Available online at www.sciencedirect.com
ScienceDirect
VIROLOGY
ELSEVIER
Virology 365 (2007) 292–301
www.elsevier.com/locate/yviro

Analysis of the immune response of Hantaan virus nucleocapsid protein-specific CD8$^+$ T cells in mice

Midori Taruishi[a], Kumiko Yoshimatsu[a], Koichi Araki[b], Megumi Okumura[a], Ichiro Nakamura[c], Kiichi Kajino[c], Jiro Arikawa[a,*]

[a] *Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine, Kita-ku, Kita-15, Nishi-7, Sapporo 060-8638, Japan*
[b] *Emory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA*
[c] *Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan*

Received 28 November 2006; returned to author for revision 2 January 2007; accepted 28 February 2007
Available online 3 May 2007

Abstract

The major histocompatibility complex (MHC) class-I restricted epitope of Hantaan virus nucleocapsid protein (N) was identified using overlapping peptides and BALB/c mice. Using the MHC tetramer derived from the epitope, we found that the level of N-specific CD8$^+$ T cells increased to approximately 20% of all antigen-specific CD8$^+$ T cells in a mouse model of transient infection. However, N-specific CD8$^+$ T cells were undetectable in a mouse model of persistent infection, both in the persistently infected phase and in the convalescent phase. Levels of CD8$^+$ T cells producing interferon-γ were weak in both the acute and convalescent phases in the persistently infected model. These results indicate that hantavirus strongly suppresses the production of N-specific CD8$^+$ T cells throughout the course of infection in persistently infected mice. Moreover, N-specific CD8$^+$ T cells were not effective in recovering persistently infected mice, despite the existence of abundant N antigen *in vivo*.

Keywords: Hantavirus; MHC tetramer; Hantavirus-specific CD8$^+$ T cell; Persistent infection

Introduction

Hantaviruses comprise the genus *Hantavirus* in the family *Bunyaviridae*. Hantaviruses are spherical, enveloped viruses, with a diameter of 80–120 nm. The hantavirus genome consists of three segments of single-stranded, negative-sense RNA. The three segments have been designated the large (L), medium (M), and small (S) segments, based on their size. The L segment encodes an RNA-dependent RNA polymerase; the M segment encodes the surface glycoprotein precursor, which is cotranslationally cleaved into two glycoprotein spikes (Gn and Gc); and the S segment encodes the nucleocapsid protein (N) (Lednicky, 2003).

To date, 22 virus species have been classified in the genus *Hantavirus* based on antigenic, genetic, and ecological characteristics. Rodents are the natural reservoirs of all but one species, the Thottapalayan virus, whose reservoir animal is the insectivore *Suncus murinus* (Carey et al., 1971). Because they are species-specific, it is generally believed that hantaviruses have coevolved with their hosts, and nearly identical phylogenetic trees can be constructed from host mitochondrial DNA sequences and viral RNA sequences (Meyer and Schmaljohn, 2000). Hantaviruses cause two serious and often fatal human diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Transmission of hantaviruses to humans does not require direct human-rodent contact. Instead, humans typically become infected by inhaling virus-contaminated aerosols of rodent excreta, such as feces, saliva, or urine (Lee and van der Groen, 1989).

Because hantaviruses have no, or an incomplete, cytopathic effect on cultured cells (Ogino et al., 2004) and are generally non-lytic (Pensiero et al., 1992; Yanagihara and Silverman, 1990; Zaki et al., 1995), the pathogenesis of HFRS and HPS may be related to immune-mediated effector responses of the host itself. Indeed, several studies have suggested that hantavirus infection can induce a vigorous cellular immune response in humans (Chen and Yang, 1990; Huang et al., 1994; Kilpatrick et al., 2004; Mustonen et al., 1994; Nolte et al., 1995;

* Corresponding author. Fax: +81 11 706 7879.
E-mail address: j_arika@med.hokudai.ac.jp (J. Arikawa).

0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2007.02.039

Temonen et al., 1996). Thus, to understand its pathogenesis, it is important to discern host immune responses to the virus, particularly hantavirus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs). The interaction of hantavirus-specific CTLs with the hantavirus remains unclear. In contrast to humans, the reservoir animals are persistently infected without signs of disease (Meyer and Schmaljohn, 2000).

In a previous study, we established a persistent infection model by inoculating mice with a sublethal dose of Hantaan virus (HTNV), the prototype of the genus *Hantavirus*, within 24 h of birth. The number of interferon (IFN)-γ-inducible HTNV-specific $CD8^+$ T cells first increased in the acute phase and then decreased to an undetectable level soon thereafter. Viral antigen was detected in the lung until 90 days after the infection, although no HTNV-specific $CD8^+$ T cells were observed at that time; T cells reappeared at 120 days, although no detectable viral antigen was present in the lung (Araki et al., 2003). A similar suppression and reappearance of HTNV-specific $CD8^+$ T cells was observed using a severe combined immune deficiency (SCID) mouse model in which spleen cells from immunocompetent BALB/c mice were passively transferred to SCID mice 14 days after infection (Araki et al., 2004a). These results indicated that disseminated HTNV infection before the induction of an immune response is important for the establishment of persistent infection and the suppression of HTNV-specific $CD8^+$ T cells in mice. HTNV-specific $CD8^+$ T cells were induced in HTNV-infected nude mice after adoptive transfer of spleen cells from immunocompetent BALB/c mice (Araki et al., 2004a), indicating that the HTNV-specific $CD8^+$ T cells appearing in SCID or nude mice were produced by the transferred cells. This suggests that the peripheral $CD8^+$ T cell response is more important than the thymus in suppressing specific T cell responses in persistently infected mice.

Although several N-specific CTL epitopes have been determined in C57BL/6 mice, as yet, none has been reported in BALB/c mice (Park et al., 2000; Maeda et al., 2004, 2005). To further analyze the $CD8^+$ T cell response, we first identified the immunodominant CTL epitope in the HTNV nucleocapsid protein (N), an abundant antigen of hantaviruses, in BALB/c mice. We then established an epitope-specific MHC tetramer assay and sequentially analyzed the epitope-specific $CD8^+$ T cell response by comparing the transient infection model and the persistent infection model.

Results

Screening for the N-specific $CD8^+$ T cell epitope in BALB/c mice

We used HTNV-infected P388D1 cells ($H\text{-}2^d$) as antigen-presenting cells to detect all antigen-specific $CD8^+$ T cells. Accordingly, we used BALB/c mice ($H\text{-}2^d$) for screening. In total, 105 15-mer peptides with 10 amino acid overlaps, encompassing the entire HTNV N protein, were divided into seven pools (Table 1). The peptide mixtures from each pool were screened for their capacity to stimulate IFN-γ production in spleen cells from BALB/c mice infected with HTNV. Based on the FACS analysis, the peptide mixture from pool 6 was able to induce IFN-γ (Fig. 1A). Approximately 20% of all antigen-specific $CD8^+$ T cells were raised as N-specific cells (Fig. 1A). Next, we examined the peptides in pool 6 (nos. 81–94) individually, and determined that peptides 83 and 84 were positive for IFN-γ induction (Fig. 1B). To identify the minimum sequence of the T cell epitope, we examined seven additional 11-mer peptides, encompassing the sequence represented in peptides 83 and 84. As shown in Fig. 1C, peptides 108 and 109 stimulated HTNV-specific $CD8^+$ T cells, while peptides 106, 107, and 110 did not. Because a 9-mer peptide is recommended for synthesis of MHC class I tetramers, we examined the shorter sequence ILQDMRNTI (N335–343), which was suggested from the results with peptides 108 and 109. As shown in Fig. 1D, this peptide induced IFN-γ (0.14%), and we thus deemed it the sole epitope for N-specific $CD8^+$ T cells in BALB/c mice ($H\text{-}2^d$).

Determination of MHC restriction in sequence NP335–343

To determine the MHC restriction of the HTNV-specific response to this peptide derived from N, an inhibition-of-peptide-stimulation assay was conducted using antibodies specific to the class I molecules D^d or K^d. The percentage of peptide-specific $CD8^+$ T cells was 0.04% with the anti-$H\text{-}2D^d$ antibody and 0.01% with the anti-$H\text{-}2K^d$ antibody (Fig. 2). Although the percentage of positive cells was lower than measured in the same experiment in Fig. 1, the peptide stimulation was only blocked by the antibody to $H\text{-}2K^d$, indicating that the epitope was $H\text{-}2K^d$-restricted. The percentage of positive cells varied depending on the condition of the cells. We obtained reproducible results from several independent experiments; therefore, we deemed this to be of significance. In addition, we also confirmed $H\text{-}2K^d$ binding affinity using the Bioinformatics and Molecular Analysis Section (BIMAS) algorithms (available at http://thr.cit.nih.gov/molbio/hla_bind/) (Parker et al., 1994). The sequence of the N335–343 peptide scored the highest in $H\text{-}2K^d$ (69.12), but lowest in $H\text{-}2D^d$ (0.54) and $H\text{-}2L^d$ (1.5). The specific motif for peptides that bind $H\text{-}2K^d$ is believed to be a nonamer, with tyrosine or phenylalanine residues in the second amino acid position, and leucine or isoleucine in the carboxyl-terminal or ninth amino acid position as dominant anchoring positions. Although the sequence that we identified does not have an aromatic residue in the second position, it is similar to that of the HIV-1 gag epitope (AMQMLKETI), which also does not contain an anchoring aromatic residue in position two (Mata et al., 1998). These results also suggest that the N335–343 peptide is $H\text{-}2K^d$-restricted. Thus, a tetramer was prepared that consisted of $H\text{-}2K^d$ molecules loaded with the N335–343 peptide; it was named NP335–343.

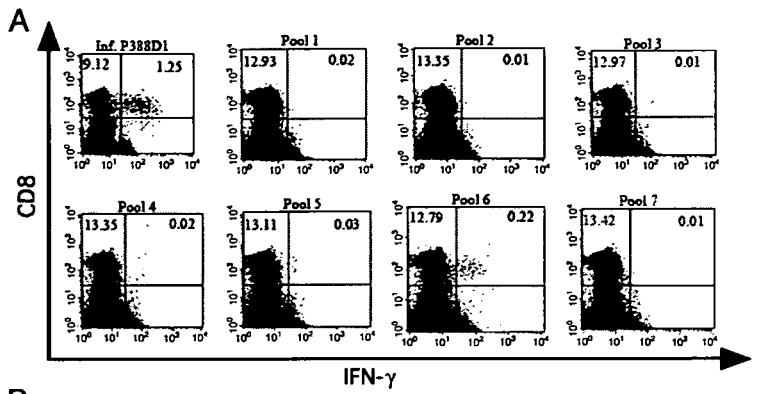
Response of NP335–343-specific $CD8^+$ T cells in a transient infection model

To evaluate the effect of NP335–343 staining on epitope-specific $CD8^+$ T cells, we first stained spleen cells from the transient infection mouse model. The frequency of NP335–343-positive cells, as a percentage of total $CD44^+$ $CD8^+$ T

Table 1
Peptide library derived from the full-length N protein: overlapping peptides used to test IFN-γ-inducing ability

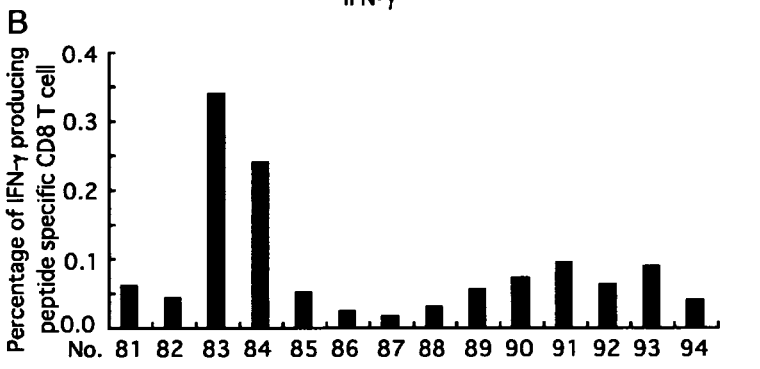
Peptide	AA position	Sequence	Peptide	AA position	Sequence	Peptide	AA position	Sequence	Peptide	AA position	Sequence
Pool 1			Pool 2			Pool 3			Pool 4		
1	1–15	MMATMEELQREINAH	17	65–79	QLADRIATGKNLGKE	33	129–143	SFVVPILLKALYMLT	49	193–207	EITPGRYRTAVCGLY
2	5–19	MEELQREINAHEGQL	18	69–83	RIATGKNLGKEQDPT	34	133–147	PILLKALYMLTTRGR	50	197–211	GRYRTAVCGLYPAQI
3	9–23	QREINAHEGQLVIAR	19	73–87	GKNLGKEQDPTGVEP	35	137–151	KALYMLTTRGRQTTK	51	201–215	TAVCGLYPAQIKARQ
4	13–27	NAHEGQLVIARQKVR	20	77–91	GKEQDPTGVEPGDHL	36	141–155	MLTTRGRQTTKDNKG	52	205–219	GLYPAQIKARQMISP
5	17–31	GQLVIARQKVRDAEK	21	81–95	DPTGVEPGDHLKERS	37	145–159	RGRQTTKDNKGTRIR	53	209–223	AQIKARQMISPVMSV
6	21–35	IARQKVRDAEKQYEK	22	85–99	VEPGDHLKERSMLSY	38	149–163	TTKDNKGTRIRFKDD	54	213–227	ARQMISPVMSVIGFL
7	25–39	KVRDAEKQYEKDPDE	23	89–103	DHLKERSMLSYGNVL	39	153–167	NKGTRIRFKDDSSFE	55	217–231	ISPVMSVIGFLALAK
8	29–43	AEKQYEKDPDELNKR	24	93–107	ERSMLSYGNVLDLNH	40	157–171	RIRFKDDSSFEDVNG	56	221–235	MSVIGFLALAKDWSD
9	33–47	YEKDPDELNKRTLTD	25	97–111	LSYGNVLDLNHLDID	41	161–175	KDDSSFEDVNGIRKP	57	225–239	GFLALAKDWSDRIEQ
10	37–51	PDELNKRTLTDREGV	26	101–115	NVLDLNHLDIDEPTG	42	165–179	SFEDVNGIRKPKHLY	58	229–243	LAKDWSDRIEQWLIE
11	41–55	NKRTLTDREGVAVSI	27	105–119	LNHLDIDEPTGQTAD	43	169–183	VNGIRKPKHLYVSLP	59	233–247	WSDRIEQWLIEPCKL
12	45–59	LTDREGVAVSIQAKI	28	109–123	DIDEPTGQTADWLSI	44	173–187	RKPKHLYVSLPNAQS	60	237–251	IEQWLIEPCKLLPDT
13	49–63	EGVAVSIQAKIDELK	29	113–127	PTGQTADWLSIIVYL	45	177–191	HLYVSLPNAQSSMKA	61	241–255	LIEPCKLLPDTAAVS
14	53–67	VSIQAKIDELKRQLA	30	117–131	TADWLSIIVYLTSFV	46	181–195	SLPNAQSSMKAEEIT	62	245–259	CKLLPDTAAVSLLGG
15	57–71	AKIDELKRQLADRIA	31	121–135	LSIIVYLTSFVVPIL	47	185–199	AQSSMKAEEITPGRY	63	249–263	PDTAAVSLLGGPATN
16	61–75	ELKRQLADRIATGKN	32	125–139	VYLTSFVVPILLKAL	48	189–203	MKAEEITPGRYRTAV	64	253–267	AVSLLGGPATNRDYL
Pool 5			Pool 6			Pool 7					
65	257–271	LGGPATNRDYLRQRQ	81	321–335	LFIAGIAELGAFFSI	95	377–391	LGQRIIVLFMVAWGK			
66	261–275	ATNRDYLRQRQVALG	82	325–339	GIAELGAFFSILQDM	96	381–395	IIVLFMVAWGKEAVD			
67	265–279	DYLRQRQVALGNMET	83	329–343	LGAFFSILQDMRNTI	97	385–399	FMVAWGKEAVDNFHL			
68	269–283	QRQVALGNMETKESK	84	333–347	FSILQDMRNTIMASK	98	389–403	WGKEAVDNFHLGDDM			
69	273–287	ALGNMETKESKAIRQ	85	337–351	QDMRNTIMASKTVGT	99	393–407	AVDNFHLGDDMDPEL			
70	277–291	METKESKAIRQHAEA	86	341–355	NTIMASKTVGTSEEK	100	397–411	FHLGDDMDPELRTLA			
71	281–295	ESKAIRQHAEAAGCS	87	345–359	ASKTVGTSEEKLRKK	101	401–415	DDMDPELRTLAQSLI			
72	285–299	IRQHAEAAGCSMIED	88	349–363	VGTSEEKLRKKSSFY	102	405–419	PELRTLAQSLIDVKV			
73	289–303	AEAAGCSMIEDIESP	89	353–367	EEKLRKKSSFYQSYL	103	409–423	TLAQSLIDVKVKEIS			
74	293–307	GCSMIEDIESPSSIW	90	357–371	RKKSSFYQSYLRRTQ	104	413–427	SLIDVKVKEISNQEP			
75	297–311	IEDIESPSSIWVFAG	91	361–375	SFYQSYLRRTQSMGI	105	416–430	DVKVKEISNQEPLKL			
76	301–315	ESPSSIWVFAGAPDR	92	365–379	SYLRRTQSMGIQLGQ						
77	305–319	SIWVFAGAPDRCPPT	93	369–383	RTQSMGIQLGQRIIV						
78	309–323	FAGAPDRCPPTCLFI	94	373–387	MGIQLGQRIIVLFMV						
79	313–327	PDRCPPTCLFIAGIA									
80	317–331	PPTCLFIAGIAELGA									

The amino acid position indicates the location of the indicated sequences in the N protein.



C

Peptide	AA pos.	Sequence	Tested
83	329-343	LGAFFSILQDMRNTI	Yes
84	333-347	FSILQDMRNTIMASK	Yes
106	329-339	LGAFFSILQDM	No
107	331-341	AFFSILQDMRN	No
108	333-343	FSILQDMRNTI	Yes
109	335-345	ILQDMRNTIMA	Yes
110	337-347	QDMRNTIMASK	No



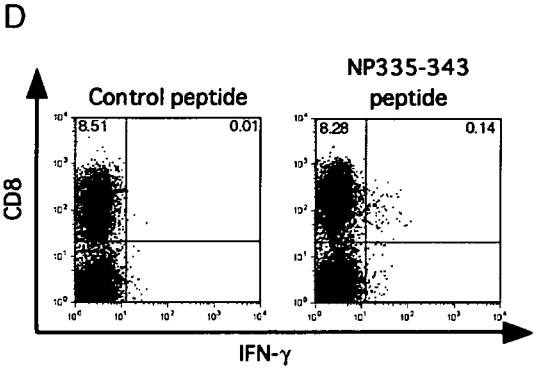


Fig. 1. Peptide screening for the N protein epitope from infected adult mice. Spleen cells were obtained from HTNV-infected BALB/c mice 10 days after HTNV inoculation. Spleen cells were cultured in the presence of brefeldin A and IL-2. IFN-γ$^+$ CD8$^+$ cells were detected using flow cytometry. The numbers are the percentages of IFN-γ$^+$ CD8$^+$ cells. (A) Spleen cells were cultured with HTNV-infected P388D1 cells (positive control) or with peptide pools (Table 1). IFN-γ positive cells were detected only with pool 6 peptides. (B) Spleen cells were cultured with peptides 81–94 from pool 6. IFN-γ-positive cells were detected using peptides 83 and 84. (C) Spleen cells were cultured with or without peptides 106–110 derived from peptides 83 and 84. IFN-γ-positive cells were detected using peptides 108 and 109. (D) Spleen cells were cultured with peptide 93 (negative control) or NP335–343 derived from peptides 110 and 112. The NP335–343 peptide stimulated CD8$^+$ cells to produce IFN-γ.

cells, increased 7 days after infection, with a peak at 10 days after infection (1.11%); it then decreased at 3 weeks after infection and continued at a low level thereafter (Figs. 3A, B). The same kinetic profile was produced by NP335–343 peptide-specific, IFN-γ-inducible CD8$^+$ T cells 3, 7, 12, and 14 days post infection (data not shown). Furthermore, these kinetics were similar to those of virus-specific, IFN-γ-inducible CD8$^+$ T cells (Fig. 3C). NP335–343-specific CD8$^+$ T cells comprised approximately 20% of all antigen-specific CD8$^+$ T cells (Figs. 3B, C). This is in good correspondence to previous data (Fig. 1A). These data indicated that the transient infection model had a normal immune response and that the tetramer NP335–343 could detect epitope-specific CD8$^+$ T cells.

Response of NP335–343-specific CD8$^+$ T cells in a persistent infection model

Next, we examined the kinetics involved in NP335–343 staining of epitope-specific CD44$^+$ CD8$^+$ T cells in a persistent infection mouse model. As a result, a very low level of NP335–343-specific CD8$^+$ T cell response was observed at all time points. NP335–343-specific CD8$^+$ T cells tended to appear just before viral antigen elimination (13 weeks after infection [0.31%]; Fig. 4A). The total number of NP335–343-positive CD44$^+$ CD8$^+$ T cells was equal to or lower than the lowest level in the transient infection model (Figs. 3C and 4C). The same tendency was seen in the NP335–343 peptide stimulation assays performed at 2, 5, 8, 10, 13, and 17 weeks (data not shown).

In our previous study with this model, we found that the IFN-γ-producing CD8$^+$ T cell response appeared in the acute phase, and was then quickly suppressed in the following viral persistent phase. IFN-γ-producing CD8$^+$ T cells reappeared at about 17 weeks after infection, when the persistent infection ended (Araki et al., 2003). We examined the kinetics of the IFN-γ-producing CD8$^+$ T cells in more detail and confirmed that the IFN-γ-producing cell response was very weak compared to that in the transient infection model. It appeared twice, at 2 weeks after infection and just before viral antigen elimination (Fig. 4C). We found suppression of IFN-γ-production and also suppression of NP335–343-specific CD8$^+$ T cell production. We looked for, but did not detect, other N-specific CD8$^+$ T cell epitopes in this model (data not shown). These results indicate that hantavirus strongly suppresses the production of N-specific CD8$^+$ T cells in the persistent infection model (Fig. 5).

Discussion

We identified an N-specific CD8$^+$ T cell epitope in BALB/c mice (H-2^d). Specifically, we found one major epitope in BALB/c mice corresponding to amino acids 335–343 of the HTNV N protein, which was H-2K^d-restricted. At least three epitopes have been found within HTNV N in C57BL/6 mice (H-

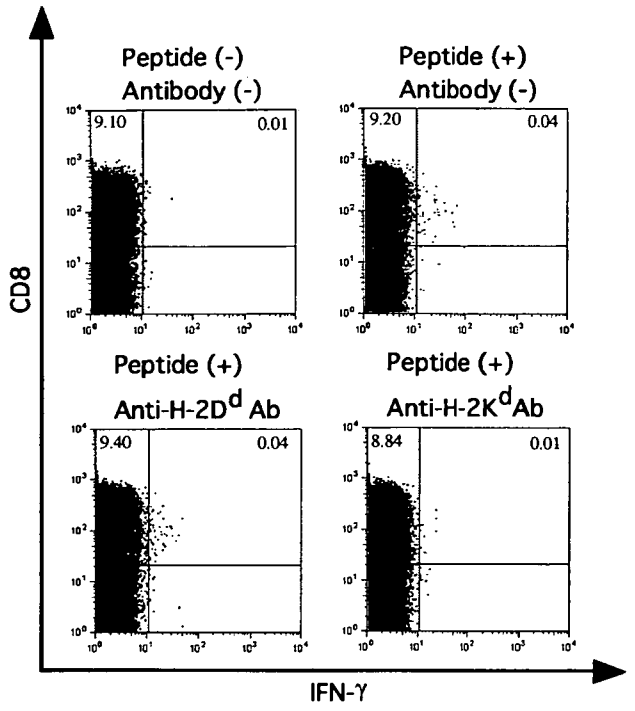


Fig. 2. The NP335–343 peptide is MHC class I H-2K^d-restricted. Spleen cells were obtained from HTNV-infected BALB/c mice 10 days after HTNV inoculation. Spleen cells were cultured in the presence of brefeldin A and IL-2. When required, anti-H-2D^d or H-2K^d antibodies were added simultaneously with or without the NP335–343 peptide. IFN-γ$^+$ CD8$^+$ cells were detected using flow cytometry. The numbers are the percentages of IFN-γ$^+$ CD8$^+$ cells.

2^b) by the BIMAS prediction algorithm (Park et al., 2000). The previously identified epitopes NP3 (amino acids 221–228: SVIGFLAL), NP4 (amino acids 328–335: LGAFFSIL), and NP7 (amino acids 422–429: SNQEPLKL) were different from our identified epitope, but NP4 does partially overlap with it. For Sin Nombre virus (SNV) infection, peptide screening in B6. PL Thy1^a/Cy mice (H-2^b) identified four epitopes, NC94–101 (SSLRYGNV), NC175–189 (minimal epitope:SMPTAQSTM), NC211–225, 217–231 (minimal epitope:SPVMGVIGF or PVMGVIGFS), and NC331–345 (minimal epitope: FAILQDMRNT or AILQDMRNTI) (Maeda et al., 2004, 2005). The NC331–345 epitope is the same as the HTNV CD8$^+$ T cell epitope (ILQDMRNTI) identified in BALB/c mice. This sequence has also been shown to elicit a strong CTL response in humans (HLA-A2.1) who have been infected with HTNV and recovered (Lee et al., 2002). In addition, this region is highly conserved in all hantavirus prototypes, suggesting that this universal region may be important in eliciting hantavirus-specific T-cell responses.

In Puumala virus (PUUV) infection, N protein may be the dominant target of CD8$^+$ CTLs in infected patients (Van Epps et al., 2002). In immunization experiments using recombinant vaccinia viruses in a mouse model, both SNV N protein and especially HTNV N protein induced IFN-γ-producing cells (Maeda et al., 2004). Therefore, N seems to be a major CTL epitope. However, approximately 20% of all antigen-specific CD8$^+$ T cells were N-specific in our study (Fig. 1A), indicating that N is not the major epitope in BALB/c mice. Gn and Gc may be major target proteins of CD8$^+$ T cells in this mouse strain. Indeed, there are several reports of antigen-specific CD8$^+$ T cells being elicited not only by N, but also by Gn and Gc in patients with PUUV, SNV, and HTNV (Van Epps et al., 1999; Kilpatrick et al., 2004; Terajima et al., 2002).

The frequency of antigen-specific CD8$^+$ T cells may be related to pathogenic severity. In SNV infection, the frequency of SNV-specific CD8$^+$ T cells was significantly higher in patients with severe HPS (7.4–44.2%) than in patients with moderate HPS (2.9–9.8%) (Kilpatrick et al., 2004). Thus, virus-specific CD8$^+$ T cells may contribute to disease outcome. In the BALB/c mice used in this study, the total HTNV-specific CD8$^+$ T cell response itself is very low (less than 2%) compared to the response to other viruses (e.g., above 20% in lymphocytic choriomeningitis virus; Fig. 1) (Woo et al., 2005; Zhou et al., 2004). The low frequency of HTNV-specific CD8$^+$ T cells in mice seems to be related to asymptomatic infection.

We synthesized the HTNV-specific MHC tetramer NP335–343, derived from the HTNV epitope identified in BALB/c mice, to analyze epitope-specific CD8$^+$ T cells. Using this tetramer, we observed that epitope-specific CD8$^+$ T cells appeared soon after infection (day 7) in a transient infection model. This corresponded to the kinetics of IFN-γ-inducible CD8$^+$ T cells. These results suggest that the tetramer NP335–343 is a useful tool to analyze epitope-specific CD8$^+$ T cells and that the immune response is normal in the transient infection model. However, a low induction of N-specific CD8$^+$ T cells was observed in the persistent infection model. Although one peak in the appearance of NP335–343-specific CD8$^+$ T cells occurred just before viral antigen elimination (13 weeks), the level was quite low, even in the convalescent phase. Mice are also killed by HTNV infection when inoculated with HTNV on day 0 (Ebihara et al., 2000; Yoshimatsu et al., 1997). Persistent hantavirus infection in mice occurs only when they are inoculated with HTNV within 24 h of birth (Araki et al., 2004b). Viruses can readily proliferate in newborn mice because the immune response may be incomplete compared to adult mice. When spleen cells from immunocompetent BALB/c mice were passively transferred to SCID mice 14 days after infection, they permitted viral persistence and suppression of virus-specific CD8$^+$ T cells. However, when spleen cells from immunocompetent BALB/c mice were passively transferred to SCID mice on the day of infection, they expressed virus-specific CD8$^+$ T cells normally (Araki et al., 2004a). These results suggest that CTL production is dependent on the host environment and is regulated by the viral antigen. It is thought that suppression of epitope-specific CD8$^+$ T cell production in this persistent infection model is also caused by large amounts of viral antigen.

CTLs are an important host–defense mechanism against many viral infections, particularly for the clearance of virus-infected cells. N-specific CTLs are strongly regulated and suppressed in this persistently infected mouse model by an unknown mechanism. Both pathogenic and nonpathogenic hantaviruses infect primary human endothelial cells (Pensiero et al., 1992; Yanagihara and Silverman, 1990) and dendritic cells (Raftery et al., 2002), as well as other cell lines from many species. Although there are no reports to show the infected cells *in vivo* in mice, infected immune cells are thought to regulate

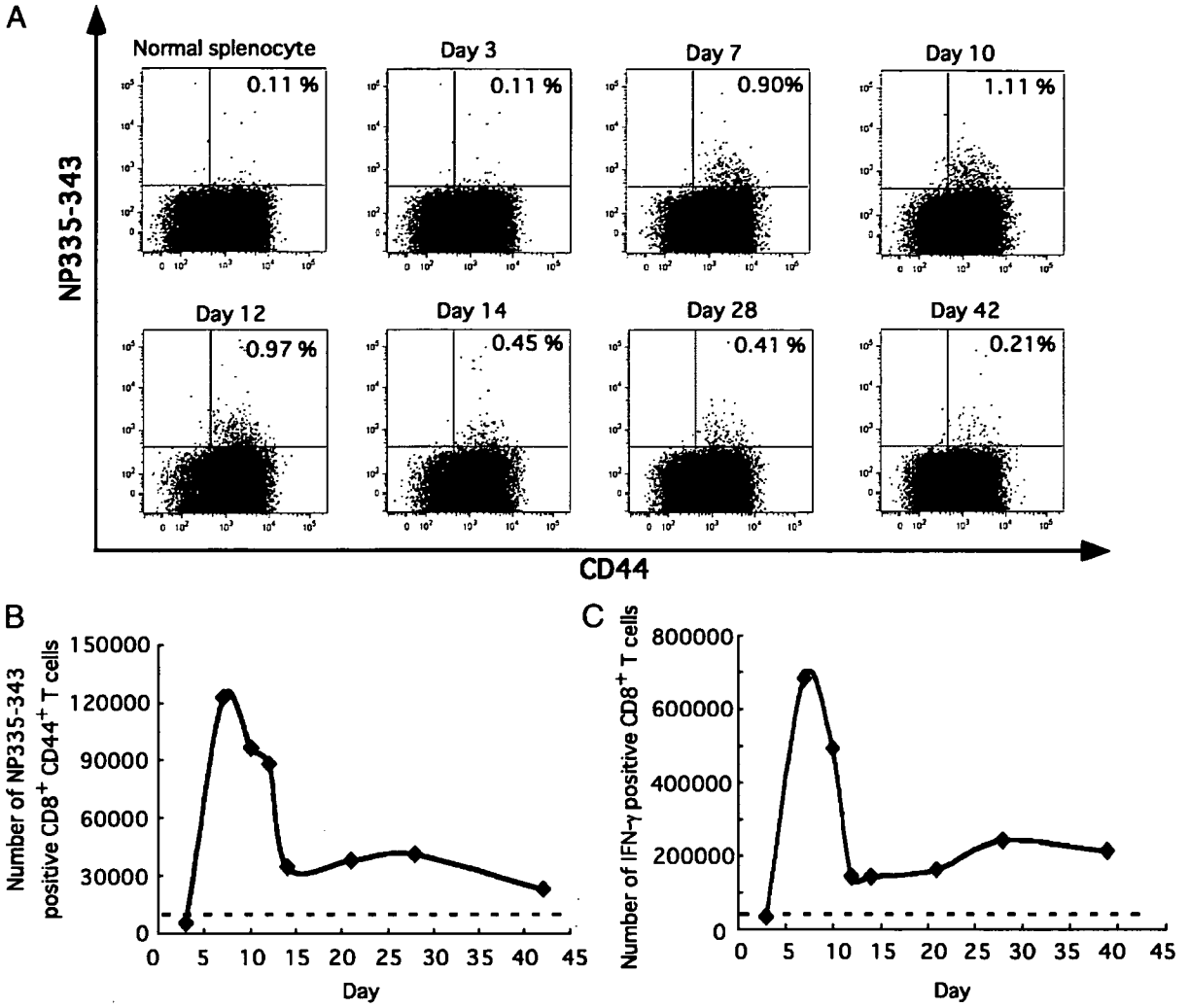


Fig. 3. Sequential analysis of NP335–343-specific $CD8^+$ T cells in a transient infection model. Spleen cells from normal adult mice and transiently infected adult mice, which were inoculated with HTNV cl-1, were stained with NP335–343. The number of $CD8^+$ T cells specific to the CTL epitope NP335–343 was determined by staining splenocytes with anti-CD8, anti-CD44, and MHC-I tetramer. (A) The dot plots are gated on total $CD8^+$ T cells, and the numbers are the percentages of tetramer-binding $CD8^+$ T cells of total $CD8^+$ T cells. (B) The representative percentage of $CD8^+$ T cells positive for NP335–343 is plotted over time following infection. The dotted line indicates the level of detection based on tetramer staining of splenocytes from naïve mice. (C) Spleen cells from BALB/c mice with or without HTNV infection were incubated with HTNV-infected P388D1 cells for 6 h in the presence of brefeldin A and IL-2. The representative percentage e of $CD8^+$ T cells producing IFN-γ is plotted over time following infection. The dotted line indicates the level of detection based on IFN-γ staining of splenocytes from naïve mice.

the alteration of these CTLs. We suspect that the alteration is triggered about 2 weeks after infection, as we have found that cells that strongly express N are detected only in the spleen of the model mice after 2 weeks of infection (unpublished data). Viral antigen is first detected in the spleen after infection (Yoshimatsu et al., 1997), and the change in CTLs occurs during this early phase. These infected cells may be immune cells, and we speculate that after infection they are involved in regulating immune responses, including the CTL response, and trigger persistent infection or viral pathogenesis. In conclusion, we demonstrated that major epitope-specific $CD8^+$ T cell production is strongly suppressed in a persistently infected mouse model. These two mouse models of transient and persistent infection are very useful for analyzing the immune response of virus-specific $CD8^+$ T cells with MHC tetramers. Using this method, further analyses should be performed to examine other protein-specific $CD8^+$ T cells, especially Gn and Gc. Although the molecular mechanism of this suppression requires further investigation, the strong suppression of the epitope-specific $CD8^+$ T cells in mice may be related to the induction of an asymptomatic infection. In particular, our findings may help improve therapies for patients with severe diseases controlled by $CD8^+$ T cells.

Materials and methods

Mice

All mice were treated according to the laboratory animal control guidelines of our institute, which conform to those of the

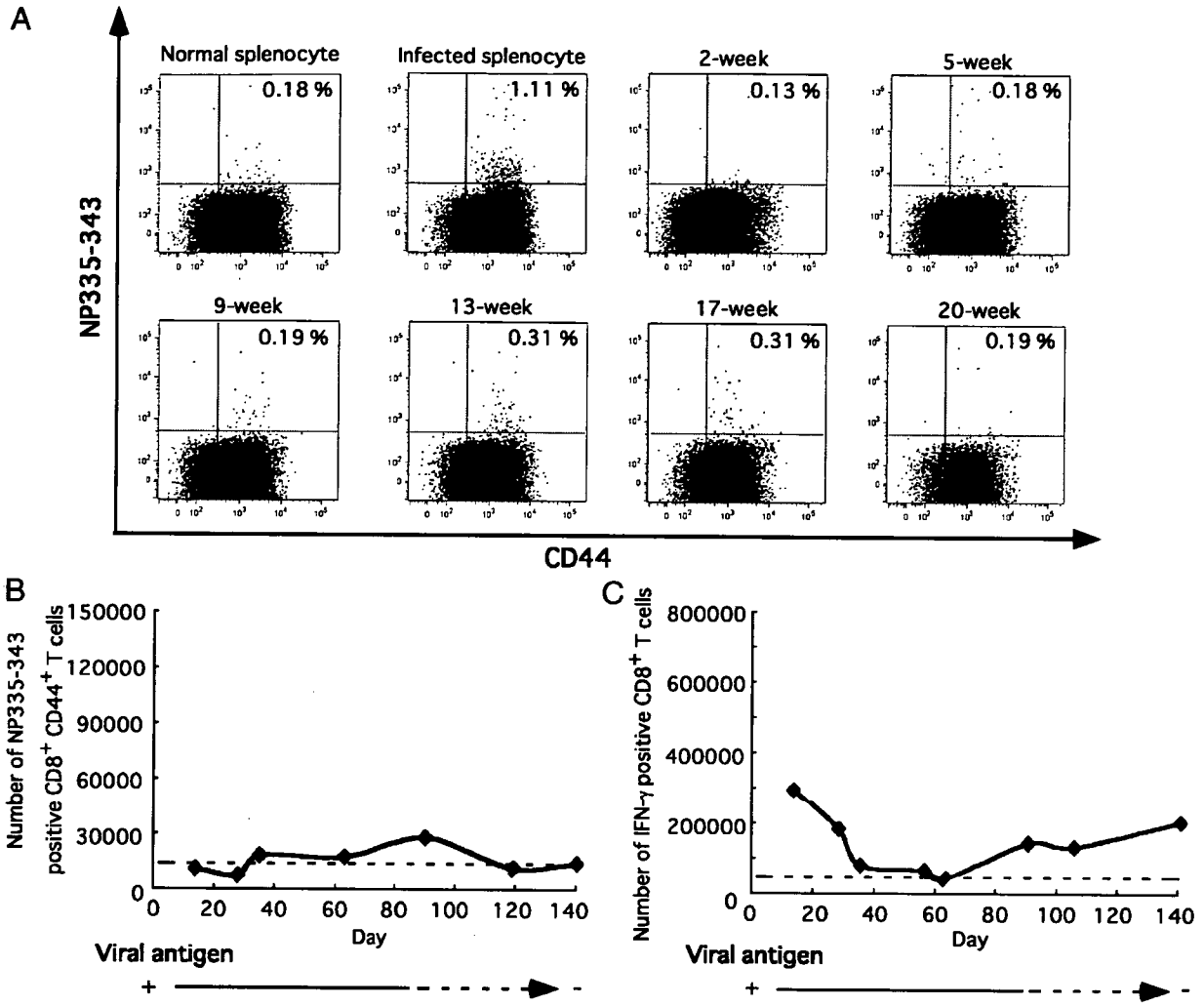


Fig. 4. Sequential analysis of NP335–343-specific $CD8^+$ T cells in a persistent infection model. BALB/c mice were inoculated with HTNV after birth (<24 h) and killed after several weeks. "Infected" indicates adult BALB/c mice that were inoculated with HTNV and recovered (positive controls). "Uninfected" denotes normal BALB/c mice (negative controls). (A) The dot plots are gated on total $CD8^+$ T cells, and the numbers are the percentages of tetramer-binding $CD8^+$ T cells of total $CD8^+$ T cells. (B) The representative percentage of $CD8^+$ T cells positive for NP335–343 is plotted over time following infection. The dotted line indicates the level of detection based on tetramer staining of splenocytes from naïve mice. (C) Spleen cells from BALB/c mice with or without HTNV infection were incubated with HTNV-infected P388D1 cells for 6 h in the presence of brefeldin A and IL-2. The representative percentage of $CD8^+$ T cells producing IFN-γ is plotted over time following infection. The dotted line indicates the level of detection based on IFN-γ staining of splenocytes from naïve mice.

U.S. National Institutes of Health (Bethesda, MD). Pregnant, 5-week-old female BALB/c mice were obtained from SLC (Hamamatsu, Japan). All experiments were performed in a class P3 facility.

Virus and viral infection of mice

HTNV cl-1 was obtained by plaque cloning from the HTNV strain 76–118 (Ebihara et al., 2000). The virus was propagated in the E6 clone of the Vero cell line (ATCC c1008) in Eagle's minimal essential medium (EMEM; Invitrogen, Carlsbad, CA), supplemented with 5% fetal bovine serum (FBS). We used two series of infected mice. For the production of persistently infected mice, BALB/c mice were subcutaneously (s.c.) inoculated with 1.3 focus-forming units (FFU) of HTNV within 24 h of birth (1.3 FFU=0.1 $NMLD_{50}$ [50% newborn mouse lethal dose]). For the production of transiently infected mice, adult (more than 5 weeks old) mice were intraperitonially inoculated with 160,000 FFU of HTNV.

Cells

The murine macrophage-like cell line P388D1 ($H\text{-}2^d$) was cultured in RPMI 1640 medium (Sigma, St. Louis, MO), supplemented with 5% FBS and 2-mercaptoethanol (2-ME, 50 μM). P388D1 cells continuously infected with HTNV were prepared as antigen-presenting cells, as described previously (Araki et al., 2003). Single-cell suspensions of spleen cells were obtained by homogenizing spleens in RPMI 1640 medium, supplemented with 10% FBS and 2-ME (50 μM). Erythrocytes were lysed with ACT solution (0.83% NH_4Cl).

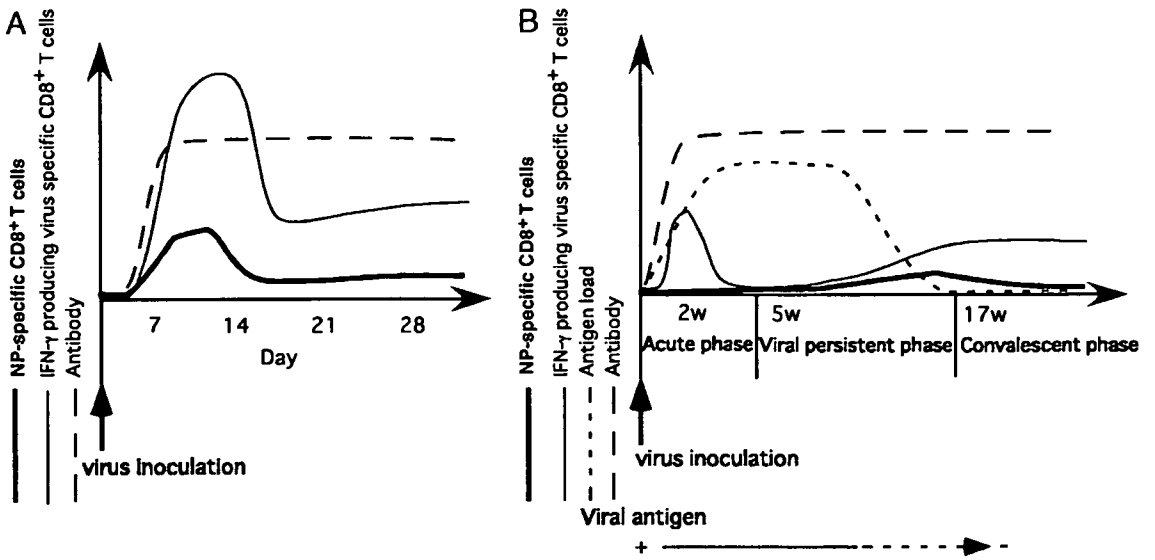


Fig. 5. Schema showing fluctuations in the numbers of $CD8^+$ T cells producing IFN-γ or that are positive for NP335–343, neutralizing antibodies titers, and antigen loads (amounts of N protein in lungs) in HTNV-infected adult or newborn mice based on data from the literature (Araki et al., 2003). (A) In transiently infected mice, NP335–343-positive $CD8^+$ T cells were normally expressed soon after infection, suggesting that transiently infected mice have normal immune responses. (B) In persistently infected mice, IFN-γ-producing virus-specific $CD8^+$ T cells were induced in the acute and convalescent phases. However, typical NP335–343-positive $CD8^+$ T cells were not detected at all time points. These results suggest that NP335–343-positive $CD8^+$ T cells were suppressed by a viral antigen.

Peptides and other reagents

Overlapping 15-mer peptides ($n=105$) with 10-amino acid overlaps, spanning residues 1–430 of the HTNV nucleocapsid protein (N), based on strain 76–118, and seven overlapping 11-mer peptides with 9-amino acid overlaps, spanning residues 329–347 of N, were purchased from Mimotope (Melbourne, Australia) and Hokkaido System Science (Sapporo, Japan). The nonamer peptide ILQDMRNTI, which was identified as the sequence of a CTL epitope, was purchased as a highly purified product from Hokkaido System Science. Stock solutions of these synthetic peptides were prepared in 100% DMSO at concentrations of 2 mg/mL for the 15-mer and 11-mer peptides and 5 mg/mL for the nonamer peptide. All were diluted to 0.2 μg/mL before use for *in vitro* stimulation. Goat anti-mouse H-$2D^d$ antibody (34-2-12) and goat anti-mouse H-$2K^d$ antibody (SF1-1.1) were purchased from BD Biosciences (Franklin Lakes, NJ) and were used for the inhibition-of-peptide-stimulation assay. The H-$2K^d$-restricted class-I peptide tetramer from the sequence ILQDMRNTI named NP335–343 was synthesized at the NIH Tetramer Facility.

IFN-γ staining for peptide screening

To detect intracellular gamma interferon (IFN-γ), spleen cells were added to 96-well V-bottomed plates at a concentration of 1×10^6 cells/well in RPMI 1640 medium, supplemented with 10% FBS, 2-ME (50 μM), murine recombinant interleukin-2 (IL-2; 40 ng/mL, Sigma), and brefeldin A (10 μg/mL, Sigma), along with peptide or HTNV-infected or uninfected P388D1 cells. The P388D1 cells were co-cultured at a concentration of 5×10^5 cells/well with the spleen cells. Peptide was added at a concentration of 0.1 to 0.2 μg/mL to the spleen cell cultures. After a 6-h incubation, cells were suspended in phosphate-buffered saline (PBS) and transferred to a 96-well plate containing ethidium monoazide bromide (EMA; 5 μg/mL, Invitrogen). After a 15-min incubation on ice in the dark, cells were washed with PBS twice and exposed to light for 10 min. Cells were washed with FACS buffer (PBS containing 1% bovine serum albumin [BSA] and 0.1% NaN_3) and stained with the anti-CD8a PE (Ly-2) antibody (eBioscience, San Diego, CA) for 30 min on ice. Cells were then washed with FACS buffer and fixed with 2% paraformaldehyde–PBS. After a 20-min incubation at room temperature, the cells were washed with FACS buffer, resuspended in permeation buffer (PBS containing 0.5% BSA, 0.5% saponin [Sigma], 0.1% NaN_3), and incubated for 10 min at room temperature. The cells were then stained with fluorescein isothiocyanate-conjugated rat anti-mouse IFN-γ antibody (Caltag Laboratories, San Francisco, CA), incubated for 30 min at room temperature, and washed twice with permeation buffer. Cells were then given a final wash with FACS buffer before analysis. Cells were analyzed using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ) and data analysis was conducted using FlowJo software (Tree Star, San Carlos, CA).

Inhibition-of-peptide-stimulation assay

Spleen cells were added to 96-well V-bottomed plates at a concentration of 1×10^6 cells/well in RPMI 1640 medium, supplemented with 10% FBS, 2-ME (50 μM), murine recombinant interleukin-2 (IL-2; 40 ng/mL, Sigma), and brefeldin A (10 μg/mL, Sigma). The NP335–343 peptide was added at a concentration of 0.2 μg/mL with or without anti-H-$2D^d$ or anti H-$2K^d$ antibodies (50 μg/mL). To remove sodium azide from the antibodies, immediately before the assay they

were dialyzed for 3 h in PBS using a microdialyzer Toru-kun (NIPPON Genetics, Tokyo). After a 6-h incubation, IFN-γ staining was carried out using the method described above.

Tetramer staining and flow cytometry

Spleen cells in several phases were stained, as described below. Cells (1×10^6 per well) were suspended in PBS and transferred to a 96-well plate containing EMA (5 μg/mL). After a 15-min incubation on ice in the dark, cells were washed with PBS twice and exposed to light for 10 min. The cells were washed with FACS buffer and stained with the anti-CD8a APC and -CD44 FITC antibodies (eBioscience) for 30 min on ice. After washing with FACS buffer, cells were stained with NP335–343 at a 1:500 dilution for 30 min at room temperature. Cells were then washed with FACS buffer and fixed with 2% paraformaldehyde–PBS. Tetramer-positive $CD44^+$ cells were analyzed with gates set on EMA-negative and CD8-positive cells. The cell samples were examined using a FACS Canto (Becton Dickinson), and data analysis was conducted with FACS Diva software (Becton Dickinson).

Acknowledgments

We thank the NIH Tetramer Facility (Atlanta, GA) for providing us with the ILQDMRNTI/H-$2K^d$ tetramer. This work was supported in part by a grant from the 21st Century COE Program, "Program of Excellence for Zoonosis Control," and in part by Grants-in-Aid for Scientific Research and the Development of Science from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, see: http://www.textcheck.com/cgi-bin/certificate.cgi?id=5X40nv.

References

Araki, K., Yoshimatsu, K., Lee, B.H., Kariwa, H., Takashima, I., Arikawa, J., 2003. Hantavirus-specific CD8(+)-T-cell responses in newborn mice persistently infected with Hantaan virus. J. Virol. 77 (15), 8408–8417.

Araki, K., Yoshimatsu, K., Lee, B.H., Kariwa, H., Takashima, I., Arikawa, J., 2004a. A new model of Hantaan virus persistence in mice: the balance between HTNV infection and CD8(+) T-cell responses. Virology 322 (2), 318–327.

Araki, K., Yoshimatsu, K., Lee, B.H., Okumura, M., Kariwa, H., Takashima, I., Arikawa, J., 2004b. Age-dependent hantavirus-specific CD8(+) T-cell responses in mice infected with Hantaan virus. Arch. Virol. 149 (7), 1373–1382.

Carey, D.E., Reuben, R., Panicker, K.N., Shope, R.E., Myers, R.M., 1971. Thottapalayam virus: a presumptive arbovirus isolated from a shrew in India. Indian J. Med. Res. 59 (11), 1758–1760.

Chen, L.B., Yang, W.S., 1990. Abnormalities of T cell immunoregulation in hemorrhagic fever with renal syndrome. J. Infect. Dis. 161 (5), 1016–1019.

Ebihara, H., Yoshimatsu, K., Ogino, M., Araki, K., Ami, Y., Kariwa, H., Takashima, I., Li, D., Arikawa, J., 2000. Pathogenicity of Hantaan virus in newborn mice: genetic reassortant study demonstrating that a single amino acid change in glycoprotein G1 is related to virulence. J. Virol. 74 (19), 9245–9255.

Huang, C., Jin, B., Wang, M., Li, E., Sun, C., 1994. Hemorrhagic fever with renal syndrome: relationship between pathogenesis and cellular immunity. J. Infect. Dis. 169 (4), 868–870.

Kilpatrick, E.D., Terajima, M., Koster, F.T., Catalina, M.D., Cruz, J., Ennis, F.A., 2004. Role of specific CD8+ T cells in the severity of a fulminant zoonotic viral hemorrhagic fever, hantavirus pulmonary syndrome. J. Immunol. 172 (5), 3297–3304.

Lednicky, J.A., 2003. Hantaviruses. A short review. Arch. Pathol. Lab. Med. 127 (1), 30–35.

Lee, H.W., van der Groen, G., 1989. Hemorrhagic fever with renal syndrome. Prog. Med. Virol. 36, 62–102.

Lee, K.Y., Chun, E., Kim, N.Y., Seong, B.L., 2002. Characterization of HLA-A2.1-restricted epitopes, conserved in both Hantaan and Sin Nombre viruses, in Hantaan virus-infected patients. J. Gen. Virol. 83 (Pt 5), 1131–1136.

Maeda, K., West, K., Toyosaki-Maeda, T., Rothman, A.L., Ennis, F.A., Terajima, M., 2004. Identification and analysis for cross-reactivity among hantaviruses of H-2b-restricted cytotoxic T-lymphocyte epitopes in Sin Nombre virus nucleocapsid protein. J. Gen. Virol. 85 (Pt 7), 1909–1919.

Maeda, K., West, K., Hayasaka, D., Ennis, F.A., Terajima, M., 2005. Recombinant adenovirus vector vaccine induces stronger cytotoxic T-cell responses than recombinant vaccinia virus vector, plasmid DNA, or a combination of these. Viral. Immunol. 18 (4), 657–667.

Mata, M., Travers, P.J., Liu, Q., Frankel, F.R., Paterson, Y., 1998. The MHC class I-restricted immune response to HIV-gag in BALB/c mice selects a single epitope that does not have a predictable MHC-binding motif and binds to Kd through interactions between a glutamine at P3 and pocket D. J. Immunol. 161 (6), 2985–2993.

Meyer, B.J., Schmaljohn, C.S., 2000. Persistent hantavirus infections: characteristics and mechanisms. Trends Microbiol. 8 (2), 61–67.

Mustonen, J., Helin, H., Pietila, K., Brummer-Korvenkontio, M., Hedman, K., Vaheri, A., Pasternack, A., 1994. Renal biopsy findings and clinicopathologic correlations in nephropathia epidemica. Clin. Nephrol. 41 (3), 121–126.

Nolte, K.B., Feddersen, R.M., Foucar, K., Zaki, S.R., Koster, F.T., Madar, D., Merlin, T.L., McFeeley, P.J., Umland, E.T., Zumwalt, R.E., 1995. Hantavirus pulmonary syndrome in the United States: a pathological description of a disease caused by a new agent. Hum. Pathol. 26 (1), 110–120.

Ogino, M., Yoshimatsu, K., Ebihara, H., Araki, K., Lee, B.H., Okumura, M., Arikawa, J., 2004. Cell fusion activities of Hantaan virus envelope glycoproteins. J. Virol. 78 (19), 10776–10782.

Park, J.M., Cho, S.Y., Hwang, Y.K., Um, S.H., Kim, W.J., Cheong, H.S., Byun, S.M., 2000. Identification of H-2K(b)-restricted T-cell epitopes within the nucleocapsid protein of Hantaan virus and establishment of cytotoxic T-cell clones. J. Med. Virol. 60 (2), 189–199.

Parker, K.C., Bednarek, M.A., Coligan, J.E., 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152 (1), 163–175.

Pensiero, M.N., Sharefkin, J.B., Dieffenbach, C.W., Hay, J., 1992. Hantaan virus infection of human endothelial cells. J. Virol. 66 (10), 5929–5936.

Raftery, M.J., Kraus, A.A., Ulrich, R., Kruger, D.H., Schonrich, G., 2002. Hantavirus infection of dendritic cells. J. Virol. 76 (21), 10724–10733.

Temonen, M., Mustonen, J., Helin, H., Pasternack, A., Vaheri, A., Holthofer, H., 1996. Cytokines, adhesion molecules, and cellular infiltration in nephropathia epidemica kidneys: an immunohistochemical study. Clin. Immunol. Immunopathol. 78 (1), 47–55.

Terajima, M., Van Epps, H.L., Li, D., Leporati, A.M., Juhlin, S.E., Mustonen, J., Vaheri, A., Ennis, F.A., 2002. Generation of recombinant vaccinia viruses expressing Puumala virus proteins and use in isolating cytotoxic T cells specific for Puumala virus. Virus Res. 84 (1–2), 67–77.

Van Epps, H.L., Schmaljohn, C.S., Ennis, F.A., 1999. Human memory cytotoxic T-lymphocyte (CTL) responses to Hantaan virus infection: identification of virus-specific and cross-reactive CD8(+) CTL epitopes on nucleocapsid protein. J. Virol. 73 (7), 5301–5308.

Van Epps, H.L., Terajima, M., Mustonen, J., Arstila, T.P., Corey, E.A., Vaheri, A., Ennis, F.A., 2002. Long-lived memory T lymphocyte responses after hantavirus infection. J. Exp. Med. 196 (5), 579–588.

Woo, G.J., Chun, E.Y., Kim, K.H., Kim, W., 2005. Analysis of immune responses against nucleocapsid protein of the Hantaan virus elicited by virus infection or DNA vaccination. J. Microbiol. 43 (6), 537–545.

Yanagihara, R., Silverman, D.J., 1990. Experimental infection of human vascular endothelial cells by pathogenic and nonpathogenic hantaviruses. Arch. Virol. 111 (3–4), 281–286.

Yoshimatsu, K., Arikawa, J., Ohbora, S., Itakura, C., 1997. Hantavirus infection in SCID mice. J. Vet. Med. Sci. 59 (10), 863–868.

Zaki, S.R., Greer, P.W., Coffield, L.M., Goldsmith, C.S., Nolte, K.B., Foucar, K., Feddersen, R.M., Zumwalt, R.E., Miller, G.L., Khan, A.S., et al., 1995. Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. Am. J. Pathol. 146 (3), 552–579.

Zhou, S., Ou, R., Huang, L., Price, G.E., Moskophidis, D., 2004. Differential tissue-specific regulation of antiviral CD8+ T-cell immune responses during chronic viral infection. J. Virol. 78 (7), 3578–3600.

Tropical Medicine and Health Vol. 35 No. 2, 2007, pp. 55-59

Proceedings of the 30th Annual Meeting of Kyushu Regional Society of Tropical Medicine Held in Nagasaki on February 9 and 10, 2007.

Hantavirus Infection - typical rodent-borne viral zoonosis

Jiro Arikawa[1,*], Kumiko Yoshimatsu[1], Truong Uyen Thang[2] and Truong Uyen Ninh[2]

INTRODUCTION

Zoonosis is a collective term for the infectious diseases transmitted from animals to humans and vice versa. Recently, various emerging infectious diseases have been identified as zoonoses. However, since the natural reservoirs for zoonosis are generally wild animals that established persistent infections without sign of disease, the eradication of zoonosis is quite difficult. An understanding of the epidemiologic and epizootiologic situation is essential, therefore, for the control and prevention of zoonosis.

Hantavirus infection is a typical zoonosis transmitted by rodents. In this paper, we briefly review the hantavirus infection and present epidemiologic and epizootiologic situation in East Asian countries.

1. Hantavirus

Hantaviruses are enveloped RNA viruses that belong to the *Hantavirus* genus of the family *Bunyaviridae*. These viruses persistently infect their rodent reservoirs without causing disease. The virus is transmitted to humans either via the inhalation of infectious aerosols generated from contaminated animal secretions or via contaminated saliva from animal bites. Hantaviruses cause haemorrhagic fever with renal syndrome (HFRS) in Euro-Asia and hantavirus pulmonary syndrome (HPS) in North and South America [1] (Fig.1, 2).

Thus far, 22 virus species have been relegated to the *Hantavirus* genus based on the criteria of 1) more than 7% difference in amino-acid identities of the complete glycoprotein precursor and N sequences, and 2) a more than fourfold difference in two-way cross neutralization tests involving species of primary reservoir animals [2].. Each *Hantavirus* species is predominantly carried by one species of rodent [3]. Phylogenetic analysis of the hantavirus genome has demonstrated three distinct clades, each com-

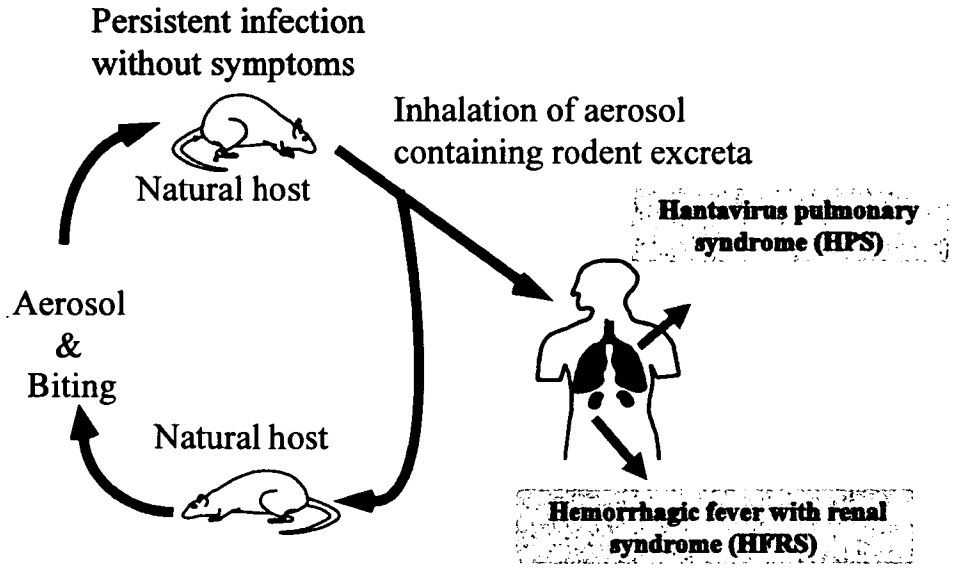


Fig. 1 Diseases caused by hantavirus

[1] *Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan*
[2] National Institute of Hygiene and Epidemiology, Hanoi, Vietnam
*Corresponding author.
E-mail address: j_arika@med.hokudai.ac.jp

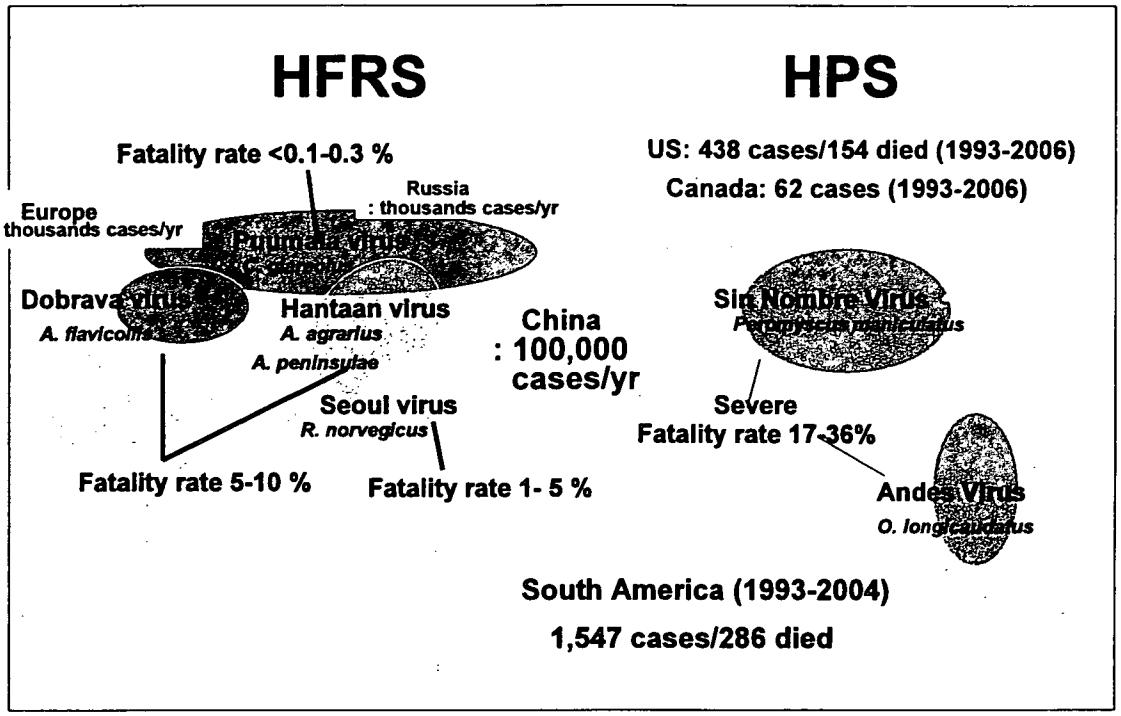


Fig. 2 Global distribution of HFRS and HPS

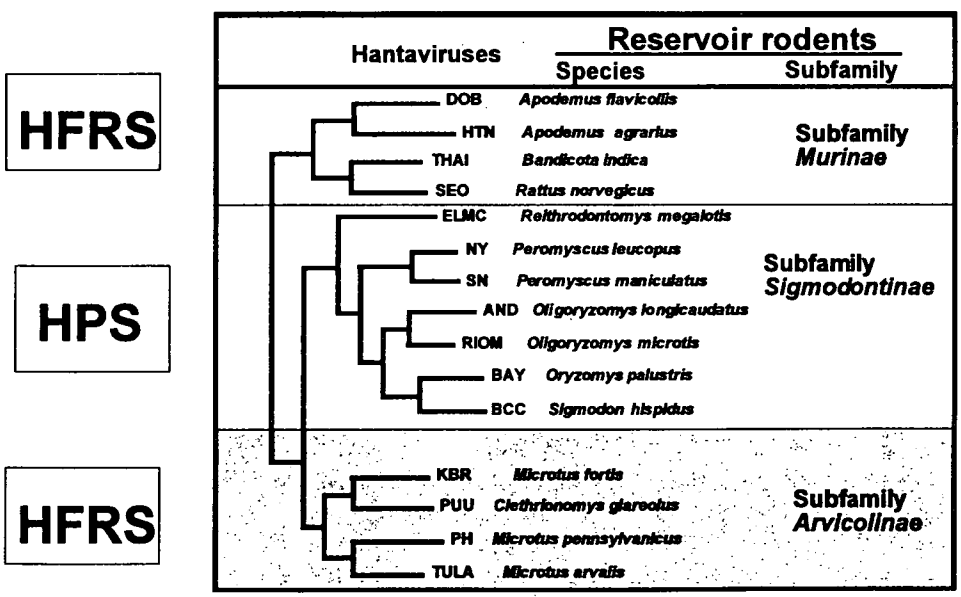


Fig. 3 Hantavirus and their rodent reservoirs

posed of viruses isolated from rodent hosts belonging to the same subfamily. For example, viral clades for the subfamilies *Murinae* (Old World rats and mice), *Arvicolinae* (voles and lemmings of the Northern Hemisphere) and *Sigmodontinae* (New World mice and rats) have been identified [4]. The phylogeny of the hantaviruses has been shown to mirror the genealogical relatedness of their host animals. Therefore, it has been suggested that hantavirus coevolved with their reservoir animals [5, 6] (Fig. 3).

2. Diseases caused by hantavirus infection

Hantavirus infection causes two different forms of severe febrile disease, i.e. hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) [1]. HFRS is characterized by systemic involvement of the capillaries and small vessels, which causes capillary leakage and haemorrhagic manifestations. Renal involvement in the

form of acute renal dysfunction as a result of interstitial haemorrhage and interstitial infiltrates is also common. After the prodromal period, the clinical course of patients with severe disease can be divided into five phases: febrile, hypotensive, oliguric, diuretic and convalescent [7]. A milder type of hantavirus infection, nephropathia epidemica (NE), is caused by PUUV and occurs in northern Europe [8]. In NE patients, although renal manifestations are common, haemorrhage is rare and the five phases typical of severe HFRS are absent. The mortality of NE patients is 0.1-0.3% and is thus much lower than the 5-10% of HFRS patients infected with HTN, SEO or DOB viruses [3].

HPS is characterized by bilateral interstitial pulmonary infiltrates, respiratory compromise usually requiring the administration of supplemental oxygen and clinical symptoms resembling those of ARDS. HPS can be divided into two phases: a prodromal phase, which usually lasts 3-5 days, and a cardiopulmonary stage marked by diffuse pulmonary edema and hypotension within 2-5 days after the onset of pulmonary symptoms. The rapid progression of interstitial pulmonary edema to alveolar edema, with severe bilateral involvement and the accumulation of pleural effusion, accounts for the 30-40% mortality associated with HPS [7].

Although the characteristic symptoms of HFRS and HPS differ, increased capillary permeability is considered to be the common underlying factor of the two diseases [9-10]. Since hantavirus is usually non-cytopathogenic in cultured cells, cell-mediated immune responses, such as activation of virus specific CD8+ T cells and increased levels of tumor necrosis factor receptor (TNF-r), interleukin (IL)-6, and IL-10 are most likely responsible for the symptoms observed in HFRS and HPS [11].

3. Epidemiology and epizootiology of HFRS in East Asian countries

Although the total number of HFRS patients is about 60,000－150,000 annually, more than 90% of these cases occur in Asian countries, including China, Russia, and Korea. Epidemiologic and epizootiologic information regarding the incidence of hantavirus infection in other East Asian countries is still limited, although patients with fevers of unknown etiology are suspected of being infected with hantavirus [12].

Seroepidemiological surveys confirmed hantavirus infections among humans and rodents in Taiwan [13][14], Hong Kong [15], Fiji [15], Malaysia [16], India [17], Indonesia [18], Singapore [19], Sri Lanka [20], Thailand [21-23] and Vietnam [24]. In Myanmar and Australia [15], positive sera were obtained only from humans, whereas in Cambodia only positive rodents were detected [25]. Nonetheless, taken together, the results clearly indicate that hantavirus infections affect humans and rodents throughout Asia.

Positive sera, mostly to SEOV, from humans and rodents were also reported in Vietnam in 1986 [24]. Since 2001, the distribution of hantavirus infection among both human and rodents has been surveyed through collaboration between Japan and Vietnam, particularly the Core University Program conducted by Nagasaki University. Eight of 308 sera obtained from healthy people residing in the Haiphong port area and in Hanam Province, in northern Vietnam, were positive for SEOV. Four of 204 serum samples obtained from patients with fevers of unknown origin (FOU) living in the northern provinces of HaNam and ThanhHoa were likewise positive. Positive sera were also detected in *Rattus* spp of rodents captured in the Haniphong port area and in HaNam and ThanhHoa provinces (Dr. Truong Uyen Ninh, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, personal communication).

A similar study has been conducted with a research group in Thailand. In Thailand, *R. norvegicus* obtained from the port area of Bangkok was reported to be infected with hantavirus, probably SEOV, transported from abroad by ship [21]. In addition, various species of inland rodents are infected with hantavirus. Amongst them, the greater bandicoot rat (*Bandicota indica*) is a major reservoir of the virus, while several species of rice-field rats, such as *R. rattus, Rattus exulans* and *Rattus losea,* are also natural reservoirs albeit to a lesser extent. The Thailand virus (THAIV), one of the distinct species of virus within the genus *Hantavirus,* was isolated from *B. indica* captured in a village near the western province of Kanchanaburi in 1985 [21]. Therefore, THAIV or related viruses appear to be distributed throughout Thailand. Ten of the 30 sera obtained from residents of the village where virus-infected *Bandicota* was captured showed antibody to hantavirus, which demonstrated that Thailand virus is able to infect humans. However, the virulency of THAIV towards humans has not been determined. Hantavirus has been suspected as one of the pathogens in fevers of unknown origin (FUOs) in Thailand. In the period 1999-2000, 115 cases of FUO were reported in patients admitted to Bangkok Hospital who were examined for antibodies to hantavirus. Paired sera from one patient showed high antibody titres to HTNV by IgG ELISA, IgM ELISA, and IFA test. Between 2002 and 2003, 260 paired sera from patients with FUO were collected in Surin Province. One of the sera showed a neutralising antibody titre to THAIV of 1:160, whilst the titres to HTNV and SEOV were less than 1:40. Furthermore, convalescent-phase serum did not contain hantavirus IgM antibody. Since the symptoms of the patient were comparable to those typical for HFRS, THAIV might be an additional causative agent of HFRS [23].

Conclusions

Epidemiological studies have shown that hantaviruses are widely distributed in Asia, both in humans and in rodents. Unlike the situation in Far East Asia, the number of hantavirus-antibody-positive sera has so far been quite small, even amongst FOU patients. Therefore, the significance of hantavirus infection as the causative agent for FOU in East Asia remains unclear, and further serological surveys amongst healthy people are needed. Nevertheless, these observations indicate that unidentified pathogens that cause FOU are prevalent in this region. To determine the new emerging infectious diseases which may cause FOU, the continuation of research both in humans and in animals is essential. For that purpose, projects such as the Core University Program for collaboration between Japanese institutions and those in endemic countries should be expanded.

Epizootiologic studies, particularly in rodents, have confirmed the close relationship between hantavirus and animals acting as reservoirs for the virus. Since it is thought that hantaviruses coevolved with their rodent hosts, an understanding of the virus' ecology may provide unique and important information about other rodent-borne pathogens as causative agents of emerging infectious diseases.

REFERENCES

1. Schmaljohn CS, Hasty SE, Dalrymple JM, LeDuc JW, Lee HW, von Bonsdorff CH, Brummer-Korvenkontio M, Vaheri A, Tsai TF, Regnery HL, Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science 1985; 227: 1041-1044.
2. Nichol ST, Beaty BJ, Elliott RM, Goldbach R, Plyusnin A, Schmaliohn CS, Tesh RB, Bunyaviridae. In Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (Eds.) Virus taxonomy: Classification and nomenclature of viruses: Eighth report of the international committee on the taxonomy of viruses., VIIIth Edn. Elsevier Academic Press., San Diego, London, Tokyo 2005; 695-716.
3. Schmaljohn C, Hjelle B, Hantaviruses - a global disease problem. Emerging Infectious Diseases 1997; 3: 95-104.
4. Plyusnin A, Morzunov SP, Virus evolution and genetic diversity of hantaviruses and their rodent hosts. Curr Top Microbiol Immunol 2001; 256: 47-75.
5. Meyer BJ, Schmaljohn CS, Persistent hantavirus infections: Characteristics and mechanisms. Trends in Microbiology 2000; 8: 61-67.
6. Plyusnin A, Vapalahti O, Lundkvist A, Hantaviruses: Genome structure, expression and evolution. J Gen Virol 1996; 77: 2677-2687.
7. Peters CJ, Simpson GL, Levy H, Spectrum of hantavirus infection: Hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Annual Review of Medicine 1999; 50: 531-545.
8. Lahdevitra J, Clinical manifestations and treatment of HFRS (Puumala virus). In Lee HW, Calisher C, Schmaljohn CS (Eds.) Manual of hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. WHO Collaborating Center for Virus Reference and Research, Seoul 1999; 28-32.
9. Cosgriff TM, Mechanisms of disease in hantavirus infection: Pathophysiology of hemorrhagic fever with renal syndrome. Rev Infect Dis 1991; 13: 97-107.
10. Kanerva M, Mustonen J, Vaheri A, Pathogenisis of Puumala and other hantavirus infection. Reviews in Medical Virology 1998; 8: 67-86.
11. Zaki SR, Greer PW, Coffield LM, Goldsmith CS, Nolte KB, Foucar K, Feddersen RM, Zumwalt RE, Miller GL, Khan AS, et al., Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. American Journal of Pathology 1995; 146: 552-579.
12. Lee HW, Epidemiology and pathogenesis of hemorrhagic fever with renal syndrome. In Elliott RM (Ed.) The Bunyaviridae. Plenum Press, New York 1996; 253-267.
13. Chin C, Chiueh TS, Yang WC, Yang TH, Shih CM, Lin HT, Lin KC, Lien JC, Tsai TF, Ruo SL, Nichol ST, Ksiazek TG, Rollin PE, Peters CJ, Wu TN, Shen CY, Hantavirus infection in Taiwan: The experience of a geographically unique area. Journal of Medical Virology 2000; 60: 237-247.
14. Liu YH, Huang JH, Hsueh PR, Luh KT, Hantavirus infection with marked sinus bradycardia, Taiwan. Emerg Infect Dis 2002; 8: 644-645.
15. Lee HW, Epidemiology and epizootiology. In Lee HW, Calisher C, Schmaljohn CS (Eds.) Manual of hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. WHO Collaborating Center for Virus Reference and Research, Seoul 1999; pp40-48.
16. Lam SK, Chua KB, Myshrall T, Devi S, Zainal D, Afifi SA, Nerome K, Chu YK, Lee HW, Serological evidence of hantavirus infections in Malaysia. Southeast Asian J Trop Med Public Health 2001; 32: 809-813.
17. Chandy S, Mitra S, Sathish N, Vijayakumar TS, Abraham OC, Jesudason MV, Abraham P, Yoshimatsu K, Arikawa J, Sridharan G, A pilot study for serological evidence of hantavirus infection in human population in south India. Indian J Med Res 2005; 122: 211-215.
18. Plyusnina A, Ibrahim IN, Winoto I, Porter KR, Gotama IB, Lundkvist A, Vaheri A, Plyusnin A, Identification of Seoul hantavirus in *Rattus norvegicus* in Indonesia. Scand J Infect Dis 2004; 36: 356-359.
19. Chan KP, Chan YC, Doraisingham S, A severe case of hemorrhagic fever with renal syndrome in Singapore. Southeast Asian J Trop Med Public Health 1996; 27: 408-410.
20. Vitarana T, Colombage G, Bandaranayake V, Lee HW, Hantavirus disease in Sri Lanka. Lancet 1988; 2.
21. Elwell MR, Ward GS, Tingpalapong M, LeDuc JW, Serologic evidence of Hantaan-like virus in rodents and man in Thailand. Southeast Asian J Trop Med Public Health 1985; 16: 349-354.
22. Suputthamongkol Y, Nitatpattana N, Chayakulkeeree M,

Palabodeewat S, Yoksan S, Gonzalez JP, Hantavirus infection in Thailand: First clinical case report. Southeast Asian J Trop Med Public Health 2005; 36: 217-220.

23. Pattamadilok S, Lee BH, Kumperasart S, Yoshimatsu K, Okumura M, Nakamura I, Araki K, Khoprasert Y, Dangsupa P, Panlar P, Jandrig B, Kruger DH, Klempa B, Jakel T, Schmidt J, Ulrich R, Kariwa H, Arikawa J, Geographical distribution of hantaviruses in Thailand and potential human health significance of Thailand virus. Am J Trop Med Hyg 2006; 75: 994-1002.
24. Rollin PE, Nawrocka E, Rodhain F, Sureau P, McCormick JB, Serological data on hemorrhagic fever with renal syndrome in southeast Asia. Bulletin de la Societe de Pathologie Exotique et de Ses Filiales 1986; 79: 473-475.
25. Reynes JM, Soares JL, Hue T, Bouloy M, Sun S, Kruy SL, Flye Sainte Marie F, Zeller H, Evidence of the presence of Seoul virus in Cambodia. Microbes Infect 2003; 5: 769-773.

Available online at www.sciencedirect.com
ScienceDirect
Comparative Immunology, Microbiology & Infectious Diseases 30 (2007) 341–356
COMPARATIVE IMMUNOLOGY MICROBIOLOGY & INFECTIOUS DISEASES
www.elsevier.com/locate/cimid

Hantavirus infection in East Asia

Hiroaki Kariwa[a], Kumiko Yoshimatsu[b], Jiro Arikawa[b,*]

[a]*Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan*
[b]*Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan*

Received 16 January 2007

Abstract

Hantaviruses are enveloped RNA viruses that belong to the *Hantavirus* genus of the family *Bunyaviridae*. These viruses persistently infect their rodent reservoirs without causing disease. The virus is transmitted to humans via the inhalation of infectious aerosols generated from contaminated animal secretions or through the contaminated saliva of animal bites. Hantaviruses cause haemorrhagic fever with renal syndrome in Euro-Asia, and hantavirus pulmonary syndrome (HPS) in North and South America. Here, we review the epidemiology and epizootiology of hantavirus infection in Asian countries.

Keywords: Zoonosis; Bunyavirus; Rodent; HFRS; HPS; Renal; Pulmonary; Persistent infection

Résumé

L'Hantavirus est un virus, enveloppé d'ARN, classé comme une espèce de Hantavirus de la famille Bunyaviridae. Ce virus infecte constamment des réservoirs rongeurs sans provoquer immédiatement la maladie. Le virus est transmis aux humains par l'intermédiaire de l'inhalation d'aérosols infectieux, qui sont fabriqués des sécrétions animales contaminées, ou par l'intermédiaire de la salive contaminée des animaux lorsque les humains sont mordus par un animal. La fièvre hémorragique sera provoquée par l'infection à Hantavirus et cette

*Corresponding author. Tel.: +81 11 706 6905; fax: +81 11 706 7879.
E-mail address: j_arika@med.hokudai.ac.jp (J. Arikawa).

0147-9571/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cimid.2007.05.011

fièvre est souvent accompagnée du syndrome rénal (HFRS) dans les régions Euro-Asies, tandis qu'elle provoque le syndrome pulmonaire à Hantavirus (SPH) dans l'Amérique du Nord et du Sud. Dans ce document, nous révisons l'épidémiologie et l'épizootiologie de l'infection à Hantavirus dans les pays asiatiques.

Mots clés: Zoonose; Bunyavirus; Rongeur; HFRS; HPS; Renal; Pulmonaire; Persistant infection

1. Introduction

Hantaviruses form a separate genus, *Hantavirus*, within the family *Bunyaviridae*. To date, 22 *Hantavirus* species have been registered within this genus based on comparisons of nucleotide sequence similarity and evolutionary classifications of viral genomes [1].

Hantavirus infection includes two different forms of severe febrile diseases, haemorrhagic fever with renal syndrome (HFRS) [2] and hantavirus pulmonary syndrome (HPS) [3]. The virus is maintained in persistently infected rodents, which therefore serve as natural viral reservoirs. Transmission of the virus to humans and rodents occurs via the inhalation of infectious aerosols generated from hantavirus-containing animal secretions [4–6] or by the contaminated saliva of animal bites [7]. Other viruses in the *Bunyaviridae* are transmitted by arthropod vectors [8].

Each *Hantavirus* species is predominantly carried by one species of rodent [9]. Phylogenetic analysis of the hantavirus genome has demonstrated three distinct clades, each composed of viruses isolated from rodent hosts belonging to the same subfamily. Thus, viral clades for the subfamilies *Murinae* (Old World rats and mice), *Arvicolinae* (voles and lemmings of the Northern Hemisphere) and *Sigmodontinae* (New World mice and rats) have been identified [10]. Thottapalayam virus (TPMV) is the only hantavirus isolated from a non-rodent host, the house musk shrew *Suncus murinus*, which was first captured in southern India in 1964 [11]. The phylogeny of the hantaviruses, including TPMV, has been shown to mirror the genealogical relatedness of their host animals, suggesting their co-evolution [12,13].

Due to the close relationship between hantavirus and rodent species, the distribution of cases of HFRS and HPS has been confined to the geographic distributions of the viral host species. Thus, since the reservoir animal species for the virus that causes HPS inhabits North and South American countries, the disease has only been reported in those regions [10]. Similarly, reservoir animals for Hantaan (HTNV) [14], Dobrava (DOBV) [15,16], and Puumala (PUUV) [17] viruses, which cause HFRS, live primarily in eastern Asia, northern and eastern Europe, central Europe, and central to northern Europe, respectively, and cases of disease caused by infections with these viruses are confined to the corresponding region. However, the HFRS-causing Seoul virus (SEOV) [18] is found worldwide, probably due to the distribution of its infected host, the brown rat, through international freight transportation. Nonetheless, HFRS resulting from SEOV infection has been confined, thus far, to Asian countries [19].

The total number of HFRS patients is about 60,000–150,000 annually. More than 90% of these cases occur in Asian countries, including China, Russia, and Korea. Epidemiologic and epizootiologic information regarding the incidence of hantavirus infection in other East Asian countries is still limited, although patients with fevers of unknown etiology are suspected of being infected with hantavirus [20].

HPS was first recognised in the southwestern United States (US) in 1993 as an acute respiratory distress syndrome (ARDS) with greater than 40% mortality [3]. Between the first case in 1993 and June 2006, 451 cases (159 deaths) in 32 states in the US [21] and 62 cases in Canada [22] have been reported. In South American countries, 1685 cases resulting in 331 deaths were recorded from 1993 to 2004 in Panama, Brazil, Bolivia, Chile, Paraguay, Uruguay, and Argentina [23]. The numerous cases of hantavirus and HPS in North and South America confirm the importance of this rodent-borne zoonosis in the New World (Table 1).

In this report, we briefly review current knowledge of hantavirus and hantavirus-mediated diseases, and then discuss hantavirus infection in humans and rodents in Asia. Finally, we assess the potential threat of an outbreak of hantavirus infection in Asian countries.

2. Hantavirus

Hantaviruses are enveloped, negative-sense RNA viruses [2]. The hantaviral particle is spherical or oval in shape with a diameter ranging from 80 to 120 nm. The genome of the virus consists of small (S), medium (M) and large (L) segments, 1696–2059, 3616–3696 and 6530–6562 nucleotides long, respectively [24]. The S, M and L genome segments encode nucleocapsid protein (N, 48–54 kDa), two external glycoproteins [Gn (68–76 kDa) and Gc (52–58 kDa)], and transcriptase protein (L, 246–247 kDa), respectively [25]. Unlike other members of the *Bunyaviridae*, hantaviruses do not encode non-structural proteins [24]. Hantavirus virions are formed on membranes of the host-cell Golgi complex, followed by budding into Golgi cisternae [26] and release of the virions by exocytosis.

Thus far, 22 virus species have been assigned to the *Hantavirus* genus based on the criteria of more than 7% difference in amino-acid identities of the complete glycoprotein precursor and N sequences, and a more than fourfold difference in two-way cross-neutralisation tests involving species of primary reservoir animals [1].

3. Diseases caused by hantavirus infection

3.1. Hantavirus diseases in humans

In the prodromal phase, HFRS and HPS produce similar flu-like symptoms, such as high fever, myalgia and headache. Subsequently, however, the symptoms associated with HFRS [27] and HPS [28] are very different.

HFRS is characterised by systemic involvement of the capillaries and small vessels, which causes capillary leakage and haemorrhagic manifestations. Renal involvement in the form of acute renal dysfunction as a result of interstitial haemorrhage and

Table 1
List of Old World and New World Hantaviruses

Virus species	Strain	Abbreviation	Principal species	Original isolation in	Geographical distribution of reservoir	Disease
Old World Hantaviruses						
Murinae subfamily-associated viruses						
Hantaan virus	Amur virus	AMRV	*Apodemus peninsulae*	Far East Russia	East Asia	HFRS
	Da Bie Shan virus	DBSV	*Niviventer confucianus*	South East China	East Asia	?
	Hantaan virus-76-118	HTNV	*Apodemus agrarius*	Korea	East Asia	HFRS
Dobrava-Belgrade virus	Dobrava-Belgrade virus	DOBV	*Apodemus flavicollis*	Slovenia/Servia	East Europe	HFRS
	Saaremaa virus	SAAV	*Apodemus agrarius*	Estonia	East Europe	HFRS
Seoul virus	Seoul virus-HR80-39	SEOV	*Rattus norvegicus*, *R. rattus*	Korea	Worldwide	HFRS
	Seoul virus-L99	SEOV	*R. losea*	Korea	Worldwide	HFRS
	Seoul virus-SR-11	SEOV	*R. norvegicus* (laboratory rat)	Japan	Worldwide	HFRS
Thailand virus	Thailand virus	THAIV	*Bandicota indica*	Thailand	South East Asia	?
Arvicolinae subfamily-associated viruses						
Puumala virus	Hokkaido virus-Kamiiso-8Cr-95	PUUV	*Clethrionomys rufocanus*	Japan	East Asia	?
	Muju virus	MUJV	*Eothenomys regulus*	Korea		?
	Puumala virus-Sotkamo	PUUV	*Clethrionomys glareolus*	Finland	Northern Europe	HFRS
Khabarovsk virus	Khabarovsk virus	KHAV	*Microtus fortis*	Far East Russia		?
Tula virus	Tula virus-Tula/Ma76/87	TULV	*Micotus arvalis*, *M. rossiaemeridionalis*			?
	Tula virus-Moravia/Ma530	TULV	*Micotus arvalis*, *M. rossiaemeridionalis*		?	
Topografov virus	Topografov virus	TOPV	*Lemmus sibiricus*			?
Not defined (suggested from *Insectivor*-associated virus)						
Thottapalayam virus	Thottapalayam virus	TPMV	*Suncus murinus*	India	East Asia?	?

Table 1 (*continued*)

Virus species	Strain	Abbreviation	Principal species	Original isolation in	Geographical distribution of reservoir	Disease
New World Hantaviruses						
Sigmodontinae subfamily-associated viruses						
Andes virus	Andes virus	ANDV	*Oligoryzomys longicaudatus*	Chile, Argentina	South America	HPS
	Bermejo virus	BMJV	*Oligoryzomys chacoensis*			
	Lechiguanas virus	LECV	*Oligoryzomys flavescens*			
	Maciel virus	MCLV	*Bolomys obscurus*			
	Oran virus	ORNV	*Oligoryzomys longicaudatus*			
	Pergamino virus	PRGV	*Akadon azarae*			
Bayou virus	Bayou virus	BAYV	*Oryzomys palustris*	Eastern USA	North America	HPS
Black Creek Canal virus	Black Creek Canal virus	BCCV	*Sigmodon hispidus*	South Eastern USA	South East USA, Northern South America	HPS
Cano Delgadito virus	Cano Delgadito virus	CADV	*Sigmodon alstoni*	Venezuela		?
El Moro Canyon virus	El Moro Canyon virus-RM-97	ELMCV	*Reithrodontomys megalotis*		Canada, USA, Mexico	?
Laguna Negra virus	Laguna Negra virus	LANV	*Calomys laucha*	Paraguay, Bolivia		HPS
Muleshoe virus	Muleshoe virus	MULV	*Sigmodon hispidus*	South East USA, Northern South America		?
New York virus	New York virus-RI-1	NYV	*Peromyscus leucopus*	USA, Canada, Mexico		HPS
Rio Mamore virus	Rio Mamore virus	RIOMV	*Oligoryzomys microtis*	South America		?
Rio Segundo virus	Rio Segundo virus	RIOS	*Reithrodontomys mexicanus*	Central America		?
Sin Nombre virus	Blue River virus-Indiana	BRV	*Pelomyscus leucopus*	Central USA		HPS
	Blue River virus-Okahoma	BRV	*Pelomyscus leucopus*			
	Monongahera virus	MGLV	*Pelomyscus maniculatus*	Eastern USA, Canada		
	Sin Nombre virus-Convict Creek 107	SNV				