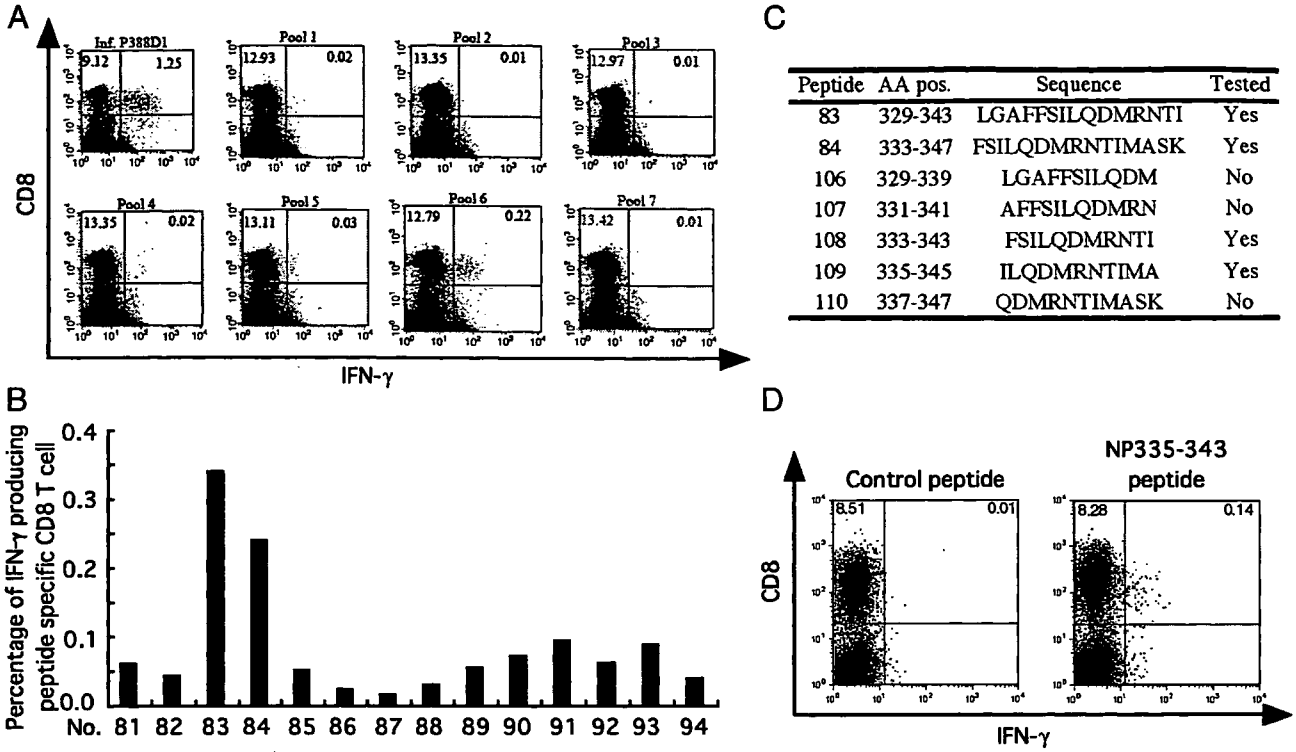

Fig. 1. Peptide screening for the N protein epitope from infected adult mice. Spleen cells were obtained from HTNV-infected BALB/c mice 10 days after HTNV inoculation. Spleen cells were cultured in the presence of brefeldin A and IL-2. IFN-γ^+ $CD8^+$ cells were detected using flow cytometry. The numbers 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-

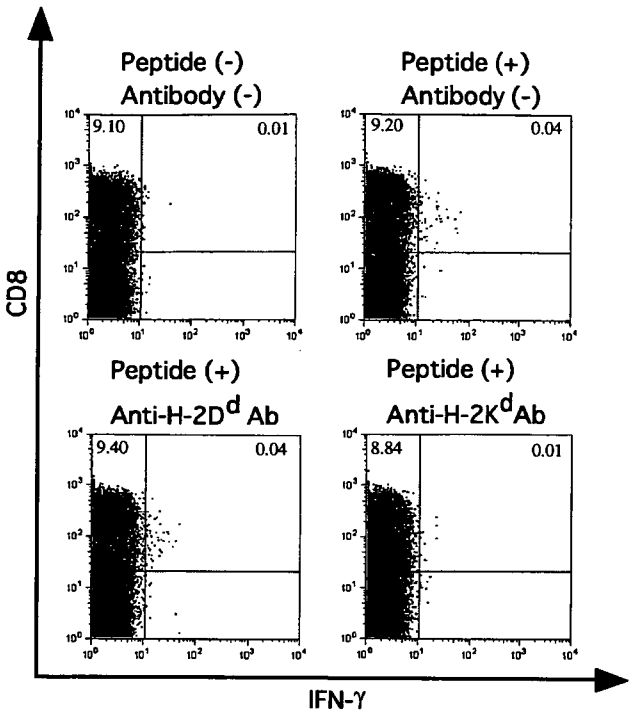

Fig. 2. The NP335–343 peptide is MHC class I H-2K^d-restricted. Spleen cells were obtained from HTNV-infected BALB/c mice 10 days after HTNV inoculation. Spleen cells were cultured in the presence of brefeldin A and IL-2. When required, anti-H-2D^d or H-2K^d antibodies were added simultaneously with 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

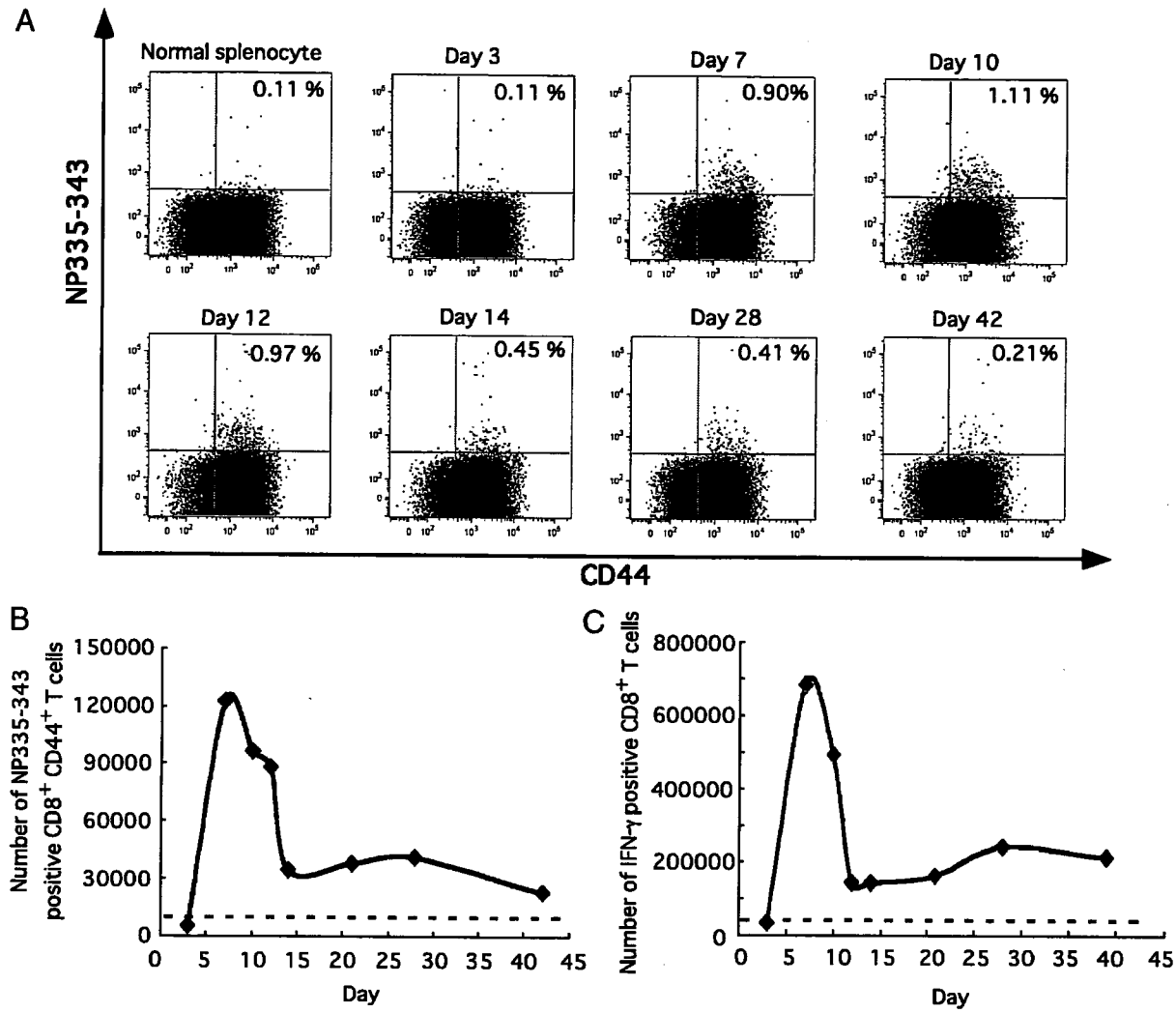

Fig. 3. Sequential analysis of NP335–343-specific $CD8^+$ T cells in a transient infection model. Spleen cells from normal adult mice and transiently infected adult mice, which were inoculated with HTNV cl-1, were stained with NP335–343. The number of $CD8^+$ T cells specific to the CTL epitope NP335–343 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

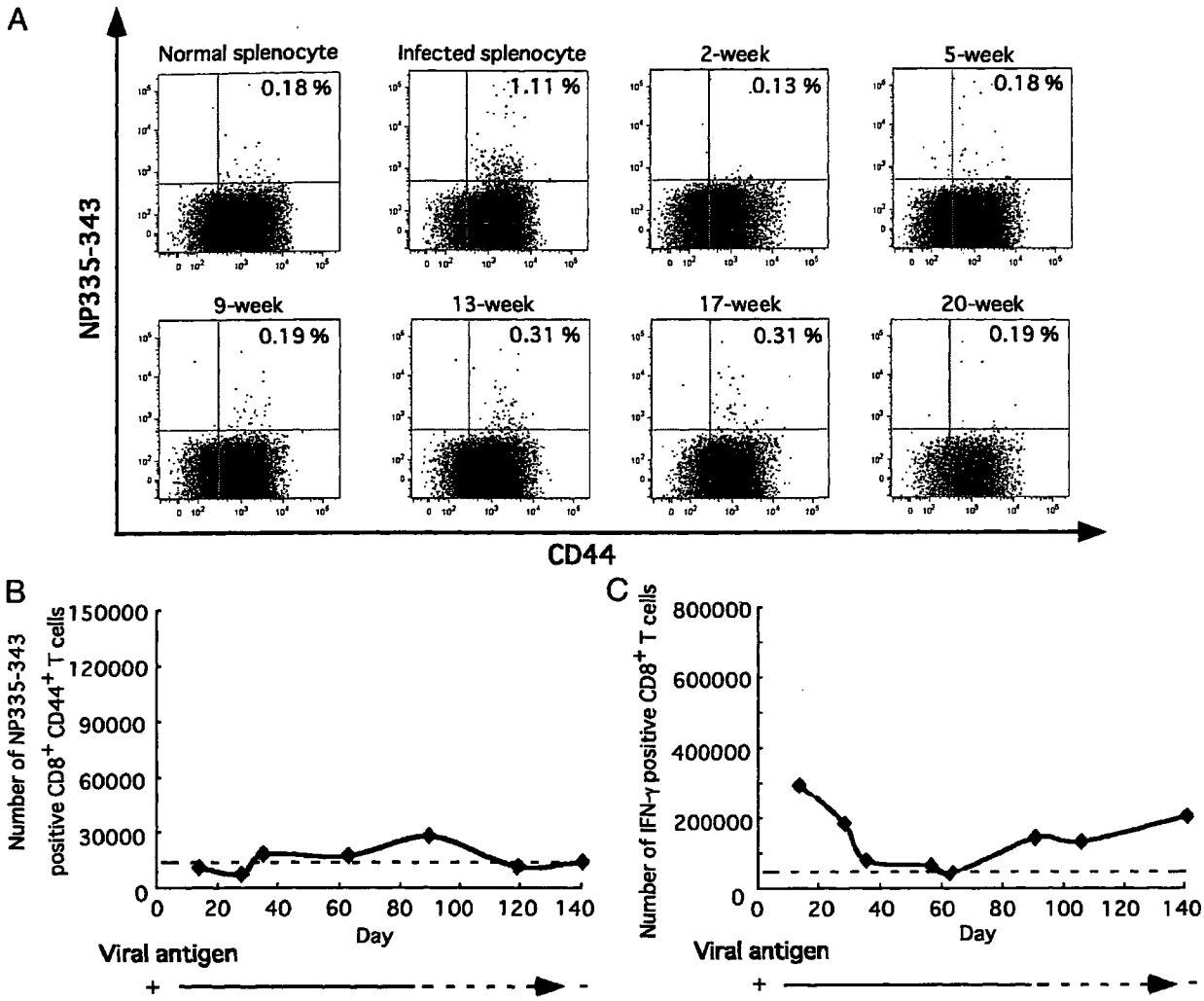

Fig. 4. Sequential analysis of NP335–343-specific $CD8^+$ T cells in a persistent infection model. BALB/c mice were inoculated with HTNV after birth (<24 h) and killed after several weeks. "Infected" indicates adult BALB/c mice that were inoculated with HTNV and recovered (positive controls). "Uninfected" 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 cl008) 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).

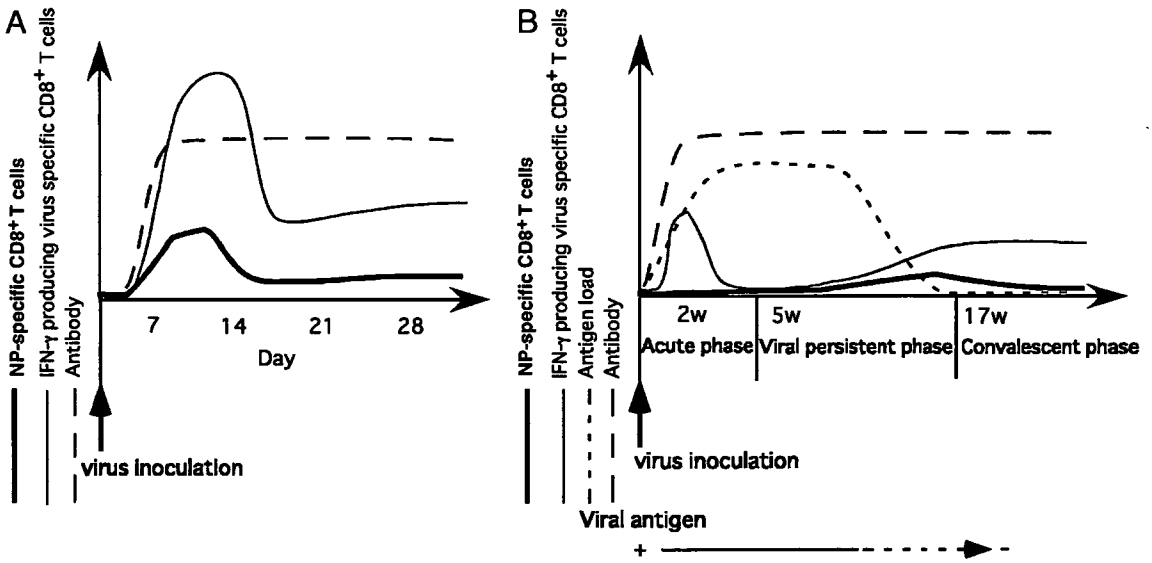

Fig. 5. Schema showing fluctuations in the numbers of $CD8^+$ T cells producing IFN-γ or that are positive for NP335–343, neutralizing antibodies titers, and antigen loads (amounts of N protein in lungs) in HTNV-infected adult or newborn mice based on data from the literature (Araki et al., 2003). (A) 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

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Hantavirus Infection - typical rodent-borne viral zoonosis

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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-

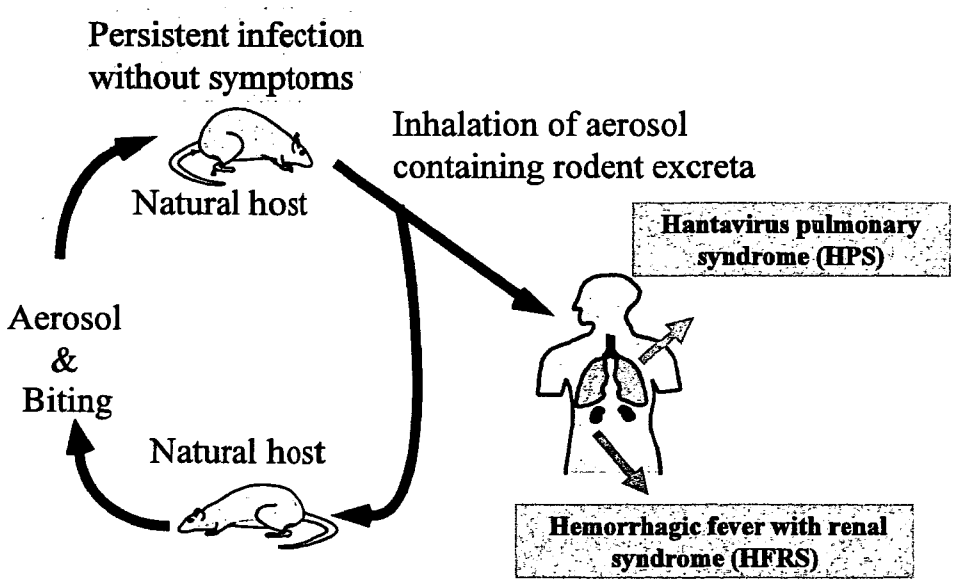

Fig. 1 Diseases caused by hantavirus

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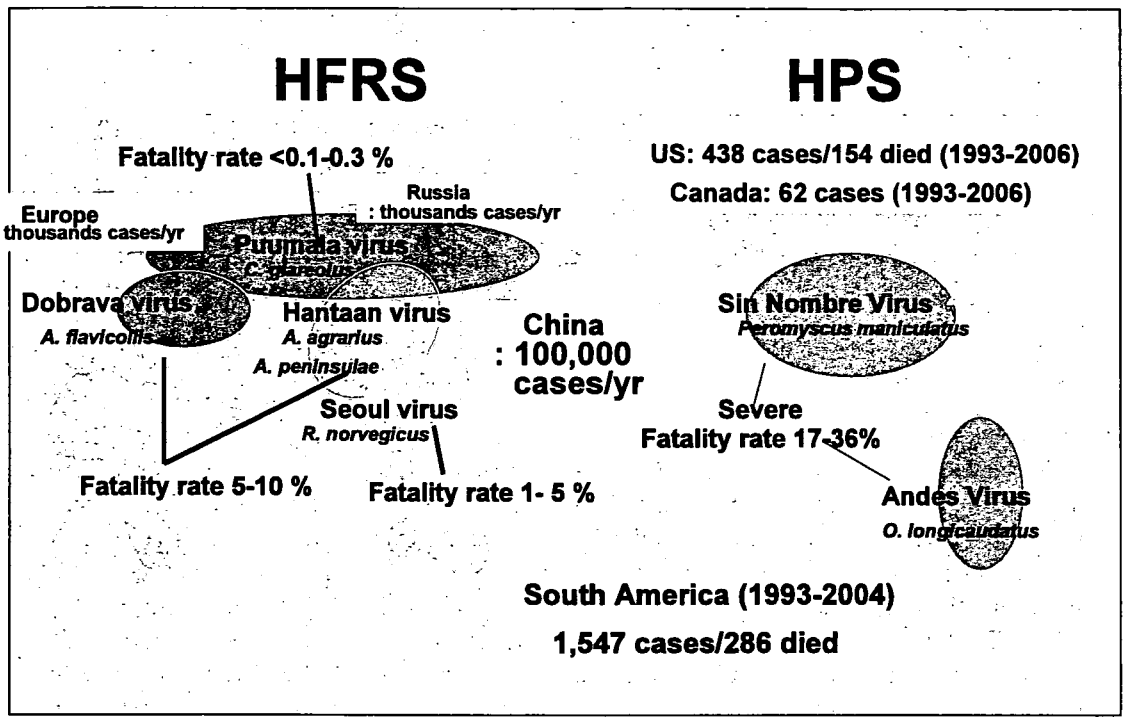

Fig. 2 Global distribution of HFRS and HPS

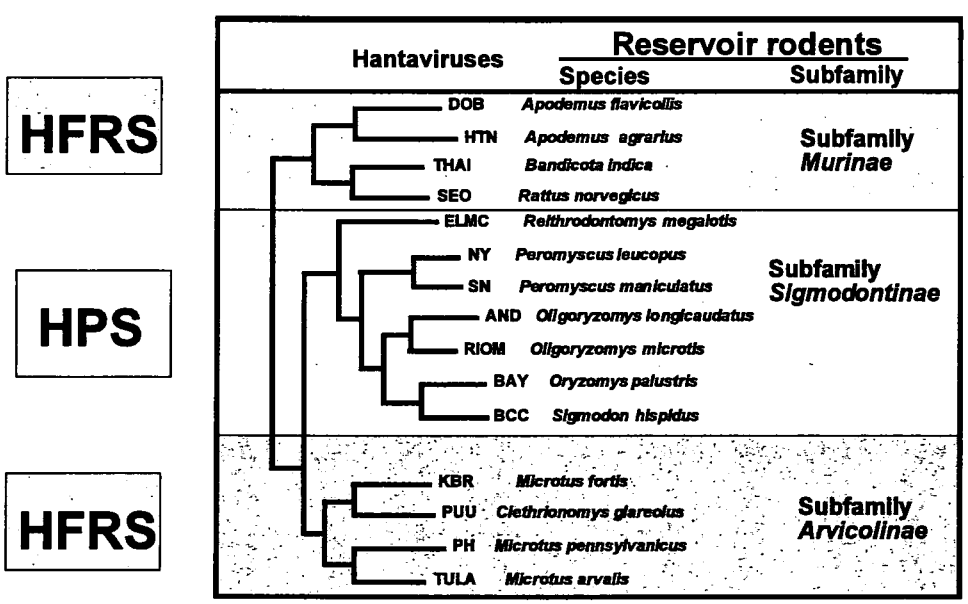

Fig. 3 Hantavirus and their rodent reservoirs

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.

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Seroepidemiological study on hantavirus infections in India

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Summary Hantaviruses are etiological agents of hemorrhagic fever with renal syndrome in many parts of Asia and Europe. There has been no documented case of hantavirus disease from India, although serological evidence exists. We investigated the prevalence of hantavirus in the Indian population and tried to identify potential risk groups for hantavirus infections. The presence of hantavirus-specific IgG antibodies was prospectively evaluated in 661 subjects belonging to different groups, i.e. patients with chronic renal disease, warehouse workers and tribal members engaged in rodent trapping. Healthy volunteer blood donors were included as a control group. Thirty-eight seropositive samples were found using a combination of a commercial ELISA followed by an indirect immunofluorescence assay. Western blot using recombinant Hantaan virus nucleocapsid antigen confirmed the presence of anti-hantavirus IgG in 28 (74%) of the 38 sera tested. This study confirms the presence of hantaviruses in India and warrants increasing awareness of the problems of emerging pathogens and the threats they may pose to the public health system.

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1. Introduction

Hantaviruses represent a group of emerging viruses. The genus *Hantavirus*, belonging to the family Bunyaviridae, comprises more than 20 species that can cause two diseases in humans: hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas (Lednicky, 2003). Almost all hantaviruses are maintained in rodents of the family Muridae, and are transmitted to humans via aerosolized urine, saliva and feces of infected rodents.

Although hantavirus infections were recognized in Asia for centuries, it was only during investigations initiated after the Korean conflict in the 1950s, during which thousands of UN soldiers were affected by HFRS, that the Hantaan virus (HTNV) serotype was isolated. The circulation of hantavirus serotypes, namely Seoul virus (SEOV) and Thailand virus (THAIV), has been demonstrated in several Southeast Asian countries, i.e. Thailand, Cambodia, Viet Nam and Indonesia (Plyusnina et al., 2004; Reynes et al., 2003; Truong et al., 2004). Recently a report from Thailand documented the first serological evidence of THAIV causing HFRS in humans (Pattamadilok et al., 2006). The Thottapalyam virus (TPMV), which was isolated from an insectivore, *Suncus murinus*, in 1964, is the only known hantavirus species indigenous to India (Cary et al., 1971).

The association of chronic renal disease and hantavirus seropositivity has frequently been speculated upon as studies conducted in the United States (Baltimore) suggested that hantavirus seropositives had higher rates of chronic renal disease and hypertensive renal disease than age-matched seronegative controls (Glass et al., 1990). Similar studies have been reported from Israel (George et al., 1998) and Egypt (Botros et al., 2004). An epidemiological study from Taiwan has reported detection of anti-hantavirus antibody in various risk groups such as garbage collectors and animal handlers (Chen et al., 1998).

Although the isolation of TPMV pre-dates that of HTNV, interest in hantaviruses was revived in India in 2005, with two reports on the serological evidence of hantavirus infections in patients with febrile illnesses (Chandy et al., 2005; Clement et al., 2006). However, seroepidemiological surveys have not been reported from India, and this study is the first attempt to investigate the epidemiology of hantavirus infections in India.

2. Materials and methods

2.1. Study population

The study subjects belonged to different groups and were recruited at the Christian Medical College, Vellore, south India. Healthy volunteer blood donors (n=360) comprised the control group. The potential risk groups included 99 sera from asymptomatic Irulas, a tribal community living in Tamil Nadu, a state in south India (the tribe members are professional rat catchers and eat rats) and 51 sera from people working in warehouses (warehouse workers) in and around Vellore. An additional group was represented by 151 serum samples from patients with chronic renal disease with serum creatinine and urea levels of >1.4 mg/dl and >40 mg/dl, respectively.

The mean (±SD) age of the subjects in the Irula group was 32 (±11.2) years, and this group included 41 males and 58 females; in the warehouse workers it was 38 (±10.17) years and all were males; and in the renal disease patients it was 43 (±12.11) years and there were 47 females and 104 males. The subjects in the control group were aged 18–60 years.

The sample size was calculated based on a previous study of hantavirus infections in India (Chandy et al., 2005). The study period was from August 2004 to May 2007. Samples from healthy blood donors and patients with chronic renal disease were collected at the Department of Clinical Virology, Christian Medical College, Vellore and were included in the study by convenient sampling. All samples collected from asymptomatic Irulas and warehouse workers were included in the study. Written informed consent was obtained before collecting blood.

2.2. ELISA and immunofluorescence assay

Serological screening was done using a commercial (ISO certified) hantavirus IgG ELISA (Focus Technologies, Cypress, CA, USA) according to the manufacturer's instructions. This ELISA uses a cocktail of hantavirus antigens of HTNV, SEOV, Puumala virus (PUUV), Sin Nombre virus (SNV) and Dobrava-Belgrade virus (DOBV) to coat the polystyrene microwells and can detect IgG antibodies against these serotypes. The screening ELISA is an indirect test in which the optical density (OD) is directly proportional to the antigen-specific IgG antibodies present in the sample. The results were obtained by comparison of the sample OD readings with reference cut-off OD readings.

Results were reported as index values relative to the cut-off calibrator. To calculate index values, each sample OD value was divided by the mean of the cut-off calibrator OD values.

Sera positive by ELISA were re-tested by an indirect immunofluorescence assay (IFA) using HTNV-infected Vero E6 cells as antigens (Yoshimatsu et al., 1993). The secondary antibody used was fluorescein-isothiocyanate-conjugated rabbit anti-human IgG (DakoCytomation, Glostrup, Denmark). For the IFA, all spots with at least half of the infected cells showing a characteristic apple green granular cytoplasmic fluorescence with ≥2+ intensity were scored positive.

Finally, a sample was considered positive if reactive by both ELISA and IFA. The screening ELISA and IFA were evaluated using a panel of positive and negative control sera kindly supplied by the European Network for Diagnostics of Imported Viral Diseases [ENIVD (Biel et al., 2003)].

2.3. Western blot analysis

Western blot analysis was performed on 38 positive sera using recombinant nucleocapsid protein (NP) of HTNV (Fojnica strain) as antigen (Razanskiene et al., 2004). Sera at dilutions of 1:1000 were applied to the membrane, and goat anti-human IgG conjugated with alkaline phosphatase (Genelabs Diagnostics Pte Ltd, Singapore Science Park, Singapore 118259, Republic of Singapore) at 1:1000

Table 1 Results of evaluation of the screening ELISA and Hantaan virus-immunofluorescence assay (IFA) using European Network for Diagnostics of Imported Viral Diseases (ENIVD) sera

ENIVD no.	Sample type	IgG level	Origin	Hantavirus strain	Serum status	ELISA result	IFA result
2	Positive serum	++	Sweden	Puumala	Convalescent	+	+
8	Positive serum	+	Kosovo	Dobrava	Convalescent	+	+
9	Positive serum	++	Sweden	Puumala	Convalescent	+	+
16	Positive serum	++	Sweden	Puumala	Convalescent	+	+
17	Positive serum	+	Finland	Puumala	Convalescent	–	–
4	Negative serum	–	Germany	–	Control	–	–
12	Negative plasma	–	Germany	–	Control	–	–
18	Negative serum	–	Germany	–	Control	–	–
19	Negative serum	–	Germany	–	Control	–	–

+, positive; ++, strong positive; –, negative.

dilutions was used as the secondary antibody. Thereafter the membrane was developed with substrate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT). In the Western blot the molecular size of the expected product was about 49 kDa.

2.4. Statistical analysis

The statistical analysis was done using EpiInfo version 6.04b (CDC, Atlanta, GA, USA) to compare two categorical variables. Percentages were calculated for categorical outcomes (positives/negatives). A P-value <0.05 was considered significant.

3. Results

ENIVD sera were used to evaluate the commercial IgG ELISA and the IFA using HTNV antigen; these assays could detect anti-Puumala virus and anti-Dobrava virus IgG-positive sera. However, one ENIVD anti-Puumala virus serum (origin Finland) could not be detected by either of these assays (Table 1).

In the initial screening, 661 serum samples were tested by a commercial IgG ELISA (Table 2). Forty-seven of the 661 sera were found to be reactive in the ELISA. The majority (38/47) of the ELISA-reactive sera were also detected by IFA using HTNV antigen. Seropositivity in the Irula tribal group (11%) was significantly higher than in the control group (4%, $P<0.05$). There was no statistically significant difference between seropositivity in the chronic renal disease patient group (7%) compared to that of the control group. The level of seropositivity in the warehouse workers (2%) was very similar to that of the control group. Sex as a demographic factor was not significantly associated with hantavirus infections in the different subject groups, suggesting that males and females are equally likely to contract hantavirus infections. The mean age of the seropositives in the renal disease patient group, the Irulas and the warehouse workers was 50 (SD ± 11.29), 29 (SD ± 8.9) and 31 years, respectively. Twenty-eight of the 38 positive sera (74%) were positive by Western blot (Table 2).

4. Discussion

Studies on hantavirus infections in India are in the early stages. The data given here represent the first attempt to characterize the epidemiology of hantavirus infections in India and strengthen previous reports on serological evidence of hantavirus infections in India (Chandy et al., 2005; Clement et al., 2006).

Forty-seven of 661 serum samples were positive by ELISA. Serology is the mainstay of diagnosis of hantavirus infections. ELISAs are highly sensitive and are the preferred diagnostic tool for serological surveys. The commercial ELISA used in this study uses a cocktail of six antigens, and can be used in areas where the circulating hantavirus species are unknown. The specificity of the hantavirus assays used in the study is acceptably good, as evaluated by the ENIVD-negative control sera. There may be problems with diagnostic sensitivity, as one ENIVD anti-Puumala IgG-positive serum was not detected by both the assays.

Table 2 Results of serological studies

Group tested	No. tested	No. positive by ELISA (%)	No. positive (ELISA and HTNV-IFA) (%)	No. positive/no. tested by Western blot (%)
Blood donors	360	19 (5)	16 (4)	11/16 (69)
Renal disease patient group	151	14 (9)	10 (7)	9/10 (90)
Irulas	99	12 (12)	11 (11)	7/11 (64)
Warehouse workers	51	2 (4)	1 (2)	1/1 (100)
Total	661	47 (7)	38 (6)	28/38 (74)

HTNV-IFA: Hantaan virus-immunofluorescence assay.

The lower seroprevalence observed in the IFA and Western blot analysis might be due to the HTNV antigen, which suggests that hantavirus species other than HTNV are circulating and causing human disease in India. Alternatively, we cannot exclude the possibility that the ELISA picked up false positives.

It has been documented that TPMV is phylogenetically and antigenically quite distinct from the other well-characterized hantaviruses (Song et al., 2007), and although antibodies against hantavirus NP are cross-reactive between different hantavirus species, we cannot speculate about the efficiency of the assays used in the study to detect antibodies against TPMV. Moreover, there may be other hantavirus species circulating in India that may be as diverse as TPMV, and in the case of an antigenic mismatch the assays used here may fail to detect seropositives. It is thus important to define the hantavirus species circulating in India and develop sensitive assays using homogeneous antigens.

In this study, the renal disease patient group appears to have a higher risk of hantavirus seropositivity compared with the control group, but the difference is not statistically significant. It is still not clear whether patients with chronic renal disease are at a higher risk of acquiring hantavirus infections or that hantavirus infections by themselves contribute to the development of chronic renal disease. These results are preliminary, and follow-up studies are needed to prove any significant association of hantavirus infections with chronic renal disease.

The tribal group in this study has a relatively high level of contact with rodents, as they are traditionally rat catchers and also eat rats. They display a high seropositivity when compared with the control group. By contrast, warehouse workers showed a low seropositivity in our study (2%). This may reflect a lower risk of contracting hantavirus infections due to the fact that many warehouses in India adopt stringent rodent-control measures.

No well-documented hantavirus case, as defined by virus isolation or molecular evidence, has been reported from India to date. However, our study confirms that one or more hantaviruses are circulating in the Indian population and indicates that the threat from emerging pathogens must be continually assessed. Studies to identify the hantaviruses that might cause problems for public health systems are important, as they will aid the development of new strategies for the prevention and control of such emerging infections.

Authors' contributions: GS and PA designed the study protocol; SC carried out the immunoassays and drafted the manuscript; KY, RGU, MM, MO and JA supplied IFA slides and Western blot strips and helped analyse results obtained; RP, GTJ, VB, JM and JM helped with the identification of subjects in the various groups and collection of samples. All authors read and approved the final manuscript. GS is guarantor of the paper.

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四類感染症

腎症候性出血熱

Hemorrhagic fever with renal syndrome (HFRS)

有川二郎

Key words：人獣共通感染症，ハンタウイルス肺症候群(HPS)，ハンタウイルス感染症，齧歯類，四類感染症

はじめに

腎症候性出血熱(hemorrhagic fever with renal syndrome: HFRS)はブニヤウイルス科のハンタウイルスを原因とする熱性疾患で，腎臓の機能障害を伴う出血熱という点が特徴である．齧歯類を自然宿主とし，糞尿中に排泄されたウイルスによってヒトに感染する人獣共通感染症である．

現在，我が国での発生はないが，近隣諸国の中では，中国，韓国，ロシアで流行が報告され，また，我が国の港湾地区のドブネズミが高率に感染していることから，輸入症例や未確認症例の存在が危惧されている．このため，本症は'感染症の予防及び感染症の患者に対する医療に関する法律'(以下，感染症法と記す)において四類感染症に分類され，診断した医師に届出の義務がある．

本稿では，HFRSについて，疫学と疾患の概要を中心に紹介する．ハンタウイルス肺症候群(HPS)もHFRSウイルスに近縁のウイルスを原因とするが，その流行は南北アメリカ大陸に限局している．HPSの稿を参照されたい．

1. 歴　　史[1)]

ユーラシア大陸全域で以前から腎臓の機能障害を伴う出血熱の存在が知られていた．それぞれの地方で風土病として，北欧では流行性腎症，中国では孫呉熱，虎林熱，二道崗熱，韓国では韓国型出血熱と呼ばれていた．

第二次世界大戦中，旧日本陸軍(関東軍)は旧満州(中国東北地方)で本症に遭遇し，1万人以上の患者と3,000人以上の死亡例が報告され，大きな問題となった．日本軍医団の研究によって，本症がウイルス性(濾過性病原体)の疾患であることが明らかにされ，流行性出血熱(epidemic hemorrhagic fever: EHF)と命名された．現在でも中国ではHFRSを流行性出血熱と呼んでいる．

1950年代の朝鮮戦争時にも国連軍兵士の間で3,000例以上の発生例があり，死亡率も5〜10%に達した．症状からEHFであることが判明したが，原因ウイルスの分離には至らなかった．

1976年，韓国の李らによって，韓国型出血熱流行地を流れるハンターン川のそばで捕獲されたセスジネズミから原因ウイルスが初めて分離された．そこで，この川の名前にちなみ，ハンターンウイルス(Hantaan virus)と命名された．このウイルスを用いた血清診断によって，上述したユーラシア大陸全域で報告されていた類似疾患がいずれもハンターンウイルスと類似のウ

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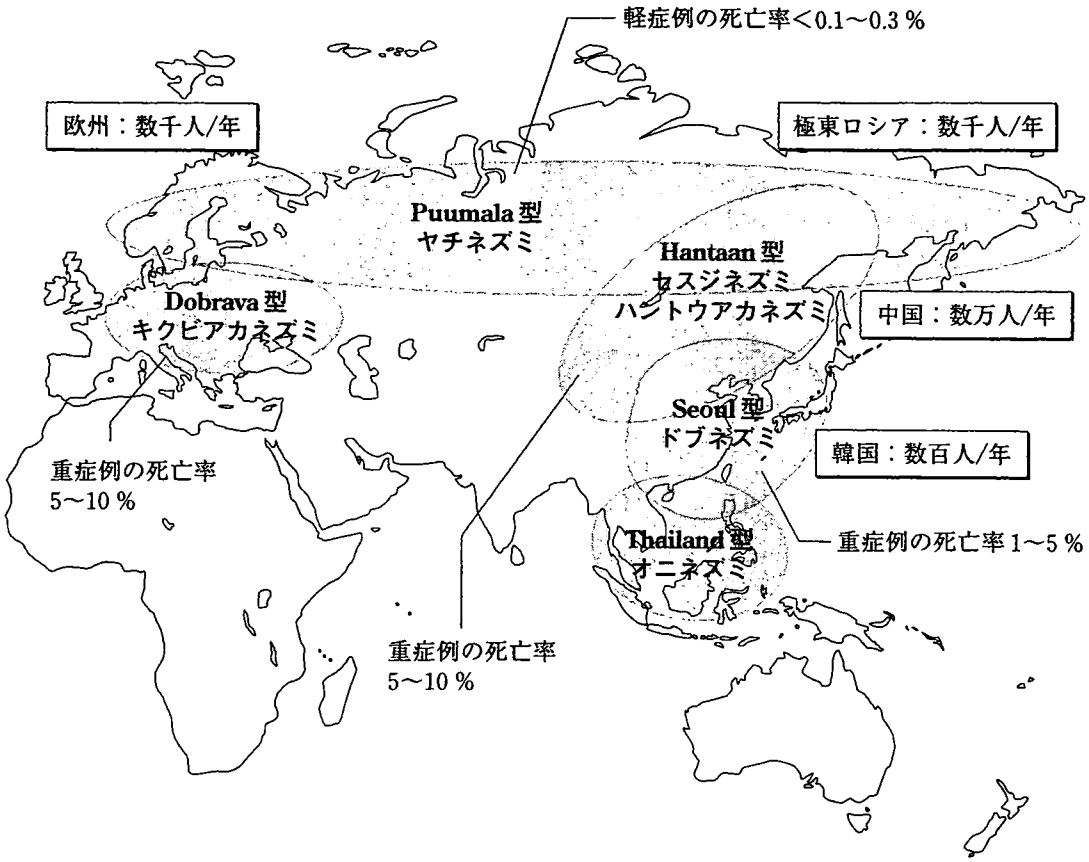

図1 HFRSの流行地域

イルスを原因とすることが明らかとなった．更に，抗原性や遺伝子性状が類似しているウイルスが次々と分離されたことから，原因ウイルスは，ブニヤウイルス科のハンタウイルス(Hantavirus)属にまとめて分類されることとなった．また，1982年，世界保健機関(WHO)は東京で研究集会を開催し，これらの関連した疾患を腎症候性出血熱と統一して呼称することを決定した．

2. 疫　　学[2)]

ハンタウイルスの中の，Hantaan(ハンターン)型，Seoul(ソウル)型，Puumala(プーマラ)型，Dobrava(ドブラバ)型がHFRSの原因となる．ウイルス型ごとに，異なった種類の齧歯類を自然宿主としている．このため，齧歯類の生息地域に一致して流行が発生している．また，ヒトへの病原性もウイルスの型によって異なる傾向がある．Hantaan型とDobrava型が最も病原性が高く，死亡例は重症例の5～10％に達する．Seoul型やPuumala型ではそれよりも低率で，特にPuumala型では軽症例がほとんどで，死亡率は多くの場合0.1％以下である．最大の流行国は中国で，年間数万人の患者が報告されている．そのほか，韓国では年間数百例，極東ロシアと欧州ではそれぞれ年間数千例の報告がある．最近，Pattamadilokらは，東南アジア諸国にはThailand(タイランド)型ウイルスがオニネズミの間に広く感染し，HFRSの原因となり得ることを明らかにした[3)](**図1**)．従来，原因不明の不明熱として診断されていた症例中にハンタウイルスを原因とするものが含まれている可能性が示唆されている．

我が国におけるハンタウイルス感染の背景と現状を**表1**にまとめた．これまでに，大阪(梅田駅近く)でのドブネズミを感染源とする流行(都市型流行)と実験用ラットを感染源とする流行(実験室型流行)を経験しているが，現在流行の発生は報告されていない．しかし，北海道のほぼ全域でPuumala型ウイルスに感染したエ

表1 我が国におけるハンタウイルス感染症の背景

年	場所	自然宿主齧歯類	患者数	死亡数
1940年代	中国（旧満州）	*Apodemus agrarius*（セスジネズミ）	1万人	3,000人
1960～70	大阪（梅田）	*Rattus norvegicus*（ドブネズミ）	119人	2人
1970～84	全国22研究機関	*Rattus norvegicus*（実験用ラット）	126人	1人
1984～98	全国19カ所，港湾地区と空港	感染したドブネズミの生息を確認	患者発生の報告なし	
1992～現在	北海道ほぼ全域	感染エゾヤチネズミ	患者発生の報告なし	
	富山県，島根県	感染アカネズミ	患者発生の報告なし	
1992年	国立大学動物実験施設	実験用ラット	抗体陽性者，5人確認（不顕性感染）	
1998年	原因不明肝炎患者血清105例中3例にハンタウイルス抗体確認			
1999年	北海道駐屯自衛隊207例中2例にハンタウイルス抗体確認			
2002年	西日本31病院，血液透析患者1,382人中14例にハンタウイルス抗体確認			

ゾヤチネズミが発見され，また，少数例ではあるが，富山と島根で捕獲されたアカネズミがHantaan型に感染していることも明らかになった[4]．更に，1975～98年までに実施された港湾区域における齧歯類の調査から，全国19カ所の港湾地区で捕獲されたドブネズミにはSeoul型ウイルスの感染が確認され，予想以上に広い範囲で齧歯類の間にハンタウイルスが存在していると考えられている[5]．

感染齧歯類が確認されているにもかかわらず，ヒトでのHFRSの流行は報告されていない．しかし，原因不明肝炎患者[6]，慢性腎不全で血液透析中患者[5]および感染エゾヤチネズミの生息する北海道での野外訓練歴の長い自衛隊隊員[7]の中に抗体陽性例が確認されている．いずれも，ウイルス分離やゲノム検出は行われていないため，ハンタウイルス感染と疾患との関連は確定されていないが，肝炎や腎不全はHFRSの症状として知られており，継続調査と，類似疾患に対する情報提供が望まれる．

3. 病原体と感染経路

ハンタウイルスはブニヤウイルス科ハンタウイルス属に分類されるRNAウイルスで，直径約80～100nmの球形でエンベロープをもつウイルスである．自然宿主となる齧歯類は高い中和抗体を保有するにもかかわらず，不顕性に持続感染し，糞尿や唾液中にウイルスを長期間排泄する．それら糞尿などを含む粉塵などにより呼吸器感染すると考えられている．このため，中国では，齧歯類の活動が活発化する春と秋に感染例が増加する．ヒトからヒトへの直接伝播は報告されていない．しかし，急性期の患者血液中には感染性ウイルスの存在が確認されており，医療現場では注意が必要である．ウイルスの感染性は消毒用アルコールで容易に失活する．トゲダニをベクターとする成績が中国で得られているが，他の流行国では節足動物をベクターとする成績は得られていない．

4. 症　　状

WHOハンタウイルスレファレンスセンター出版の診断マニュアル[8]，感染症法に基づく届出の基準[9]を基に，病態ならびに臨床診断に有効とされる所見を**表2**にまとめた．

HFRSは腎臓の機能障害を伴う出血熱という