused by WN virus infection. These reports and our results suggest that IL-6 relates virus clearance and pathogenicity in WN virus infection.

In a previous report, IP-10 and RANTES mRNAs were highly up-regulated in the brains of mice infected with the NY strain of WN virus and were not expressed in the brains of mice infected with the Eg 101 strain, which is also a non-glycosylated WN virus strain (Shirato et al., 2004a). This previous report suggested that IP-10 and RANTES influenced the pathogenesis of the E protein-glycosylated NY strain of WN virus. In the present study, IP-10 and RANTES mRNAs were up-regulated in murine peritoneal macrophages, as compared with the levels in mock-infected mice, until the examined time point. However, no difference was observed between E protein-glycosylated and non-glycosylated virus infections. This result suggests that IP-10 and RANTES were expressed in macrophages infected with WN virus, regardless of E protein glycosylation status.

The results of the present study showed that TNFα was up-regulated in peritoneal macrophages infected with E protein-glycosylated WN virus but not in those infected with non-glycosylated virus. This finding suggests that TNFα induced by E protein-glycosylated WN virus infection is involved in the difference in neuroinvasiveness between E protein-glycosylated and non-glycosylated WN virus infections.

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