an urgent need for assessing pretreatment angiogenic status and evaluating the response to this therapy in clinical studies. In the present study, we found that ^{18}F-FDG uptake not only can reflect the angiogenic status in lung adenocarcinomas but also, and more importantly, can reflect active angiogenesis in lung adenocarcinomas. These properties may have further applications in assessing and monitoring antiangiogenic therapy in the near future.

CONCLUSION

In contrast to CD31-MVD, CD105-MVD reflects active tumor angiogenesis and is a better indicator of prognosis in patients with lung adenocarcinomas. ^{18}F-FDG uptake correlated significantly with the active angiogenesis determined by CD105-MVD. This property may have applications in assessing and monitoring antiangiogenic therapy.

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Soochong Virus: An Antigenically and Genetically Distinct Hantavirus Isolated From *Apodemus peninsulae* in Korea

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Hantaan (HTN) virus, the etiologic agent of clinically severe hemorrhagic fever with renal syndrome (HFRS), was first isolated in 1976 from lung tissue of a striped-field mouse (*Apodemus agrarius*) captured in Songnae-ri, Gyeonggi Province, Republic of Korea. Found primarily in mountainous areas, the Korean field mouse (*A. peninsulae*) is the second-most dominant field rodent species found throughout Korea. A new hantavirus, designated Soochong (SOO), was isolated in Vero E6 cells from four *A. peninsulae* captured in August 1997 at Mt. Gyebang in Hongcheon-gun, Mt. Gachil, Inje-gun, Gangwon Province, and in September 1998 at Mt. Deogyu, Muju-gun, Jeollabuk Province. The entire S, M, and L genomic segments of SOO virus, amplified by RT-PCR from lung tissues of seropositive *A. peninsulae* and from virus-infected Vero E6 cells, diverged from HTN virus (strain 76–118) by 15.6%, 22.8%, and 21.7% at the nucleotide level and 3.5%, 9.5%, and 4.6% at the amino acid level, respectively. Phylogenetic analyses of the nucleotide and deduced amino acid sequences, using the maximum parsimony and neighbor-joining methods, indicated that SOO virus was distinct from *A. agrarius*-borne HTN virus. SOO virus shared a common ancestry with Amur virus from Far East Russia, as well as with H5 and B78 hantaviruses, previously isolated from HFRS patients in China. Cross-focus-reduction neutralizating antibody tests showed that SOO virus, which is the first hantavirus isolated in cell culture from *A. peninsulae*, could be classified as a new hantavirus serotype. ***J. Med. Virol. 78:290–297, 2006.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: *Apodemus peninsulae; hantavirus;* HFRS; soochong virus

INTRODUCTION

Hantaviruses, members of the family *Bunyaviridae*, are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) [Lee et al., 1978; Nichol et al., 1993]. They are negative-sense, single-stranded RNA viruses possessing large (L), medium (M), and small (S) genomic segments that encode the viral polymerase, envelope glycoproteins (G1, G2), and nucleocapsid (N) protein, respectively [Schmaljohn et al., 1986, 1987; Schmaljohn, 1990]. Hantaan (HTN) virus, the etiologic agent of clinically severe HFRS in Far East Asia and Russia, was first isolated from lung tissues of the striped-field mouse (*Apodemus agrarius*) captured in Songnae-ri, Gyeonggi Province, Korea [Lee et al., 1978].

Hantaviruses show co-evolution and co-speciation with specific rodent species, for example, HTN virus with *A. agrarius*, Seoul (SEO) virus with *Rattus norvegicus* and *R. rattus*, Puumala (PUU) virus with *Clethrionomys glareolus*, Prospect Hill (PH) virus with *Microtus pennsylvanicus*, Dobrava–Belgrade (DOB)

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virus with *A. flavicollis*, Tula (TUL) virus with *M. arvalis*, Khabarovsk (KBR) virus with *M. fortis*, Sin Nombre (SN) virus with *Peromyscus maniculatus*, New York (NY) virus with *P. leucopus*, Black Creek Canal (BCC) virus with *Sigmodon hispidus*, Bayou (BAY) virus with *Oryzomys palustris*, El Moro Canyon (ELMC) virus with *Reithrodontomys megalotis*, and Andes (AND) virus with *Oligoryzomys longicaudatis* [Lee et al., 1978, 1982, 1998; Brummer-Korvenkontio et al., 1980; Avsic-Zupanc et al., 1992; Gligic et al., 1992; Nichol et al., 1993; Plyusnin et al., 1994; Song et al., 1994, 2004].

HTN virus is the primary etiologic agent of HRFS in Korea. Approximately 100–300 HFRS cases are reported annually with a mean mortality rate of 4.5%. About 70% of these HFRS patients are infected with HTN virus, 20% with SEO virus, and the remaining 10% of cases by unidentified agents. To identify other HFRS-causing hantaviruses, surveillance of populations of Korean field mice (*A. peninsulae*) was performed. The Korean field mouse, which inhabits mountainous areas throughout Korea, is the second-most dominant field-rodent species in Korea. We now report the isolation and characterization of Soochong (SOO) virus, an antigenically and genetically distinct hantavirus isolated from Korean field mice captured in Korea.

MATERIALS AND METHODS

Rodent Trapping and Serology

Korean field mice were live caught at Mt. Gyebang (1,577 m) in Hongcheon-gun and Mt. Gachil (1,241 m) in Inje-gun, Gangwon Province, on August 6–7, 1997, and Mt. Deogyu (1,614 m) in Muju-gun, Jeollabuk Province, on September 16–19, 1998 (Fig. 1). Rodent sera were screened for IgG antibodies against hantavirus by the indirect immunofluorescent antibody (IFA) technique, using slides spotted with HTN virus-infected Vero E6 cells. Lung and spleen tissues were frozen at −70°C until used for virus isolation and RNA extraction.

Virus Isolation

Subconfluent monolayers of Vero E6 cells (ATCC CRL 1586), grown in 25-cm^2 flasks, were inoculated with 5% suspensions of lung and spleen tissues prepared in DMEM without fetal bovine serum (FBS). Inocula were allowed to adsorb for 2 hr followed by centrifugation for 2 hr at 670*g* at 25°C. Subsequently, the cells were maintained with DMEM supplemented with 5% heat-inactivated FBS. Cells were subcultured at 10- to 14-day intervals, at which time an aliquot of cells was examined for hantavirus antigens by IFA, using convalescent-phase sera from patients with Korean hemorrhagic fever, and rat and mouse sera specific for HTN, SEO, PUU, and PH viruses. Hantavirus antigen-positive Vero E6 cell cultures were examined for hantaviral genomic sequences by RT-PCR using a consensus primer sets for HTN-SEO viruses [Xiao et al., 1991, 1992].

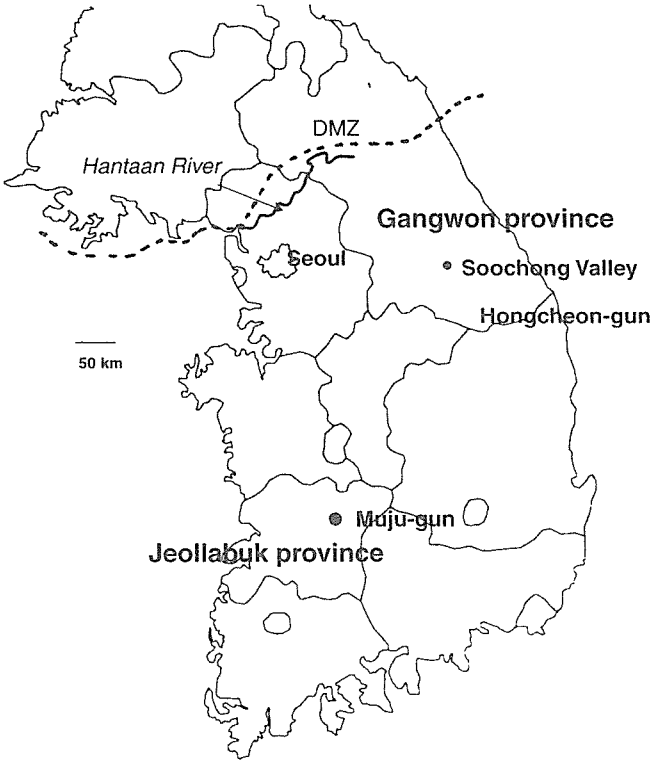

Fig. 1. Map of Korea, showing *A. peninsulae* capture sites, which led to the isolation of Soochong virus.

Antigenic Characterization of Hantaviruses by IFA Using Monoclonal Antibodies (MAbs)

A panel of MAbs that recognize the glycoproteins G1 and G2 and nucleocapsid protein of HTN or SEO viruses was employed in a standard IFA format [Arikawa et al., 1989; Yoshimatsu et al., 1996]. Briefly, Vero E6 cells, infected with hantaviruses (HTN 76–118, H5, and SOO-3) and cultured for 5 days, were trypsinized, suspended in MEM with 5% FBS and spotted onto 24-well slides. After incubation at 37°C for 4 hr, the slides were fixed with cold acetone, air-dried and used as antigen slides. Serially diluted MAbs (1:1 to 1:1,000 for MAbs that were derived from culture supernatant and 1:100 to 1:100,000 for MAbs that were derived from mouse-derived ascitic fluid) were spotted onto virus-infected Vero E6 cells and incubated for 1 hr at 37°C. After three washes with phosphate-buffered saline (PBS), fluorescein isothocyanate (FITC)-conjugated goat antibody to mouse IgG (ICN Pharmaceuticals, Inc., Aurora, OH) was added, incubated at 37°C for 1 hr, followed by three washes with PBS. Specific binding was detected by fluorescence microscopy.

Focus-Reduction Neutralization Test

Endpoint titers of neutralizing antibodies were determined by the focus-reduction neutralization test (FRNT). Mouse immune sera, which were prepared against H5, Bao14, and HTN 76–118 strains, and sera from antibody-positive *A. peninsulae* were used to

analyze the serological relationships between SOO and other hantaviruses [Lokugamage et al., 2002, 2004]. H5 and B78, isolated from Chinese HFRS patients, and Bao14, isolated from *A. agrarius* in China, were previously known as HTN virus strains [Liang et al., 1994; Wang et al., 2000; Lokugamage et al., 2002]. Serially two-fold dilutions of sera (100 μl) were incubated for 1 hr at 37°C with equal volumes of stock viruses (100 focus-forming U/100 μl); 100 μl of the virus-serum mixtures were then inoculated onto Vero E6 cell monolayers grown in eight-well slides. After adsorption for 1 hr at 37°C, the inocula were removed and MEM containing 1.5% carboxymethyl cellulose (CMC) was layered onto the cells. The slides were incubated in a CO_2 incubator at 37°C for 5–7 days. Monolayers were then washed with PBS, fixed with methanol at room temperature, and air-dried. Mouse immune serum to HTN 76–118 strain was added to the fixed Vero E6 cells, incubated for 1 hr at 37°C, followed by three washes with PBS. FITC-conjugated antibody to mouse IgG was added to the cells and incubated at 37°C for 1 hr. FITC-stained foci were counted under a fluorescence microscope. The FRNT titer was determined as the highest dilution of serum that showed 80% or greater reduction of focus formation.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA, extracted using RNAzol (GIBCOBRL, Gaithersburg, MD) from lung tissues of 16 hantavirus-seropositive *A. peninsulae* captured in 1997 and 1998 and from Vero cells infected with four strains of SOO virus, was reverse transcribed using the superscript II RNase H^- reverse transcriptase kit (GIBCOBRL). SOO virus sequences were then amplified by RT-PCR, using previously described and newly designed oligonucleotide primers [Xiao et al., 1991, 1992; Song et al., 2000, 2004]. These primers afforded the amplification of the entire S, M, and L segments of SOO virus. PCR products were cloned using the TOPO-TA cloning system (Invitrogen Corp., San Diego, CA), while plasmid DNA was purified by the QIAprep-spin Plasmid kit (QIAGEN, Inc., Chatsworth, CA). DNA sequencing was performed in both directions from at least three clones of each PCR product, using the dye primer cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) on an automated sequencer (Model 373A).

PCR Amplication of Mitochondrial DNA (mtDNA)

Total DNA was extracted from rodent liver tissues using the QIAamp Tissue Kit (QIAGEN). To study the phylogenetic relationship of *A. peninsulae* from various geographic regions, the cytochrome b region of mtDNA was amplified by PCR, using previously described universal primers that permitted amplification of 482-bp products [Bibb et al., 1981; Smith and Patton, 1991]. PCR was carried out in 50-μl reaction mixtures, containing 200 μM dNTP and 1.25 U of rTaq polymerase (Takara, Shiga, Japan). PCR fragments were amplified with an initial denaturation at 95°C for 4 min followed by 40 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min in a PTC-200 DNA Engine Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA). PCR amplicons were cloned, and sequenced, as indicated above.

Phylogenetic Analysis

SOO virus sequences from four Vero E6 cell-culture isolates were aligned and compared with previously published hantavirus sequences [Liang et al., 1994; Baek et al., 1998; Yashina et al., 2000, 2001], using the Clustal W method (Lasergene program version 5, DNASTAR, Inc., Madison, WI). The GenBank accession numbers for the S segment of SOO virus strains SOO-1, SOO-2, SOO-3, and SOO-4 were AY675349, AY675350, AY675351, and AY675352; for the M segment of strains SOO-1, SOO-2, and SOO-3, they were AY675353, DQ056293, and DQ056295; and for the L segment of strains SOO-1 and SOO-2, they were DQ056292 and AY675354, respectively. Phylogenetic trees were constructed by the neighbor-joining method, unweighted pair-group method of assortment (UPGMA), and maximum parsimony PAUP (Phylogenetic Analysis Using Parsimony, version 4). Topologies were evaluated by bootstrap analysis of 1,000 iterations [Swofford, 2003].

RESULTS

Serology and Virus Isolation

Using the IFA test, IgG antibodies against HTN virus were detected in 24% (6/25) of *A. peninsulae* captured on Mt. Gyebang and Mt. Gachil in Gangwon Province in August 1997, and in 35% (10/29) of *A. peninsulae* captured on Mt. Deogyu in Jeollabuk Province in September 1998 (Table I).

Characteristic hantavirus-specific fluorescence was detected by IFA in Vero E6 cells at 80 days after inoculation with 5% lung homogenates from hantavirus-seropositive *A. peninsulae*. The new hantavirus isolates in cell culture from *A. peninsulae*, designated SOO virus, were serially passed in Vero E6 cells. SOO virus strains SOO-1 and SOO-2 were isolated from *A. peninsulae* captured at Gangwon Province, Northeastern Korea, and strains SOO-3 and SOO-4 from Korean

TABLE I. Field Survey of Hantavirus Infection in Rodents at Mt. Gyebang at Mt. Gachil, in Hongcheon-gun, Gangwon Province, and Mt. Deogyu in Muju-gun, Jeollabuk Province, Republic of Korea, 1997–1998

Rodent species	Number of rodents captured	Number of seropositive (%)
Apodemus agrarius	25	2 (8.0)
Apodemus peninsulae	54	16 (29.6)
Eothenomys regulus	7	1 (14.3)
Micromys minutus	1	0 (0)

TABLE II. Antigenic Characterization by Cross-FRNT (80% reduction)

Antiserum	Animal	Virus			
		H5	HTN Bao14	HTN 76–118	SOO-3
H5[a]	Mouse	80	80	40	320
#61[b]	*A. peninsulae*	160	20	20	640
#63[b]	*A. peninsulae*	320	40	40	640
HTN Bao14	Mouse	40	1280	320	160
HTN 76–118	Mouse	<40	320	320	80

[a]Antisera against H5, HTN Bao14, and 76–118 were made from 5 weeks old ICR mice. Viruses (1×10^2–1.6×10^3 FFU) inoculated by subcutaneously and the mice were bled after 2 months. The underlined numbers are FRNT titers against homologus antisera and highest titer.
[b]Seropositive *A. peninsulae* were captured in the suburbs of Vladivostok, Russia.

field mice captured at Jeollabuk Province, South-Central Korea.

Antigenic Characterization by Monoclonal Antibodies

The antigenic characterization of HTN (strain 76–118), H5 and SOO (strain SOO-3) viruses was performed using a panel of MAb to glycoproteins G1, G2, and NP, which included two MAb to G1-1 (6D4 and 10F11), three to G1-b (16D2, 3D5, and 2D5), two to G2-a (HCO2 and 16E6), one to G2-b (EB06), one to G2-c (11E102-2), three to G2-d (17G6, 3D7, and 5B7), one to G2-e (20D3), six to G2-f (8E10, 23G10-1, 1C6, 7G6, 3B6, and 18F5), one to NP-1 (ECO2), and three to NP-III (C16D11, C24B4, and F23A1) [Arikawa et al., 1989; Lokugamage et al., 2004]. MAb 2D5 may recognize a specific epitope on G1-b of *A. agraius*-borne HTN virus, as evidenced by the strong reaction to HTN 76–118 and the absent reaction to H5 and SOO-3.

Focus-Reduction Neutralization Test

FRNT showed that SOO virus was antigenically distinct from *A. agrarius*-borne HTN virus 76–118 (Table II). The titers of anti-H5 serum and #61 and #63 sera against SOO virus strain SOO-3 were 8- to 32-fold higher than that against HTN virus 76–118. The titer of the anti-HTN virus 76–118 serum against HTN 76–118 was four-fold higher than that against SOO-3. These results suggest that *A. peninsulae*-borne SOO and H5 viruses are antigenically distinguishable from *A. agrarius*-borne HTN virus.

Sequence Analysis of Soochong Virus

The complete S segment, sequenced for the cell-culture isolates of SOO virus strains SOO-1, SOO-2, SOO-3, and SOO-4, was 1,695 nucleotides in length, with a predicted nucleocapsid protein of 430 amino acids starting at nucleotide position 37. Also, the SOO virus had a 371 nucleotide-length 3′ noncoding region (NCR). The intra-strain genetic divergence of the entire S segment of the Northeastern strains (SOO-1 and SOO-2) and South-central strains (SOO-3 and SOO-4) was 1.1% and 1.2% at the nucleotide level, respectively (Table III), whereas the divergence between the Northeastern and South-central strains was 12%–13% and 1.7%–2.9% at the nucleotide and amino acid levels, respectively. Six amino acid differences were observed between the SOO-1 and SOO-2 strains, and one amino acid differed between the SOO-3 and SOO-4 strains. Also six amino acids (positions 233, 241, 251, 271, 322, and 357) differed between the Northeastern and South-central strains. The entire S genomic segment of SOO virus SOO-1 strain diverged from HTN virus 76–118 strain by 15.6% and 3.5% at the nucleotide and amino acid levels, respectively.

TABLE III. Percent Nucleotide and Amino Acid Sequence Homologies of the Entire S, M, and L Segmenst Between Soochong Virus Strain SOO-1 and Other *Apodemus* Rodent-Borne Hantaviruses

Virus species/strain	S segment		M segment		L segment	
	1290 nt	430 aa	3615 nt	1135 aa	6533 nt	2151 aa
Soochong SOO-2	98.9	98.6	98.6	98.4	98.6	99.4
Soochong SOO-3	89.0	98.4	89.8	96.4	ND	ND
Soochong SOO-4	88.9	98.1	87.4	95.9	ND	ND
Soochong Liu	91.5	98.6	87.0	96.8	ND	ND
Amur H5	91.5	98.8	87.0	96.9	ND	ND
Amur SLAP61	91.2	99.8	ND	ND	ND	ND
Amur AP708	90.8[a]	99.5	ND	ND	ND	ND
Hantaan 76–118	84.4	96.5	80.7	91.1	81.4	95.6
Dobrava Greece	72.8	83.4	71.6	77.0	75.0	85.5

ND, not determined because of insufficient sequence data.
[a]Entire coding region of S segment.

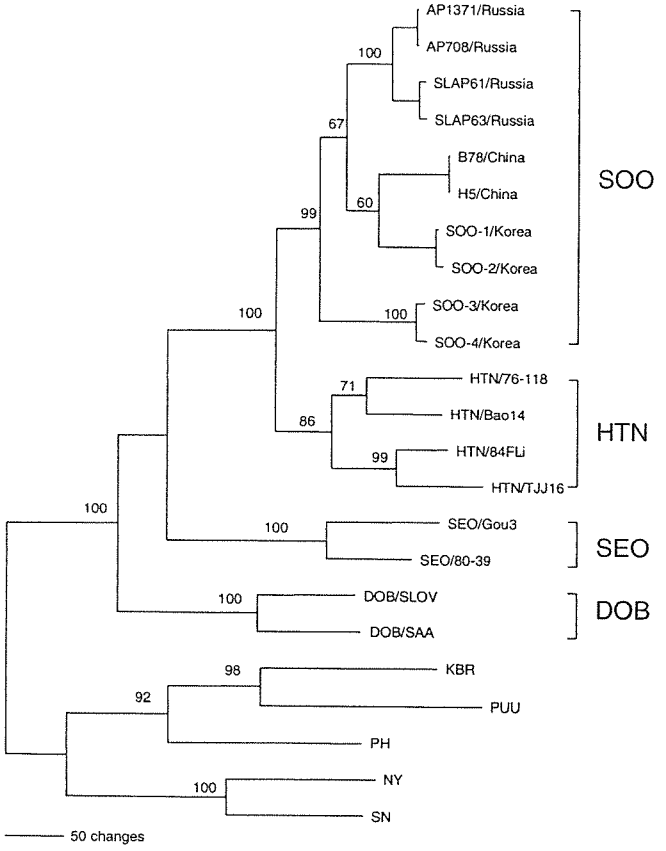

Fig. 2. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire coding region of the S segment of hantaviruses. The phylogenetic position of Soochong (SOO) virus strains is shown in relationship with hantavirus isolates from an HFRS patient in China (H5 and B78) and with Amur virus from *A. peninsulae* captured in Russia (SLAP61, SLAP63, AP708, and AP1317). Branch lengths are proportional to the number of nucleotide substitutions, while vertical distances are for clarity only. The numbers at each node are bootstrap probabilities (expressed as percentages), as determined for 1,000 iterations by PAUP version 4.0b.

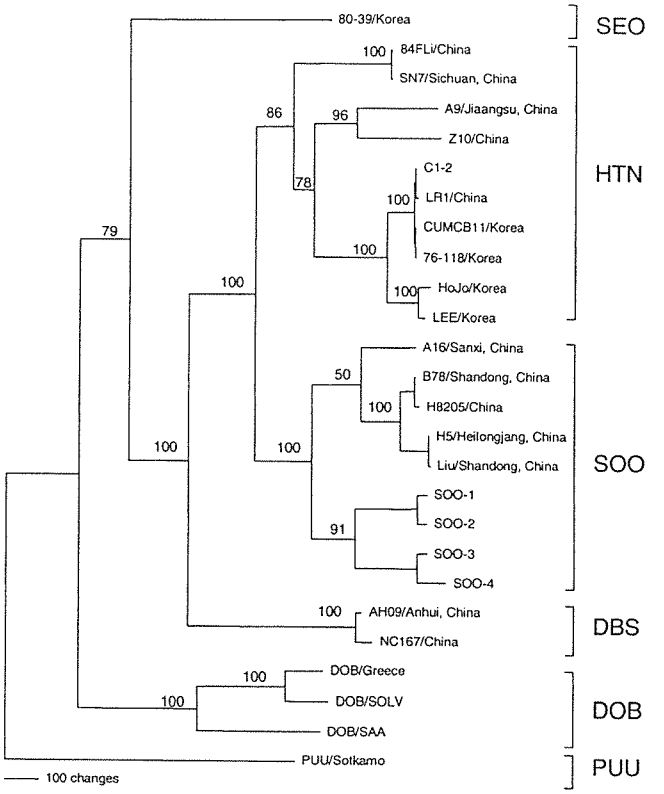

Fig. 3. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire M segment except 18-nucleotides of both ends of hantaviruses.

The full-length M genomic segment of SOO viruses was 3,615 nucleotides, with a predicted glycoprotein of 1,135 amino acids. Also, the SOO virus had 5′- and 3′-NCR of 40 and 166 nucleotides, respectively. The intra-strain genetic divergence of the entire M segment of Northeastern and South-central isolates was 1.5% and 3.2% at the nucleotide level, respectively, whereas the divergence between the Northeastern and South-central strains was 10.8%–14.1% and 3.5%–24.3% at the nucleotide and amino acid levels, respectively. The entire M genomic segment of SOO virus diverged from HTN virus 76–118 by about 22.6%–23.1% at the nucleotide level and 8.3%–9.5% at the amino acid level.

The entire L genomic segment of SOO viruses was 6,533 nucleotides, with a predicted coding capacity of 2,151 amino acids. The SOO 5′- and 3′-NCR was 37 and 39 nucleotides. The complete L genomic segment of SOO virus SOO-1 and SOO-2 strains diverged from HTN virus 76–118 by 21.8%–21.9% at the nucleotide level and 4.6% at the amino acid level.

Phylogenetic Analysis

Phylogenetic analyses of the nucleotide sequences of the S, M, and L segments of SOO virus and other hantaviruses, using the maximum parsimony and neighbor-joining methods, indicated that SOO virus was distinct from HTN virus and was more closely related to B78 and H5 viruses from China and Amur virus from Far East Russia (Figs. 2–4). Topologies were supported by bootstrap analysis of 1,000 iterations. A neighbor-joining tree, based on the 268-nucleotide partial M segment of hantaviruses (nucleotide position 2034–2301 compared to SOO-1 strain) showed that SOO virus shared a common ancestry with Amur virus from Russia and H5 virus from China, and formed a geographic-specific cluster of *A. peninsulae*-borne hantavirus strains (Fig. 5).

Phylogenetic analysis based on a 424-nucleotide cytochrome b region of mtDNA sequences showed that *A. peninsulae*, *A. agrarius*, and *A. flavicollis* were distinct species that co-evolved with their hantaviruses (Fig. 6).

DISCUSSION

The number of HFRS cases in Korea has decreased significantly since a hantavirus vaccine (Hantavax®) was approved for use by the Korean Food and Drug Administration in 1990 [Cho et al., 2002]. However,

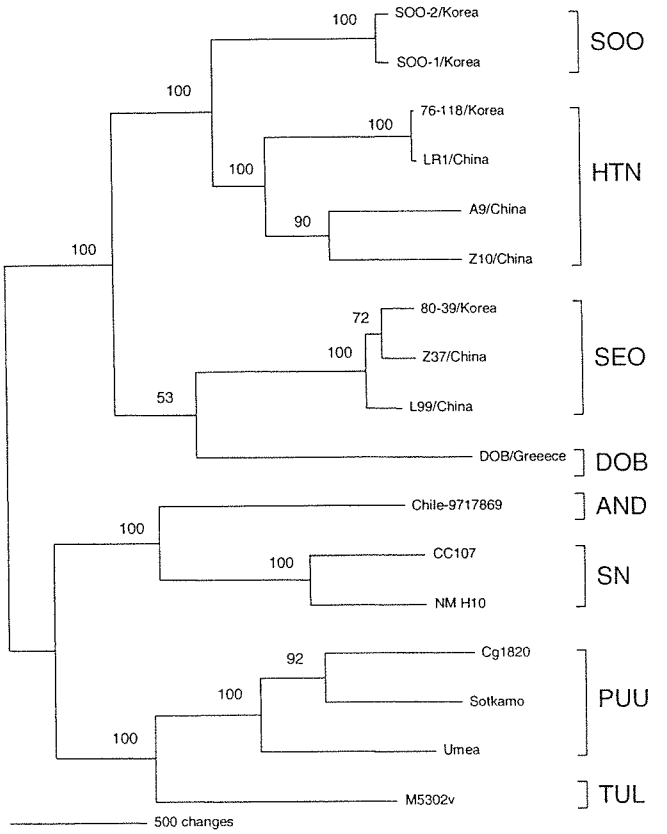

Fig. 4. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire L segment of hantaviruses.

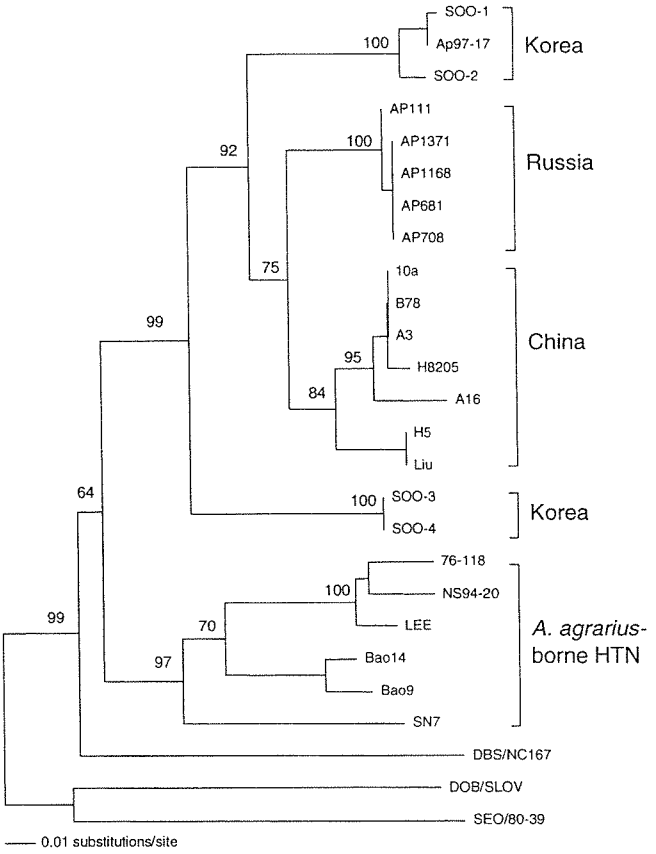

Fig. 5. Neighbor-joining trees, rooted at the midpoint, based on the 268-nucleotide partial M segment of hantaviruses (nucleotide position 2034–2301 compared to SOO-1 strain). Ap97-17 strain was amplified from *A. peninsulae* captured at Mt. Gyebang, Gangwon Province, Korea, 1997.

other factors, including ecological and environmental changes may also be responsible for this declining trend of HFRS. A recent case-control study reported that the effectiveness of Hantavax™ depended principally on the number of doses received (with protection rates of 25%, 46%, and 75% for one, two and three doses, respectively) in Korea [Park et al., 2004]. Despite these epidemiological trends, HFRS continues to be regarded as one of the principal acute febrile diseases during the autumn season among military personnel and civilians in Korea [Sachar et al., 2003]. Of the approximately 100–300 HFRS cases (with 4.5% overall mortality) occurring annually in Korea, HTN virus is the primary etiologic agent, accounting for 70% of all cases, with 20% being attributed to SEO virus, and the remaining 10% to as yet undefined hantaviruses.

Each genetically distinct hantavirus appears to have co-evolved with one or a few closely related rodent species. Phylogenetic clustering of murid rodent-borne hantaviruses and their rodent reservoir hosts lend further support to the concept that hantaviruses co-evolved with rodents. *Apodemus* mice are the most common rodents inhabiting woodlands, tall grasses, rice paddies, and broadleaf forests in the temperate zone of the Palearctic region. Two or more species may be sympatric, or coexist in the same forest (e.g., *A. agrarius* and *A. peninsulae* in Korea). The Korean field mouse (*A. peninsulae*) has a wide geographic distribution, extending from eastern and southern Siberia, Manchuria, northeastern and central China, Korea, and as far easterly as Sakhalin and Hokkaido [Nowak, 1999]. This is the second-most dominant rodent species in Korea, and is primarily found in forested mountainous areas >500 m in elevation.

Four strains of SOO virus, SOO-1, SOO-2, SOO-3, and SOO-4, were isolated in Vero E6 cells from lung tissues of *A. peninsulae* captured in two geographically distant provinces at separate times. These SOO virus strains are the first hantaviruses isolated in cell culture from *A. peninsulae*. Although genetically similar hantaviruses, H5 and B78, have been isolated from HFRS patients in China [Liang et al., 1994], no report is currently available on the isolation of Amur virus from *A. peninsulae* in Russia. The antigenic characterization by a panel of MAb to glycoproteins G1, G2, and NP showed that MAb 2D5, which recognizes an epitope on the G1-b region, could distinguish the *A. peninsulae*-borne SOO hantavirus from the *A. agrarius*-borne HTN virus. Also, MAb 16D2 of the G1-1b region reacted with HTN 76–118 and H5 viruses, but not with SOO virus, suggesting that there is a distinct epitope between SOO

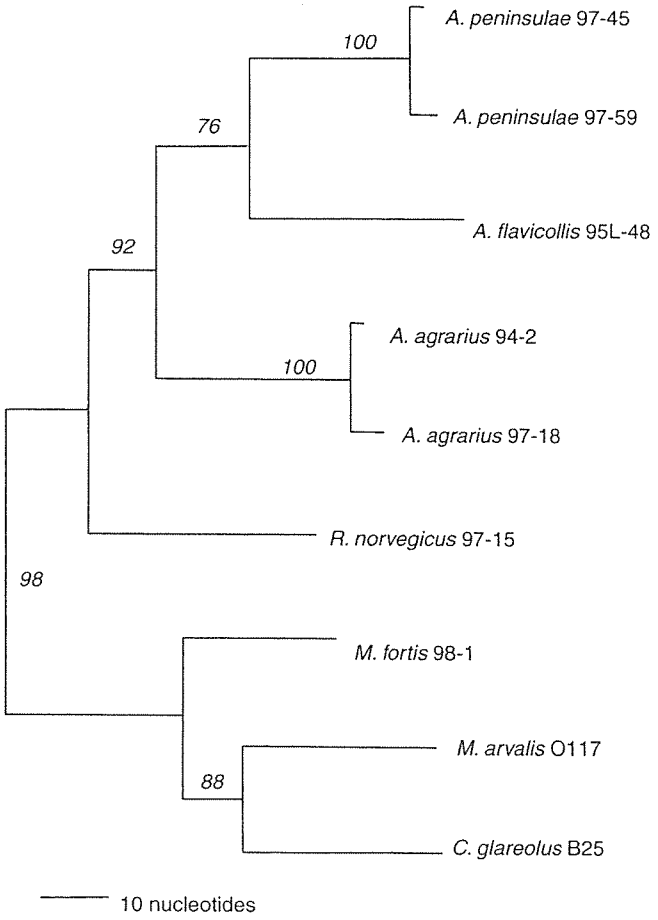

Fig. 6. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on a 424-nucleotide region of the cytochrome b gene of mtDNA from reservoir rodent species of hantaviruses. mtDNA sequences were amplified from liver tissues of *A. peninsulae* 97-45 (SOO-1) and 97-59 (SOO-2), *A. agrarius* 94-2 and 97-18, *R. norvegicus* 97-15 and *M. fortis* 98-1 captured in Korea; *A. flavicollis* 95-L48 and *M. arvalis* O117 captured in Poland; and *C. glareolus* B25 from Sweden.

and H5. The cross-neutralization test using anti-HTN 76–118 serum showed that the titer to HTN 76–118 and Bao14 were 320. However, the NT titer to SOO-3 and H5 were 80 and <40, respectively. These cross-NT results and previously reported M segment sequences suggest that *A. peninsulae*-borne SOO and Amur viruses are antigenically distinguishable from *A. agrarius*-borne HTN virus [Lokugamage et al., 2004].

In our previous report on the genetic diversity of HTN virus in Korea, H5 was an outgroup of all other Korean and Chinese HTN virus strains [Song et al., 2000]. Phylogenetically, the Northeastern SOO virus strains (SOO-1 and SOO-2) were distinguishable from the South-central SOO virus strains (SOO-3 and SOO-4) in Korea. SOO virus isolated from the Korean field mouse in Korea shared a common ancestry with Amur virus strains, including SLAP61, SLAP63, AP708, and AP1371 from *A. peninsulae* of Far East Russia, as well as with H5 and B78 viruses from HFRS patients in Heilongjiang and Shandong, China [Liang et al., 1994; Wang et al., 2000; Lokugamage et al., 2002]. Thus, *A. peninsulae*-borne hantaviruses were evolutionarily distinct from HTN and DOB viruses harbored by *A. agrarius* and *A. flavicollis*, respectively. The high seropositivity rates among *A. peninsulae* captured in certain mountainous regions in Korea suggest that HFRS caused by SOO virus may be under-reported among nearby residents or among individuals who might visit such areas for recreational or occupational purposes.

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A pseudotype vesicular stomatitis virus containing Hantaan virus envelope glycoproteins G1 and G2 as an alternative to hantavirus vaccine in mice

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Abstract

We examined whether a vesicular stomatitis virus (VSV) pseudotype bearing the hantavirus envelope glycoproteins (GPs) G1 and G2 (VSVΔG*HTN) could be used as a safe and effective alternative to native hantavirus. Mice were immunized with purified particles of VSVΔG*HTN. After the second immunization, all mice produced anti-GP antibody as detected in ELISA and a neutralization test. After the third immunization, the mice were challenged with Hantaan virus. Neither anti-NP antibody production nor Hantaan virus-specific CD8 T-cell reactions were detected in these mice. The present study demonstrated the potential of using a pseudotype VSV system as a tool for developing a hantavirus vaccine.

Keywords: Envelope; Hantavirus; Pseudotype

1. Introduction

Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) are rodent-borne viral zoonoses caused by viruses in the *Hantavirus* genus of the *Bunyaviridae* family. Among the 22 currently registered virus species within the genus, the Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava virus, and Puumala virus (PUUV) are causative agents of HFRS in Eurasia, while the Sin Nombre virus, Andes virus, New York virus, and other related viruses are known to cause HPS in North and South America [1].

Each virus species is carried by a specific rodent species in nature, thus restricting the virus' prevalence to the regions populated by these rodents [1,2]. HFRS has been reported throughout Eurasia and particularly in China, where tens of thousands of cases are reported annually [3]. In addition, thousands of cases have been reported in Europe and Far East Asia [4], and several hundred have been reported in both North and South America [1]. The mortality rates of HFRS and HPS patients are 0.1–10% and around 40%, respectively [1]. Thus, both HFRS and HPS are important zoonoses from a public health perspective.

Hantaviruses contain a single-stranded, negative-sense RNA genome that is divided into three segments, designated large (L), medium (M), and small (S) [5]. The L segment encodes RNA-dependent RNA polymerase. The S segment encodes nucleocapsid protein (NP), and the M segment encodes a glycoprotein (GP) precursor that is cotranslationally cleaved into the envelope proteins G1 and G2 [5]. The G1 and G2 proteins form projections on the virion surface, induce low-pH dependent cell fusion [6], and are the targets of neutralizing antibodies [7,8].

Inactivated HTNV, SEOV, or PUUV vaccines prepared from the viruses grown in suckling mouse brain or tissue culture cells have been developed in Korea and China [9–11]. Although limited efficacy has been observed, attempts have been made to develop recombinant protein-based vaccines

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that reduce or eliminate biohazard risks during preparation as well as achieve higher efficacy. Immunizations with recombinant G1 and G2 proteins expressed by baculovirus and vaccinea virus induce very low or negligible amounts of neutralizing antibody [12–14]. Although the antibody titers are high, this low efficacy for inducing neutralizing antibody may be attributable to the incorrect conformation of the recombinant G1 and G2 compared to the native proteins.

We recently expressed the G1 and G2 of HTNV using a mammalian cell expression vector. In addition, by supplying the G1 and G2 proteins *in trans* to the infected cells with recombinant vesicular stomatitis virus (VSV), whose envelope G protein was altered to enhanced green fluorescent protein (eGFP), we generated the pseudotype VSV (pVSV), which possesses the HTNV envelope proteins G1 and G2 (VSVΔG*HTN) [15]. Infection by VSVΔG*HTN was inhibited by neutralizing monoclonal antibodies (MAbs) and patient sera. The neutralizing antibody titers were virtually identical to those measured with native virus. These results indicated that the structure and function of the expressed G1 and G2 effectively mimics the native viral proteins. Based on these results, we examined the applicability of pVSV as a possible tool for producing inactivated vaccine that may induce neutralizing antibody more efficiently than the previously examined recombinant proteins.

2. Materials and methods

2.1. Viruses and cells

HTNV strain 76–118 was propagated in the Vero cell E6 clone (ATCC C1008). The 293T cell is derived from the human embryonic kidney cell line 293 and contains the simian virus 40 large T antigen. The 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 0.45% glucose and 10% heat-inactivated fetal calf serum (FCS). The recombinant baculovirus for expressing HTNV viruses G1 and G2 was kindly supplied by Dr. Connie S. Schmaljohn [12] and was propagated in high five™ cells.

2.2. Mice

Specific-pathogen-free, 6-week-old female BALB/c/slc mice were obtained from SLC (Hamamatsu, Japan). All mice were treated in accordance with the laboratory animal control guidelines of our institute, which conform to those of the US. National Institutes of Health. All experiments were conducted in a BSL class P3 facility.

2.3. Production of pseudotype VSV

Pseudotype VSV containing HTNV G1 and G2 (VSVΔG*HTN) or the G protein of VSV (VSVΔG*G) was prepared in 293T cells as previously described [15]. Briefly, 36 h after the transfection of 293T cells with expression vectors based on pCAGGS/MCS containing the coding information of the glycoproteins of HTNV, SEOV, or G protein of VSV, the cells were infected with VSVΔG*G at a multiplicity of infection (MOI) of one for 1 h at 37 °C. VSVΔG*G, whose genome had the enhanced GFP gene instead of the G protein, was kindly provided by Dr. Michael A. Whitt. The 293T cell monolayer was then washed with 1% heat-inactivated FCS–PBS three times, and culture medium was added. After a 24-h incubation at 37 °C in a CO_2 incubator, the culture supernatant was clarified by low-speed centrifugation and stored at −80 °C.

2.4. Purification of pseudotype VSV

The culture supernatant containing pseudotype VSV was purified by ultracentrifugation at 100,000 rpm for 1 h through a 20–60% sucrose cushion in TNE buffer (10 mM Tris, 135 mM NaCl, 2 mM EDTA) with a type-50 titanium rotor in a Beckman L-80 ultracentrifuge (Beckman Instruments, Palo Alto, CA). Virions were recovered from the sucrose cushion and measured as the hemagglutination (HA) activity of goose erythrocytes [16,17].

2.5. Titration of pseudotype VSV

Vero E6 cells grown on eight-well slides were infected with 50 μl of serially diluted virus stock. After a 1-h adsorption period, the inoculum was removed; fresh culture medium was added, and the cells were incubated at 37 °C in a CO_2 incubator. At 16 h postinfection, the cells were fixed with 10% formalin in PBS for 10 min at room temperature, washed with distilled water, and air-dried. Fluorescent GFP-expressing cells were counted under a fluorescence microscope. Because pseudotype VSV is unable to produce infectious progeny, the numbers of GFP-positive cells were regarded as infectious units (IU).

2.6. Immunization and sample collection

Eight mice were immunized subcutaneously three times at intervals of 3 and 2 weeks after the first immunization (Fig. 1) with purified particles of VSVΔG*HTN

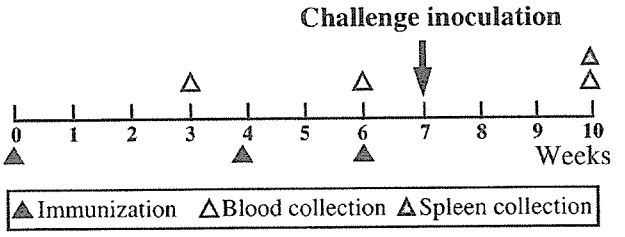

Fig. 1. Schedule of experiment. Immunizations with VSVΔG*HTN, VSVΔG*G, or PBS are shown as closed triangles. The collection of serum samples is shown as open triangles.

(1×10^7 IU/mouse/50 μl) mixed with 50 μl of CpG DNAadjuvant (ImmunEasy Mouse Adjuvant; Qiagen GmbH, Hilden, Germany). The adjuvant contained immunostimulatory CpG™ DNA short oligonucleotides that had unmethylated cytosine–guanine dinucleotides within a certain base context. Four mice were immunized with VSVΔG*G as controls. Fifty microliters of blood was collected from the mice under ether anesthesia, at the intervals described in Fig. 1 by retro-orbital plexus puncture. The blood was immediately mixed with 200 μl of PBS and centrifuged to remove blood cells. The supernatant represented an approximately 1:10 dilution of serum. The sera were inactivated by exposure at 56 °C for 30 min. All serum specimens were stored at −30 °C prior to antibody titration. After 3 weeks of HTNV inoculation, the spleen was collected under ether anesthesia. Spleen single-cell suspensions were obtained using previously published methods [18] and stored at −80 °C in freezing medium (Cell Banker; Jujin, Tokyo, Japan) prior to $CD8^+$ T cell analysis.

2.7. HTNV infection

Seven groups of six, 6-week-old female BALB/c mice were inoculated intraperitoneally with various doses (10^{-3} to 10^{-8} dilution) of HTNV diluted in PBS, and the antibody responses were examined 3 weeks after inoculation. Based on the ratio of seropositive mice, the ID_{50} of HTNV was determined as 0.127 focus-forming units (FFU). Mice were inoculated with 4.0 FFU of HTNV in the challenge experiment.

2.8. Purification and papain digestion of MAb clone 11E10-2-2

MAb clone 11E10-2-2, which recognizes G2 of HTNV [7], was purified by protein A column chromatography (Affi–Gel, MOPS II kit; Bio-Rad, Hercules, CA). To prepare the Fab fragment of the MAb, purified IgG was digested with papain (P-3125; Sigma–Aldrich, St. Louis, MO) as previously described [19]. Briefly, papain digestion was performed with an enzyme-to-antibody ratio of 1:400 in standard PBS buffer containing 3 mM EDTA and 10 mM-cysteine at 37 °C. The reaction was stopped with the addition of fresh iodoacetamide solution to a final concentration of 10 mM followed by incubation in the dark at 4 °C for 30 min. The digested MAb was then passed through protein A columns under the same experimental conditions used for the purification of IgG. The unbound fraction (Fab fragment) and bound fractions (Fc fragment and undigested IgG) were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

2.9. ELISA

To detect antibody specific to HTNV GP, ELISA was performed using the Fab fraction of MAb 11E10-2-2, which was directed to HTNV GP as the capture antibody. The 96-well microtiter plates (FALCON 3915; Falcon, Franklin Lakes, NJ) were coated with Fab of MAb 11E10-2-2 (50 μg/ml) in PBS and incubated at 4 °C overnight. After three washes with PBS containing 0.05% Tween-20 (PBS-T; Wako, Osaka, Japan), High Five™ cell lysate containing recombinant GP as previously described [20] was added to the plates for 1 h at 37 °C. After washing three times with PBS-T, the wells were filled with Block Ace (Yukijirushi, Tokyo, Japan), incubated at room temperature for 30 min, and washed again. The mouse sera were diluted at 1:200 with ELISA buffer containing PBS-T with 0.5% BSA (A-4503; Sigma–Aldrich) and were then added to the wells. The plates were incubated at 37 °C for 1 h and washed three times with PBS-T. Bound antibodies were detected with 0.5 μg/ml protein A–horseradish peroxidase conjugate (Biogenesis, San Leandro, CA) in ELISA buffer. Wells were developed with 100 μl of substrate solution, which contained 10 mg *o*-phenylenediamine (Sigma–Aldrich) plus 6 ml of 0.02% H_2O_2 (Wako). After a 15-min incubation in the dark at room temperature, the absorbance at 450 nm was read using a microplate spectrophotometer (Spectra Max 340; Molecular Devices, Sunnyvale, CA). For ELISA to detect antibody specific to NP, recombinant HTNV NP was used as the antigen. The ELISA procedure was as previously described [21].

2.10. Focus reduction neutralization test

The focus reduction neutralization test (FRNT) using HTNV and Vero E6 cells was performed as previously described [22]. The FRNT titer was expressed as the reciprocal of the highest dilution that gave a reduction of greater than 80% in the number of infected cell foci.

2.11. Detection of HTNV-specific $CD8^+$ T cells

To detect HTNV-specific $CD8^+$ T cells, we used flow cytometry to assay for intracellular gamma interferon (IFN-γ) of $CD8^+$ T cells stimulated by incubation with HTNV-infected antigen-presenting cells, as previously described [18].

3. Results

3.1. ELISA and FRNT antibody responses in mice immunized with pseudotype VSV

The antibody responses of mice immunized with pseudotype VSVs were measured by ELISAs for specific antibodies to HTNV GP (Fig. 2) or to HTNV NP (Fig. 3). Although FRNT antibody was not detected in any mice except one at 3 weeks after the first immunization with VSVΔG*HTN, a low level of anti-GP antibody was detected in a few mice compared to control mice immunized with VSVΔG*G or PBS (Table 1, Fig. 2). After the second immunization, all mice

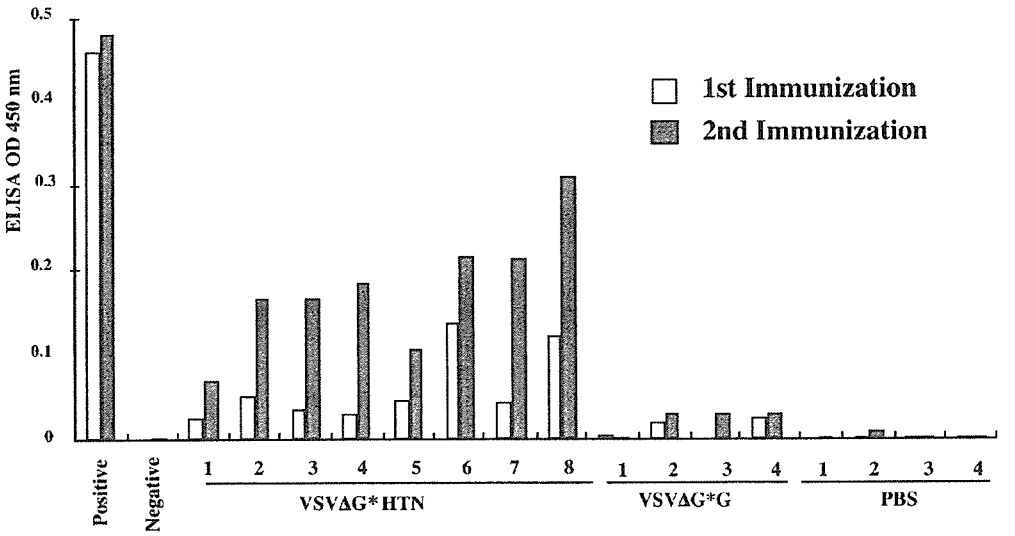

Fig. 2. Antibody responses against hantavirus GP before challenge inoculation. Anti-GP antibodies were measured using ELISA; the recombinant GP was expressed in insect cells by the baculovirus vector system as described in Section 2. The positive control was serum from a hyperimmune mouse experimentally inoculated with HTNV. The negative control was serum from an uninfected mouse.

immunized with VSVΔG*HTN possessed both FRNT antibody and anti-GP antibody as detected by ELISA (Table 1, Fig. 2). However, NP-specific antibody induction was not detected in any of the mice inoculated with VSVΔG*HTN, VSVΔG*G, or PBS alone (Fig. 3). These results indicated that VSVΔG*HTN was able to induce GP-specific antibody in mice.

The neutralizing activity of the GP-specific antibody was measured using the 80% FRNT. As summarized in Table 1, all eight mice immunized with VSVΔG*HTN developed FRNT antibody at titers ranging from 1:40 to 1:160 within 2 weeks after the second immunization. All control mice inoculated with either VSVΔG*G or PBS alone were negative for FRNT antibody.

3.2. Evaluation of protective immunity induced by pseudotype VSV for preventing HTNV challenge in mice

To assess the protective immunity, mice were challenged with HTNV at 1 week after the third immunization (Fig. 1). Although exceptional fatal infection models in adult mice have been reported [23,24], hantavirses are generally non-pathogenic to mature rodents. In this study, we used a general HTNV strain. Therefore, it is difficult to evaluate protective immunity. To differentiate the antibody response induced by immunization from that induced by HTNV infection, seroconversion against NP accompanied with the establishment of infection was used as an index for protection (Fig. 2). In addition, the HTNV-specific $CD8^+$ T cell response was also

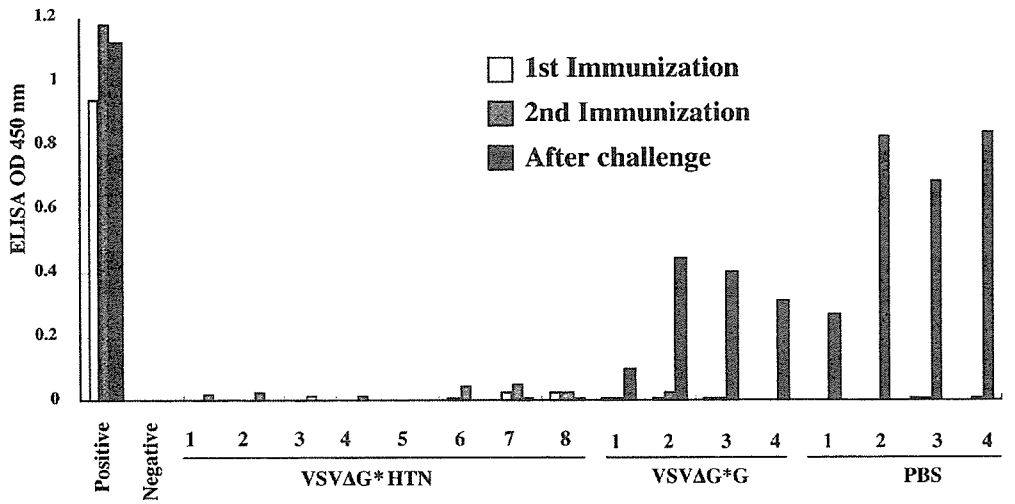

Fig. 3. Antibody responses against HTNV N protein after challenge inoculation. Anti-N protein antibodies were measured using ELISA; recombinant NP was expressed in *Escherichia coli* as described in Section 2. The positive control was serum from a hyperimmune mouse experimentally inoculated with HTNV. The negative control was serum from an uninfected mouse.

Table 1
Neutralizing antibody responses of mice immunized with VSVΔG*HTN

Antigen	Mouse ID	Titer for 80% FRNT			
		1st imm.	2nd imm.	Post-challenge	Protection
VSVΔG*HTN	1	<40	40	40	Yes
	2	<40	40	80	Yes
	3	<40	40	80	Yes
	4	<40	40	80	Yes
	5	40	80	80	Yes
	6	<40	160	160	Yes
	7	<40	160	80	Yes
	8	<40	80	40	Yes
VSVΔG*G	1	<40	<40	40	No
	2	<40	<40	160	No
	3	<40	<40	160	No
	4	<40	<40	80	No
PBS	1	<40	<40	80	No
	2	<40	<40	80	No
	3	<40	<40	80	No
	4	<40	<40	80	No

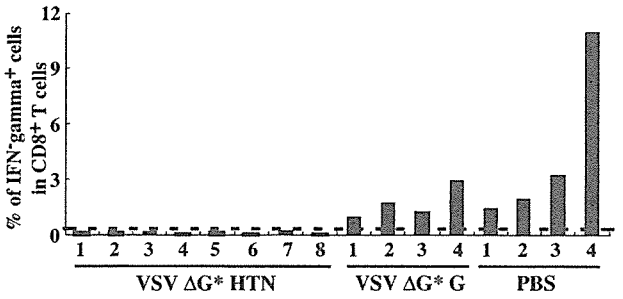

Fig. 4. HTNV-specific $CD8^+$ T cell responses after challenge inoculation. HTNV-specific $CD8^+$ T cells were counted as IFN-γ-producing cells; values are expressed as percent of total splenic $CD8^+$ T cells. A horizontal broken line indicates the limitation of the background level in this assay.

measured as an index of protection. All mice immunized with VSVΔG*HTN remained negative for anti-NP antibody in ELISA, and no detectable level of HTNV-specific $CD8^+$ T cells was observed in spleen cells at 3 weeks after the challenge (Fig. 4), indicating that the mice were protected from HTNV infection. In contrast, seroconversion was detected in VSVΔG*G-immunized mice and in PBS control mice. Furthermore, a significant number of HTNV-specific $CD8^+$ T cells was observed only in the control mice. These results indicate that protective immunity was not induced in these mice, confirming that immunization with VSVΔG*HTN conferred protective immunity.

4. Discussion

In general, virus proteins that are targeted by vaccines are observed on the outer surfaces of the virions or the envelope proteins. In addition, it is well-known that the structure of virus like particles (VLPs) in combination with envelope proteins strengthens their immunogenicity. Therefore, the combination of VLPs and envelope proteins was expected to be an effective material for vaccine development. VLPs with recombinant hepatitis B surface proteins expressed in yeast provided a remarkably successful application for vaccine development [25]. Chimeric HBc particles with the hantavirus NP sequence have been reported to induce high antibody titer to NP, as well as to confer protective immunity in mice models [26]. However, similar applications have not been reported with hantavirus GPs.

In our previous study, we succeeded in producing VSV pseudotypes bearing hantavirus GPs, and these GPs were considered to have structures similar to those of native hantavirus GPs based on neutralization pattern profiling of Mabs and polyclonal antibodies [15]. Furthermore, because the pseudotype VSV lacks a gene for GP, it also lacks replication activity. Therefore, we anticipated that the pseudotype virion could be applicable as a safe alternative to authentic virion for use in vaccines. In this study, we used the particle of pseudotype VSVΔG*HTN as a type of VLP and examined its antigenicity in a mouse model.

Epitope analyses of GPs using MAbs against G1 and G2 showed that both G1 and G2 were associated with the FRNT-related epitope. The FRNT-related epitope was composed of partially overlapping epitopes on G1 and G2 [7,27,28] and might be related to the inhibition of the membrane fusion step [6]. In this study, we measured only anti-GP2 antibody in the ELISA because of the difficulty with the methodology. Because the FRNT epitopes on G1 and G2 overlap each other, it was expected that anti-G2 antibody was related to the rise in anti-G1 antibody. Actually, anti-G2 antibodies were correlated to FRNT titers as shown in Table 1.

As observed in this study, the pseudotype VSVΔG*HTN was able to induce neutralizing antibody with titers comparable to those reported for inactivated vaccines in humans as well as for recombinant proteins in the mouse model [10,11,29]. Furthermore, the pseudotype VSVΔG*HTN conferred protective immunity for hantavirus challenge in the mouse model. The fact that an HTNV-specific $CD8^+$ T-cell response was not observed in immunized mice after challenge also confirmed that the induced neutralizing antibody alone was effective for protection from the challenge inoculation. This is the first successful application of pseudotype VSV as a type of VLP for the induction of protective immunity. Few studies on the application of pseudotype virus as a vaccination antigen have been reported. In hepatitis type C virus (HCV), pseudotype virus was developed and applied to analyze virus–cell interactions and the assembly of virus particles [30,31]. Beyene et al. [32] tried to apply pseudotype VSV incorporating the envelope glycoprotein of HCV to vaccine development. In their experiment, neutralization antibody against pseudotype virus was successfully induced; however, there is no system for detecting neutralizing and protective activity against authentic HCV in vitro or in vivo [32].

Although an inactivated hantavirus vaccine has been developed and a protective efficacy comparable to those

of other virus vaccines has been reported, low efficacy for the induction of neutralizing antibody remains an important aspect to be overcome [9–11,29]. Further studies are needed to clarify the potential of the pseudotype VSV with HTNV GPs as a useful tool for the development of hantavirus vaccine. It has been reported that the M protein of VSV strongly induces budding. The characteristics of VSV M protein suggest that the pseudotype VSV system could be applied to other viruses for which VLP development has been unsuccessful.

Owing to the structural complexity of HTNV GP, a component vaccine made from recombinant GP proteins has not been developed. However, DNA vaccine trials have been reported [8] in which the DNA vaccine was able to induce a significantly high level of FRNT antibody in animals; this indicates that the recombinant GP expressed in mammalian cells possesses sufficient antigenicity to induce FRNT antibody. These results also imply that the fixation step during the preparation of inactivated hantavirus vaccine may be responsible for the preservation of antigenicity of the inactivated vaccine. The pseudotype virion used here could be developed as a useful tool with which to evaluate fixation methods for virus particles. By comparing the immunogenicity of unfixed and fixed pseudotype virions, we were able to estimate the damage done to GP molecules by fixation during the preparation of the hantavirus vaccine. Careful examination of the appropriate fixation conditions for inactivated vaccines may lead to higher efficacy.

The pseudotype VSV used in this study can replicate to express reporter genes, such as GFP, and exhibit a cytopathic effect. Therefore, for the practical application of pseudotype virion to vaccine, the development of VLPs lacking a replication system and containing HTNV GPs and VSV M protein should be pursued.

Acknowledgments

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GEOGRAPHICAL DISTRIBUTION OF HANTAVIRUSES IN THAILAND AND POTENTIAL HUMAN HEALTH SIGNIFICANCE OF *THAILAND VIRUS*

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Abstract. Phylogenetic investigations, sequence comparisons, and antigenic cross-reactivity studies confirmed the classification of *Thailand virus* (THAIV) as a distinct hantavirus species. The examination of sera from 402 rodents trapped in 19 provinces of Thailand revealed that five greater bandicoot rats (*Bandicota indica*) and one lesser bandicoot rat (*B. savilei*) from four provinces were focus reduction neutralization test (FRNT) antibody-positive for THAIV. One of 260 patients from Surin province in Thailand (initially suspected of having contracted leptospirosis, but found to be negative) showed symptoms compatible with hemorrhagic fever with renal syndrome (HFRS). The serum of this patient showed high titers of hantavirus-reactive IgM and IgG. FRNT investigations confirmed virus-neutralizing antibodies against THAIV. These observations suggest that THAIV or THAI-like viruses occur throughout Indochina and may represent an additional causative agent of HFRS.

INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) is a rodent-borne viral zoonosis caused by certain members of the viruses in the genus *Hantavirus* of the family *Bunyaviridae*.[1] The hantaviral species that have been causally associated with HFRS are *Hantaan virus* (HTNV), *Seoul virus* (SEOV), and *Dobrava/Belgrade virus* (DOBV) that are carried by the members of the rodents in the subfamily *Murinae* (Old World rats and mice), while the *Puumala virus* (PUUV) is carried by the members of the rodents in the subfamily *Arvicolinae* (voles and lemmings) of the family *Muridae*. Other hantaviral species that are not known as the causative agents of HFRS include *Tula virus* (TULV) and *Topografov virus* (TOPV) in Europe, *Khabarovsk virus* (KHAV) in far east Russia, and *Prospect Hill virus* (PHV) in the United States that are carried by rodents in the subfamily *Arvicolinae*. The *Thailand virus* (THAIV) is the only hantavirus species carried by the rodent in the subfamily *Murinae* in Thailand. *Thottapalayam virus* (TPMV) is the only hantavirus isolated from mammals in the Insectivore in India.[2]

The species of hantaviruses isolated from the rodents in the same subfamily; HTNV, SEOV, DOBV, and THAIV from rodents of subfamily *Murinae* and PUUV, TULV, TOPV, KHAV, and PHV from rodents of subfamily *Arvicolinae*, showed strong antigenic cross reactivity defined by antibody binding assays such as IFA and ELISA. Neutralization test is required to serologically distinguish among hantavirus species originated from rodents classified to the same subfamily.[3]

The THAIV strain Thai749 was originally isolated by Elwell et al. (1985) from a greater bandicoot rats (*Bandicota indica*) trapped in the vicinity of a small farm village in the western province of Kanchanaburi,[4] Thailand. Subsequent phylogenetic studies based on the nucleotide sequence of M segment of THAIV revealed that the THAIV is placed at the position most closely related to SEOV and grouped with other viruses from rodents classified to *Murinae*.[5] Thai749 strain is antigenically distinct from other hantavirus species.[3] However, only part of the nucleotide sequence information in the S segment of the THAIV is available so far.[5] For further understanding of THAIV of the relationship among other hantaviruses, nucleotide sequence information of entire S segment as well as further antigenic characterization is required.

It has been well characterized that a single rodent species or phylogenetically closely related rodent species are the principal host of a single hantavirus species.[6] The rodent fauna of Thailand includes 35 murine species in 7 genera and 1 arvicoline species, *Eothenomys melanogaster*.[7] A previous seroepizootiologic study of hantavirus infection conducted at central, northeastern, and near Bangkok areas revealed that greater bandicoot rat as a main reservoir and several species of rice field rats such as *Rattus rattus*, *exulans*, and *losea* are also natural reservoirs to a lesser extent in Thailand.[4] To extend our knowledge of the geographical distribution and natural host association of the hantaviruses in Thailand, we have continued further seroepizootiologic study, particularly by including the THAIV as antigen for serological screening.

Although the hantavirus infection spread in various species of rodents and wider areas in Thailand, epidemiologic information regarding to the human infection with hantavirus is quite limited. Suputthamongkol et al.[8] reported the first clinical case report of hantavirus infection in Thailand. However, the causative hantavirus species was not further characterized in the report. Since the clinical symptoms of leptospilosis and other febrile illness are similar to HFRS, undiagnosed HFRS cases would be existing among the patient with febrile illnesses of unknown etiology in Thailand.

In this study, we have examined antigenic and genetic prop-

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erties of THAIV to provide new insights into the relatedness of THAIV to other hantavirus species and confirm the classification of THAIV as a distinct hantavirus species. In addition, serological surveillance of hantavirus infection among rodents indicated the prevalence of THAIV infection mainly among giant bandicoot rats and *Rattus* species in Thailand. Finally, we present the first case of an infection with THAIV or a hantavirus antigenically more closely related to THAIV than to HTNV, SEOV, or DOBV in a human who showed symptoms consistent with HFRS.

MATERIALS AND METHODS

Viral strains and cells. *Hantaan virus* (HTNV) strain 76-118 and SEOV strain SR-11 were used as representative strains of the HTNV and SEOV species, respectively. The THAIV strain Thai749 was a gift from Dr. P.W. Lee of the WHO Collaborating Center for Virus Research for Hantaviruses in Korea. All of the viruses were propagated in Vero cells (clone E6; ATCC C1008) prior to molecular and antigenic characterizations or use in FRNT. The DOBV strains Slovenia[9] and Saaremaa-DOE,[10] SEOV strain Gou3,[11] and HTNV strain *Da Bie Shan virus* (DBSV)-NC167 isolated from *Niviventer confucianus* captured in a mountainous region near Dabishan, Anhui Province, China,[11] were used for antigenic comparisons.

Monoclonal antibodies (MAbs). Clones that produce MAbs directed against the HTNV envelope glycoproteins and N protein were prepared as previously described.[12,13]

Nucleotide sequence determination and phylogenetic analysis. Hantaviruses possess a negative-sense RNA genome that consists of 3 segments, which are designated as large (L), medium (M), and small (S). The L segment encodes the RNA-dependent RNA polymerase. The M segment encodes a glycoprotein precursor that is co-translationally cleaved into the G1 and G2 envelope glycoproteins, and the S segment encodes the nucleocapsid (N) protein.[1] The nucleotide sequence of the M segment has been published, but not in L and S segment. Total RNA was isolated from THAIV-infected Vero E6 cells, and hantavirus-specific cDNA was synthesized, as previously described.[11] To amplify the partial M genome segment that corresponds to nucleotides (nt) 2000–2300, the primer pair THLM1910F, (5′-AAAAGCAGATGTTACAT-3′) and THLM2364R (5′-TTTTCAAGTGACACTT-3′) was used. The entire S genome segment was amplified as 2 overlapping PCR products nt 1–1220 and nt 1025–1885 by using the two primer pairs CS1 (5′-TAGTAGTAGACTCCCT-AAAGAGCTAC-3′) and GS6 (5′-AGCTCIGGATCCAT-ITCAT-3′), as well as GS4 (5′-GAIIGITGTCCACCAA-CATG-3′) and CS8 (5′-TAGTAGTAGGCTCCCTAAA-AAGACAA-3′).[11,14] The PCR product of the expected size derived from the partial M segment was purified using a PCR purification kit (Qiagen, Hilden, Germany) and sequenced with the same primers that were used for the PCR amplification. The PCR products derived from the S genome segment were cloned into an *E. coli* vector using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA). Two clones of each amplification product were sequenced with M13-forward and -reverse primers. The sequencing reaction was performed with dye terminator reactions using a BigDye Terminator Cycle Sequencing Kit version 3.1 (Perkin Elmer, Applied Biosystems Division, Foster City, CA). The samples were sequenced on model 3100 DNA Sequencing System (Perkin Elmer, Applied Biosystems Division). The sequences obtained from 2 independent clones for each PCR amplification product were found to be identical. Although the almost complete S segment nucleotide sequence (except the extreme 5′- and 3′-termini covered by the amplification primers) was obtained, only the entire N protein coding sequences that allowed unambiguous alignment were used for the phylogenetic analysis.

The sequences were aligned using CLUSTALW[15] with the default parameters. The reliability of the alignment was checked using DotPlot analysis implemented in the BioEdit (Carlsbad, CA) software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The alignment was tested for phylogenetic information by likelihood mapping analysis.[16] In the subsequent phylogenetic analyses, the maximum likelihood (ML) and neighbor-joining (NJ) phylogenetic trees were calculated. To reconstruct the ML phylogenetic trees, a quartet puzzling algorithm implemented in the TREE-PUZZLE 5.2 package[16,17] was applied. The Tamura-Nei and Hasegawa-Kishino-Yano evolutionary models were used for the tree reconstructions. Missing parameters were reconstructed from the datasets. NJ trees with the Tamura-Nei evolutionary model were constructed using the PAUP* 4.0 Beta 10 software package (Sunderland, MA).[18] In addition, bootstrap analysis with 1,000 replicates was performed to evaluate the statistical support of the topology for the derived tree. The resulting evolutionary trees were then visualized using TreeView (Glasgow, UK) v.1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/ treeview.html). The accession numbers of the sequences used in the phylogenetic analysis are listed in the legend to Figure 1. The sequence of the S segment of the THAIV strain Thai749 has been deposited into the GenBank nucleotide sequence database with accession number AB186420.

Indirect immunofluorescent antibody (IFA) assay. Since the HANTADIA assay showed weak agglutination pattern in some of the sera, we also used IFA test for screening test. The indirect immunofluorescent antibody (IFA) assay was performed as described previously.[19] Briefly, acetone-fixed smears of Vero E6 cells infected with hantaviruses were used as antigens. For the antigenic comparison of THAIV with other hantaviruses by using the MAbs (Table 1), HTNV strains 76-118, AMRV-H5, and DABV-NC167, SEOV strains SR-11 and Gou3, DOBV strains Slovenia, and Saaremaa-DOE, and THAIV strain Thai749 were used. Fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (H and L chains) (Zymed Laboratories Inc., South San Francisco, CA) was used as the secondary antibody. The serum specimens that showed characteristic fluorescence in the infected Vero cells but negative with uninfected Vero cells were regarded as positive.

Focus reduction neutralization test (FRNT). The endpoint titers of neutralizing antibodies against HTNV strain76-118, SEOV strain SR-11, and THAIV strain Thai749, were determined by FRNT, as described earlier.[20] For this purpose, we selected seropositive sera from human and rodent sera. Human sera and rodent sera derived from trapping point #1 to #13 (Figure 2, Table 2) positive by ELISA were selected. And rodent sera derived from trapping point #14 to #22 positive by both HANTADIA and IFA were selected for further investigation. However a *Rattus rattus* serum from Phetchaburi was not used for FRNT assay because its amount was not suffi-

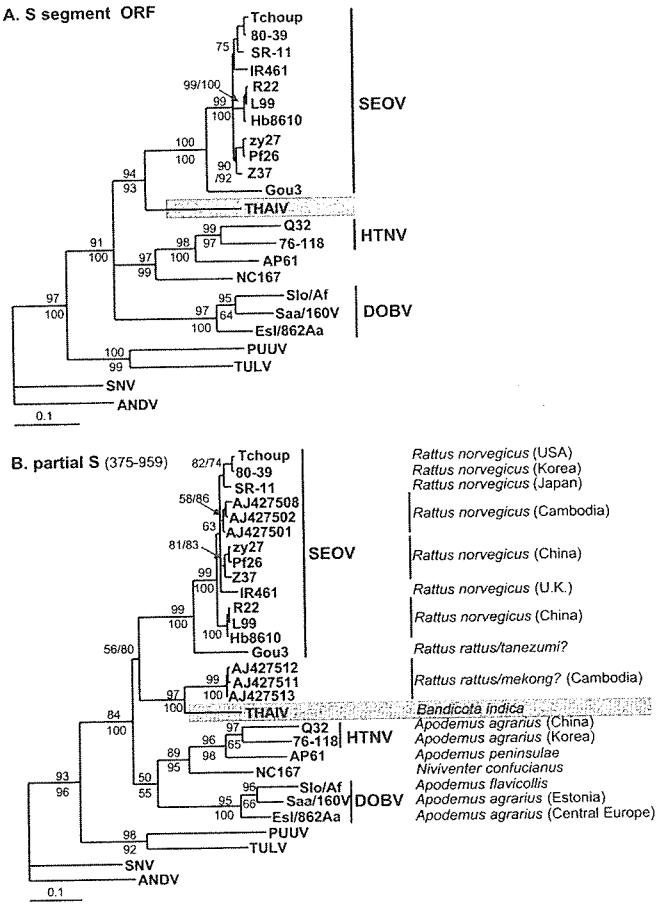

FIGURE 1. Maximum likelihood (ML) phylogenetic trees of THAIV and other Murinae-associated hantaviruses based on: (**A**) complete N protein coding nt sequences (S segment ORF); and (**B**) partial S segment nucleotide sequences of nt 375–959 (585 nts). The ML trees (Tamura-Nei evolutionary model) were calculated using TREE-PUZZLE package. The values above the branches represent PUZZLE support values. The values below the branches are the bootstrap values of the corresponding NJ tree (Tamura-Nei evolutionary model) calculated with the PAUP* software from 1,000 bootstrap replicates. THAIV is marked by a gray box. The S segment sequences that were analyzed included THAIV (AB186420), SEOV/SR11 (M34881), SEOV/Tchoupitoulas (AF329389), SEOV/80-39 (AY273791), SEOV/IR461 (AF329388), SEOV/R22 (AF488707), SEOV/L99 (AF488708), SEOV/Hb8610 (AF288643), SEOV/zy27 (AF406965), SEOV/Pf26 (AY006465), SEOV/Z37 (AF187082), Gou3 (AB027522), HTNV/76-118 (M14626), HTNV/Q32 (AB027097), Amur virus AMRV/AP61 (AB071183), DBSV/167 (AB027523), DOBV/Slo/Af (L41916), DOBV/Esl/862Aa (AJ269550), Saaremaa/160V (AJ009773), PUUV/CG1820 (M32750), Tula virus strain Moravia/5302v (Z69991), Sin Nombre virus strain NM H10 (L25784), and Andes virus strain Chile-9717869 (AF291702). In the lower tree (B), the partial sequences of Cambodian hantavirus strains detected in *Rattus rattus* (AJ427511-AJ427513) and in *R. norvegicus* (AJ427501, AJ427502, AJ427508) were added to the dataset. The natural rodent species (subfamily Murinae) of the corresponding hantavirus strains are listed.

cient. Briefly, 100 μL of serial 2-fold dilutions of serum were mixed with an equal volume of virus suspension containing 200 focus-forming units (FFU) of virus at 37°C for 1 hr. Fifty microliters of the mixture was then inoculated onto Vero E6 cell monolayers in 96-well tissue culture plates (IWAKI 3860-096, Asahi Technoglass Co., Tokyo, Japan). After adsorption for 1 hour at 37°C, the wells were overlaid with medium that contained 1.5% carboxymethyl cellulose. After being incubated for 7 days in a CO_2 incubator, the monolayers were fixed with acetone-methanol (1:1) and dried. The foci of the virus-infected cells were detected by staining with a polyclonal antiserum from a rabbit that was immunized with the truncated N protein (amino acids 1–244) of HTNV, followed by the addition of horseradish peroxidase-labeled goat antibodies and substrate. The FRNT titer was expressed as the reciprocal of the highest serum dilution that resulted in a > 80% reduction in the number of infected cell foci.

Rodent sera and antibody detection. In total, serum samples from 402 different rodents were collected from 22 locations in 19 provinces of Thailand from 1995–1998 (Figure 2, Table 2). Distinction of rodent species examined in the present study followed morphologic criteria including dental morphology and coloration of phage outlined by Corbet and Hill,[21] Musser and Brothers,[22] and Marshall.[7] The blood samples were taken after the animals were anesthetized with CO_2 and taxonomically identified; the weight, sex, and locality of collection were recorded. Then, the animals were euthanized with CO_2. Most of the captured rodents were brought to the Institute. The cadavers were incinerated at the Institute. Serum samples derived from trapping sites #1 to #13 were tested in an indirect IgG ELISA using yeast-expressed His-tagged SEOV, strain 80-39, recombinant N protein.[23] Briefly, polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 2 μg/ml recombinant N protein from SEOV diluted in 0.05 M carbonate buffer (pH 9.8). Blocking of the plates was accomplished by the addition of 3% bovine serum albumin (BSA)/0.05% Tween-20 in PBS followed by the addition of rodent serum samples diluted 1/200 with 1% BSA/0.05% Tween-20 and horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (Sigma Chemical Co., St. Louis, MO). To detect immunoreactivity, the *o*-phenylenediamine (OPD) substrate was added, and the reaction was stopped by the addition of 100 μL of 1 M H_2SO_4. Finally, the optical density (OD) was measured at 492 nm (reference, 620 nm). The final OD value for each serum sample was calculated as the difference of the OD values for antigen-containing and antigen-free wells. These final OD values for serum dilutions of 1/200 were regarded as positive if they exceeded the cutoff value of 0.270 determined by investigation of non-infected and experimentally SEOV-infected rats.[23,24] The serum samples derived from trapping sites #14 to #22 were screened using a commercial agglutination test based on inactivated HTNV antigen (HANTADIA®; Korea Green Cross Corp., Seoul, Korea) and an indirect immunofluorescent antibody (IFA) test. In HANTADIA screening, sera were screened by the manufacturer's instructions at 1:40 dilution. Serum specimen that showed clear agglutination was regarded as positive. In IFA test, the sera were examined at 1:40 dilution with HTNV strain 76-118-infected Vero E6 cell smears as antigen. As negative control, each serum sample was tested with uninfected Vero E6 cells. The serum specimen that showed characteristic fluorescence in the infected Vero E6 cells but negative with uninfected Vero E6 cells was regarded as positive. Antibody-positive sera from both screenings were confirmed by Western blotting using recombinant hantavirus N proteins of HTNV strain 76118 as previously described.[23,25,26] As positive controls, 3 serum samples from Wistar rats that were experimentally in-

TABLE 1
Antigenic profiling with N-, G1-, and G2-specific MAbs of THAIV and other murinae-associated hantaviruses*

			HTNV			SEOV		DOBV		THAIV
Proteins	Epitope	MAbs	HTNV 76118	AMRV H5	DBSV NC167	SEOV SR-11	SEOV Gou3	DOBV Slovenia	DOBV Saaremaa	THAIV Thai749
N	Cross-reactive	ECO2	+++	+++	+++	+++	+++	+++	+++	+++
	Genus-common	E5G6	+++	+++	+++	+++	+++	++	++	+++
	HTNV-specific	BDO1	+++	+++	+++	–	–	–	–	–
	SEOV-specific	DCO3	–	–	–	+++	+++	–	–	–
G1/G2	G1a	6D4	+++	+++	+++	–	–	+++	+++	+++
	G1b	3D5	+++	+++	+++	–	–	–	–	–
	G2a	HCO2	+++	+++	+++	+++	+++	–	–	+++
	G2b	EBO6	+++	+++	+++	+++	+++	±	±	–
	G2c	11E10	+++	+++	+++	–	–	+++	–	–
	G2d	3D7	+++	++	+++	+++	+++	+++	+++	+++
	G2e	20D3	+++	+++	–	+	+	++	++	+++
	G2f1	1G8	+++	++	+++	+++	+++	+++	+++	+++
	G2f2	7G6	+++	++	+++	+++	+++	++	+++	+++

Binding profiles of clones data not shown in this table were basically same results as representative clones and previous reports.[13] All the used clones were listed as below. Cross-reactive clones for N protein: ECO2, FDO3, KAO6, ECO1, GBO4, C16D11, and F23A1; Genus-common epitope binding clone: E5G6; HTNV-specific clones for N protein: BDO1, C24B4, and G5; SEOV-specific clone for N protein: DCO3.

Clones for glycoprotein epitope G1a: 6D4, 8B6, and 10F11; G1b: 3D5, 2D5 and 16D2; G2a: HCO2 and 16E6, G2b: EBO6; G2c: 11E10; G2d: 3D7; G2e: 20D3, 17G6 and 5B7; G2f1: 1G8, 8E10, 1C6, 23G10-2, and 3B6; G2f2: 7G6, 23G10-1, and 18F5. Designations: –, $< 10^2$; +, 10^2; ++, 10^3; +++, 10^4; ±, weak positive reaction at dilution of 1:100.

fected with SEOV strain SR-11 were used.[26] As negative controls, sera from 5 wild-trapped, non-infected rats from Japan were used.[24]

Human sera and methods for antibody detection. Screening for anti-hantavirus IgG and serotyping were performed by ELISA tests, as previously described,[20] using recombinant entire and truncated N protein antigens expressed by recombinant baculovirus. Briefly, serum specimens were screened with the dilution of 1:200. As a negative control antigen, bornavirus P24 antigen expressed by baculovirus was used. Recombinant N proteins of HTNV (strain 76-118), and PUUV (strain Sotkamo) and truncated N proteins of HTNV (strain 76-118), SEOV (strain SR-11), and DOBV (strain Saaremaa-DOE) were expressed from baculovirus vectors. The screening for virus-reactive IgM was performed with the μ-capture ELISA, as described previously.[27] Positive results were confirmed by IFA testing using SEOV-infected Vero E6 cell antigen and by Western blotting using recombinant HTNV antigen. Three types of positive control sera from HFRS patients who had been previously diagnosed by FRNT as being infected with HTNV, SEOV, and PUUV, and negative human control sera (NHS), which were confirmed to contain no antibodies against hantaviruses, were used.[20,28]

RESULTS

Genetic characterization of Thailand virus. The nucleotide sequences of the entire M genome segment and partial S genome segment of the THAIV strain Thai749 have been published (GenBank accession numbers L08756 and U00471[5]). Partial M segment sequence of the THAIV obtained in this study was completely identical with the published sequence. To characterize genetically the THAIV strain Thai749 in more detail, we cloned and sequenced entire S genome segment except primer binding region (GenBank accession number AB186420). The sequences of 2 independent clones for each of the PCR amplification products were found to be identical. The deduced amino acid sequence identity on comparison of the N protein of THAIV to those of SEOV, HTNV, and DOBV are calculated as 86.5%, 83.7%, and 81.6%, respectively. The previously determined values for sequences of THAIV glycoprotein precursors[5] showed amino acid sequence identity to those of SEOV, HTNV, and DOBV as 73.3%, 71.3%, and 71.2%, respectively. Thus, the N protein amino acid sequence information also meet one of the criteria set forth in the Eighth Report of the International Committee on Taxonomy of Viruses for species demarcation within the genus *Hantavirus* (more than 7% difference).[1,29] The phylogenetic analysis (Figure 1A) based on the nucleotide sequence of the N protein-encoding open reading frame (ORF) of the S genome segment revealed that THAIV was clearly placed in a distinct lineage within a single cluster with SEOV, HTNV, and DOBV, which are associated with the rodent reservoirs classified into the murid subfamily *Murinae*. Since *B. indica* is classified to the *Murinae* subfamily, the observed lineage of THAIV is in accordance with the host-virus co-evolution theory for hantaviruses.[30,31] As shown in Figure 1B, phylogenetic analysis based on a partial nucleotide sequence (nt 375–959) in the central region of the S segment, which contains the highly variable region, reveals that THAIV is most closely related to Cambodian virus strains isolated from *R. rattus*.[32]

Antigenic characterization of Thailand virus using monoclonal antibodies. To clarify the antigenic characteristics of THAIV, 34 MAbs, including 12 against the N protein and 22 against the G1 or G2 envelope proteins, were used to compare the antigenic profiles of the THAIV prototype strain Thai 749 and other hantaviruses using IFA (Table 1). The antigenic profiles of HTNV strains 76118 and Amur virus (AMRV)-H5 were taken from a previous report.[33] Among the MAbs directed against the N protein, cross-reactive clones to HTNV, SEOV, and DOBV-types were also reactive against THAIV. On the other hand, the HTNV-type specific and SEOV-type specific clones for N protein were not reactive against THAIV. Similarly, HTNV-type specific anti-G1 MAb (3D5) did not react to THAIV. However, the rest of clones showed variable cross reactivities among the 4 types of viruses. Therefore, in spite of the close antigenic relationships between hantaviruses that are associated with *Murinae* reservoir hosts, the antigenicity of THAIV was distinct from the