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# Analysis of Immune Responses Against Nucleocapsid Protein of the Hantaan Virus Elicited by Virus Infection or DNA Vaccination

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**Even though neutralizing antibodies against the Hantaan virus (HTNV) has been proven to be critical against viral infections, the cellular immune responses to HTNV are also assumed to be important for viral clearance. In this report, we have examined the cellular and humoral immune responses against the HTNV nucleocapsid protein (NP) elicited by virus infection or DNA vaccination. To examine the cellular immune response against HTNV NP, we used H-2K$^b$ restricted T-cell epitopes of NP. The NP-specific CD8$^+$ T cell response was analyzed using a $^{51}$Cr-release assay, intracellular cytokine staining assay, enzyme-linked immunospot assay and tetramer binding assay in C57BL/6 mice infected with HTNV. Using these methods, we found that HTNV infection elicited a strong NP-specific CD8$^+$ T cell response at eight days after infection. We also found that several different methods to check the NP-specific CD8$^+$ T cell response showed a very high correlation among analysis. In the case of DNA vaccination by plasmid encoding nucleocapsid gene, the NP-specific antibody response was elicited 2 ~ 4 weeks after immunization and maximized at 6~8 weeks. NP-specific CD8$^+$ T cell response reached its peak 3 weeks after immunization. In a challenge test with the recombinant vaccinia virus expressing NP (rVV-HTNV-N), the rVV-HTNV-N titers in DNA vaccinated mice were decreased about 100-fold compared to the negative control mice.**

***Key words***: cellular immune response, DNA vaccine, Hantaan virus, nucleocapsid protein

The Hantaan virus (HTNV) is a member of the genus *Hantavirus* of the family *Bunyaviridae* and a causative agent of hemorrhagic fever with renal syndrome (HFRS). More than 100,000 cases of HFRS are reported annually, with a mortality rate between 2% and 10% (Lee, 1989; Ruo *et al.*, 1994; Hjelle *et al.*, 1995).

HTNV is a spherical, enveloped virus with a genome consisting of three segments of single-stranded, negative-sense RNA. The three segments are designated as large (L), medium (M), and small (S) segments that encode RNA–dependent RNA polymerase, two envelope glycoproteins (G1, G2) and nucleocapsid protein (NP), respectively (Tamura *et al.*, 1989).

It is well known that neutralizing antibodies to G1 and G2 play major roles in protection against HTNV infection (Zhang *et al.*, 1988; Schmaljohn *et al.*, 1990; Yoshimatsu *et al.*, 1993). These circulating neutralizing antibodies are thought to prevent the primary infection of the virus in host animals, but do not contribute to the clearance of the virus that has already multiplied in the host cells. Kariwa *et al.* found that HTNV could replicate or survive for a certain period in adult mice, in spite of the presence of specific antibodies (Kariwa *et al.*, 1995). Although a high level of a neutralizing antibody was present in suckling mice inoculated with HTNV, the virus persisted in the animal for several weeks (Nakamura *et al.*, 1985b). Therefore, it seems that efficient protection against HTNV infection could not be guaranteed by neutralizing antibodies alone, and thereby needs the cellular immune system. The NP of HTNV is highly immunogenic and genetically more conservative than the envelope protein. Natural HTNV infections of rodents and humans result in the induction of strong N-specific antibody and T cell responses (Khaiboullina and St Jeor, 2002).

The DNA vaccine has been shown to elicit both humoral and cellular immune responses, conferring protection against some viral, bacterial and parasitic pathogens (Davis and McCluskie, 1999; Jung *et al.*, 2004). In the past few years, DNA vaccines have also been studied against Hantavirus (Hooper *et al.*, 1999; Bucht *et al.*, 2001; Custer *et al.*, 2003). These approaches are almost defined in M gene (G1, G2). HFRS DNA vaccines containing the M genome express the G1 and G2 glycoproteins and elicit high-titer neutralizing antibodies in rodents and non-human primates (Hooper *et al.*, 1999; Kamrud *et al.*, 1999; Hooper *et al.*, 2001). The Seoul virus (SEOV)

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and HTNV M genome segment-based DNA vaccines protect against the SEOV, HTNV, and Dobrava virus (DOBV), but not the Puumala virus (PUUV), using a hamster infection model (Hooper *et al.*, 1999; Kamrud *et al.*, 1999; Hooper *et al.*, 2001). There are reports that candidate DNA vaccines comprised of ~500-nucleotide stretches of the SNV M genome segment, or the full-length S gene, were immunogenic in mice (Bharadwaj *et al.*, 1999). These conferred some protection against infection with SNV using a deer mouse infection model (Bharadwaj *et al.*, 2002). The protection was assumed to be cell mediated because there was no overwhelming evidence that these constructs elicited a neutralizing, or otherwise protective antibody response.

In this study, we examined the HTNV NP specific $CD8^+$ T cell responses in H-$2K^b$ mice infected with HTNV and those immunized with DNA vaccine encoding nucleocapsid gene, in addition to humoral immune responses against NP of HTNV. We also tested the protective immunity of the DNA vaccine using the recombinant vaccinia virus expressing NP.

## Materials and Methods

### *Mice, virus and cells*

C57BL/6 mice were purchased from Charles River Laboratories (USA). The studies used 5 ~ 6 week old female mice. BHK, Sf9, Vero E6 and 143B TK(-) cells were obtained from American Type Culture Collection (ATCC; USA). BHK and Vero E6 were cultured in Dulbecco's Modified Eagle Medium (DMEM), the Sf9 cell was in Grace insect cell media and the 143B TK(-) cell was in alpha-MEM. All media were supplemented with 10% FBS. The HTNV strain 76-118 was obtained from Dr. Lee (Lee and van der Groen, 1989). After the virus passed five times through Vero E6 cells grown in monolayer, viral stocks were prepared from the culture medium as described (Elliott *et al.*, 1984). A titer of virus stock was determined by foci formation in Vero E6 cells followed by staining with peroxidase-antiperoxidase (PAP) as previously described (Tanishita *et al.*, 1984).

### *Construction of DNA vaccine encoding nucleocapsid gene*

Total cellular RNA was isolated from HTNV-infected Vero E6 cells using TRIsol (Invitrogen, USA) by standard methods. cDNA was synthesized and amplified using a One-step RT-PCR kit (Invitrogen, USA) with N-gene specific primer (5-HTNV-N: GCGAATCCATGGCAACTATGGAGGAATTACAGAGG, 3-HTNV-N: GCGAATTCTTAGAGTTTCAAAGGCTCTTGGTTGGA). PCR products were digested with *Eco*RI and cloned into *Eco*RI site of pcDNA3.1 (Invitrogen, USA).

Plasmid DNA containing the N gene (pcDNA3-N) was purified using endotoxin-free DNA purification kits (Qiagen, USA) according to the manufacturer's directions.

### *Infection of mice with live HTNV*

C57BL/6 mice inoculated with 1 X $10^5$ FFU/Head of cell-cultured HTNV via intra-peritoneal administration. Mouse sera were collected by retro-orbital puncture each day in order to check the NP-specific humoral immune response. After eight days, splenocytes were isolated by ficoll density gradient and the NP-specific cellular immune response was assayed. All experiments with infectious viruses were conducted in the BSL3 containment facilities.

### *Immunization*

For DNA vaccination, mice were injected with pcDNA3-N into both quadricep muscles with 50 μg DNA each. Empty pcDNA3 was used as a negative control. Three days before vaccination, mice were injected with 0.25% bupivacane into both quadriceps muscles. After vaccination, mouse sera were collected by retro-orbital puncture at two-week intervals for antibody titration. Mice were sacrificed every week after immunization for spleen isolation.

### *Expression and purification of His-tagged nucleocapsid protein using the baculovirus system*

Nucleocapsid gene encoding His-tagged NP were amplified from pcDNA3-N by PCR using specific primers (5-HTNV-N: GCGAATTCATGGCAACTATGGAGGAA T-TACAGAAG, 3-HIS-N: GCGAATTCTTAATGGTGATGGTGATGATGGAGTTTCAAAGGCTCTTGGTTGG-A) and cloned into the *Eco*RI site of pBacPAK8 (Clontech, USA). Cotransfection with baculovirus genomic DNA into Sf9 cells were performed as recommended by Clontech (USA).

Recombinant baculovirus carring the N gene were selected by plaque assay and confirmed by Western blotting with an anti-NP antibody. Sf9 cells were infected with recombinant virus at a multiplicity of infection of 10 and cultured for three days. Expressed His-tagged NP was extracted from the cell by 8 M urea and purified by $Ni^{2+}$-NTA agarose resin as recommended (Qiagen, USA).

### *Enzyme-linked immunosorbent assay (ELISA) for the detection of anti-NP antibody*

ELISA was performed to determine specific antibodies to the NP of Hantaan virus. Flat-bottomed 96-well plates (Nunc, Denmark) were coated with 0.1 μg/well of purified NP in 0.05 M carbonate buffer (pH 9.6) at 4°C for 16 h. The plates were blocked with 10% FBS in PBS for 2 h at 37°C. After washing with PBS containing 0.05% Tween-20 (PBS-T), the plates were incubated at 37°C for 1 h with sera from the immunized mice using 50 fold diluted in PBS containing 2% BSA. The plates were washed and incubated at 37°C for 1 h with horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Sigma) diluted in 1:5000. After washing with PBS-T, TMB solution (KPL) was added and the reaction was left

at room temperature for approximately 30 min. The reaction was stopped by the addition of 0.5 N $H_2SO_4$ and the optical density at 450 nm was measured.

***Enzyme linked immunospot (ELISPOT) assay***

Spleen cells were isolated and IFN-γ ELISPOT assays were performed at 8 days after HTNV inoculation, and every week following DNA vaccination. MultiScreen-IP plates (Millipore, USA) were coated with 100 μl of anti-mouse IFN-γ Ab (5 μg/ml in PBS) and were incubated at 4°C overnight. The plates were then blocked with PBS-10% FBS for 2 h at room temperature. Splenocytes ($1 \sim 5 \times 10^5$ cells) isolated from the spleens were added to wells with 5 μg/ml M6 (HTNV-NP221-228: SVIG-FLAL), N1 (HTNV-NP328-335: LGAFFSIL) peptides (Park *et al.*, 2000; Chang *et al.*, 2001) and incubated at 37°C for 24 h. After incubation, the cells were removed and 100 μl of biotinylated anti-mouse IFN-γ Ab (2 μg/ml in PBS-T) was added and kept for 2 h at room temperature. Then streptavidin-HRPO (BD-Pharmingen, USA) was added and kept for 2 h at RT. Finally, the plates were treated with 3-amino-9-ethyl-carbazole (10 mg of AEC in 0.1 M Sodium acetate buffer (pH 5.0)) peroxidase substrate at room temperature for 10 min. The reaction was stopped under running distilled water. The spots were counted using an ELISPOT reader system (AID Elispot, Germany).

***Intracellular cytokine staining (ICCS) assay***

For the analysis of intracellular IFN-γ cytokine, splenocytes were incubated for 5 h at 37°C in RPMI containing 10% FBS, 2 μg/ml of brefeldin A (Sigma, USA) and 50 μg/ml peptides (M6, N1). The cells were then washed and stained with PE-conjugated anti-CD8 Ab at 4°C for 20 min. Then, the cells were washed in PBS and fixed with 4% paraformaldehyde in PBS for 20 min. After washing, the cells were permeabilized with 0.5% saponin (Sigma, USA) in PBS for 10 min. After washing, the cells were stained with FITC-conjugated anti-mouse IFN-γ Mab for 30 min. The cell samples were analyzed with a FACSCalibur (Becton Dickinson, USA), and the data analysis was conducted with CellQuest (Becton Dickinson, USA).

***$^{51}Cr$-release assay***

CTL function was measured with a $^{51}Cr$ release assay as previously described (Feltkamp *et al.*, 1994). Briefly, Splenocytes were cultured for five days in RPMI1640 medium supplemented with 10 μg/ml M6, N1 peptide. Peptide-pulsed target cells were prepared by incubating EL-4 cells with synthetic peptide (10 μg/ml) for 4 h in a $CO_2$ incubator, then washed extensively with a PBS buffer to eliminate unbound peptides. Subsequently, the cells were incubated with 100 μCi $^{51}Cr$ for 4 h at 37°C. They were brought into contact with each other by centrifugation for 2 min and incubated for 4 h in 96-well round-bottom plates. The specific lysis was calculated as follows: (Experimental release - spontaneous release) / (100% release - spontaneous release) × 100. All assays were performed in triplicate.

***Tetramer binding assay***

The H-2K$^b$ tetramer with M6 or N1 peptide was made as previously described (Altman *et al.*, 1996; Ogg *et al.*, 1998). Refolding was performed at 10°C in the presence of 25 μg/ml peptide and protease inhibitors (pepstatin A 1 μg/ml, leupeptin 1 μg/ml, PMSF 0.4 mM). Soluble monomeric complexes were purified by gel filtration over a Superdex 200HR column (GE Healthcare Bioscience, USA) and enzymatically biotinylated by overnight incubation with purified biotin protein ligase (BirA) at room temperature. Unbound biotin was removed by gel filteration, and the purified monomers were tetramerized by incubation with PE-labeled streptavidin (Molecular Probes, USA) at a molar ratio of 4:1.

***Generation of recombinant vaccinia virus expressing HTNV NP (rVV-HTNV-N)***

Nucleocapsid gene was excised from pcDNA3-N and blunted with a klenow fragment (NEB) and cloned into *Sma*I site of pMJ601 vector under the control of the synthetic late promoter. This construct, along with a wild type vaccinia virus (Strain Western Reverse), was transfected into BHK cells using a GenePoter (GeneTherapySystem, Italy). The recombinant vaccinia virus was selected by β-gal staining in 143B TK(-) cell cultured in the presence of 100 μg/ml BrdU. Expression of the NP was confirmed by immunoblotting of the recombinant vaccinia virus (rVV-HTNV-N) infected cells. The titer of stock virus was calculated by infection of the human 143B TK(-) cell.

***Challenge studies using the recombinant vaccinia virus***

The virus challenge experiment was performed four weeks after immunization. Mice were challenged with $1 \times 10^6$ PFU/head of rVV-HTNV-N via i.p. administration. After five days, the ovaries were harvested and homogenized using a pellet pestle motor (Sigma, USA), and the sample adjusted to a final volume of 200 μl. The titer of rVV-HTNV-N in the ovaries was analyzed using a human 143B TK(-) cell.

## Results

***Immune responses after HTNV infection***

IFN-γ is a potent immunostimulatory and anti-viral cytokine. The frequency of IFN-γ secreting cells stimulated by CTL epitope was evaluated by ELISPOT. Eight days after HTNV infection, splenocytes were isolated by ficoll density gradient. IFN-γ secreting cells in stimulated with M6 peptide were approximately 1,000 spot forming cells/1×

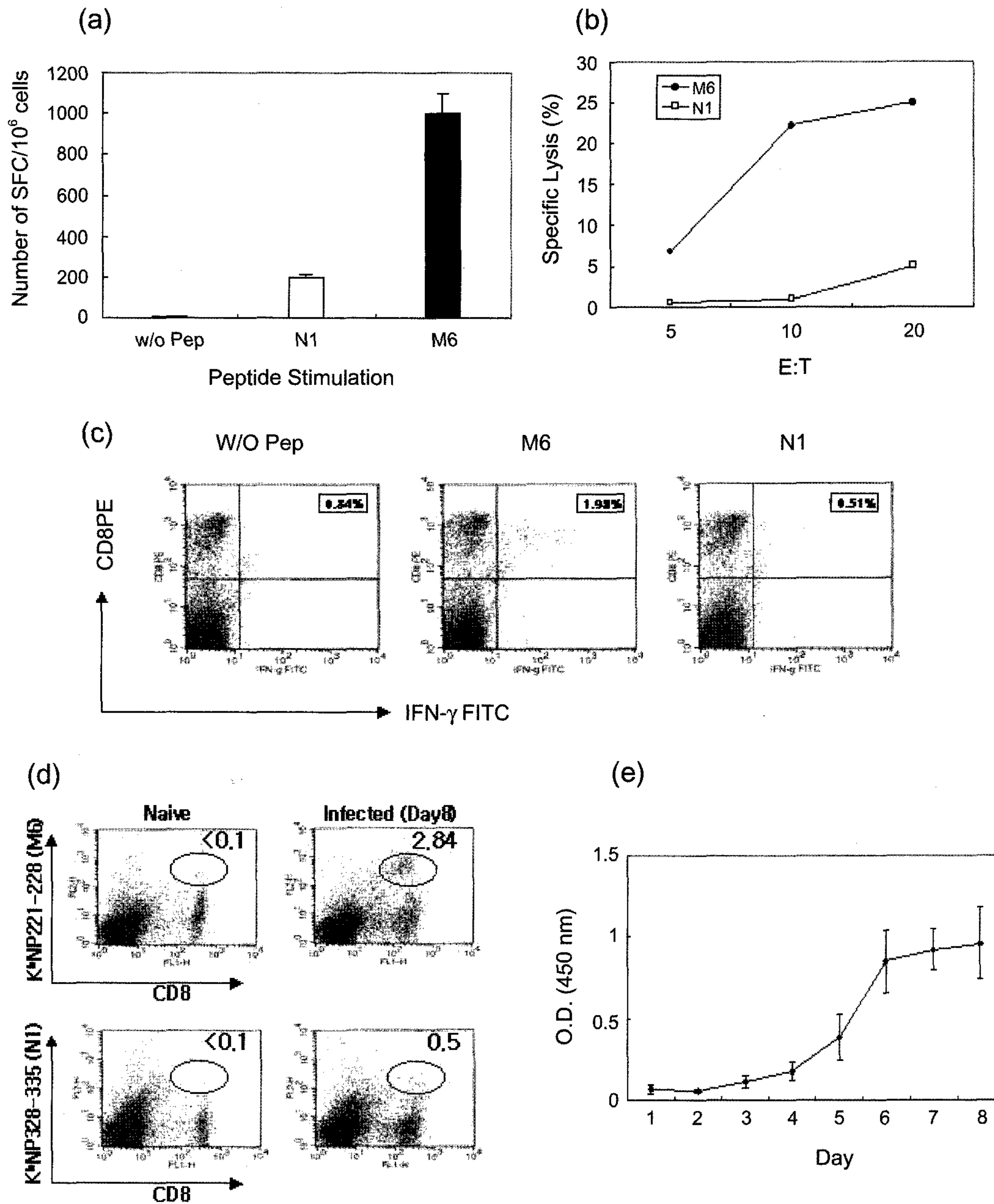


**Fig. 1. Immune responses after HTNV infection in C57BL/6 mice.** Mice were inoculated with $1\times10^5$ FFU/Head of cell-cultured HTNV via intraperitoneal administration. Splenocytes were harvested at 8 days after inoculation in order to check the cellular immune response and serum was collected by every day in order to check the humoral immune response as described in Materials and Methods. **(a)** ELISPOT Assay for HTNV-specific $CD8^+$ T cell producing IFN-γ. Splenocytes at $10^6$ cells/well were tested in the presence of 5 μg/ml CTL epitope peptides on NP, M6 or N1. W/o Pep is no peptide treated group for control. **(b)** $^{51}$Cr release assay. The cytotoxic activities (Percentage specific lysis) of splenocytes against M6, N1 peptide pulsed target cell at an effector-to-target cell ratio of 5:1, 10:1, and 20:1. Splenocytes were restimulated *in vitro* with 10 μg/ml peptides, $^{51}$Cr release assay was performed on EL-4 target cells pulsed with same peptide. **(c)** Intracellular cytokine staining assay. Splenocytes were restimulated with 5 μg/ml peptides (M6, N1) for 5 h. And then stained with PE-conjugated anti-CD8 and FITC-conjugated anti-mouse IFN-γ Abs. Percentages of IFN-$\gamma^+$ $CD8^+$ cells were shown in the right upper quadrants. **(d)** $K^b$-Tetramer (M6, N1) binding assay: Splenocytes were restimulated with $K^b$/β2m/M6 or $K^b$/β2m/N1 tetramer together with PE-conjugated anti-CD8 and FITC-conjugated anti-mouse IFN-γ Abs in the presence of 25 μg/ml each peptide. **(e)** Analysis of the NP-specific antibody response in ELISA using the homologous antigen. Sera were taken at indicated time after infection and tested after 50 fold dilution.

$10^6$ splenocytes. The cells stimulated with N1 peptide were relatively lower level than the M6 peptide stimulated group, but were consistently higher than the negative control group (Fig. 1a).

For CTL assay, splenocytes were stimulated *in vitro* for five days with a M6 or N1 peptide. The cultures were then

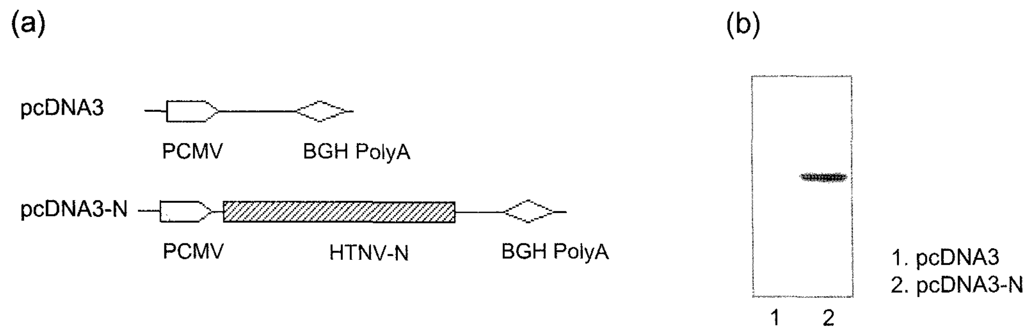


**Fig. 2. Construction of plasmid encoding nucleocapsid gene and confirmation of NP expression *in vitro*.**
**(a)** Schematic presentation of the DNA vaccine structure. HTNV nucleocapsid gene was cloned into pcDNA3.1 vector (pcDNA3-N). PCMV indicates CMV promoter, BGH polyA indicates bovine growth hormone polyadenylation signal. **(b)** Confirmation of HTNV NP expression *in vitro*. Western blot analysis was performed with the lysates of plasmid transfected BHK cells using an anti-NP monoclonal antibody. Lane 1, pcDNA3; Lane 2, pcDNA3-N.

tested for specific lysis of EL-4 target cells with peptide. Fig. 1 (b) shows that M6 peptide was sufficient to induce a specific and strong CTL response against peptide-pulsed target cells.

The frequency of M6 peptide specific T cells was further determined *ex vivo* by tetramer staining using the enriched $CD8^+$ T cell population. We stained specific T cells with $H\text{-}2K^b$ tetramers bearing the M6 or N1 peptide epitope. The frequency of specific cells determined by tetramer staining was 2.84% of $CD8^+$ T cells in M6 peptide and 0.5% of $CD8^+$ T cells in N1 peptide, while uninfected mice had below 0.1% of tetramer positive $CD8^+$ T cells (Fig. 1d).

In addition, the frequency of IFN-γ producing cells was analyzed by intracellular cytokine staining upon 5 h stimulation with peptide. The results indicate that approximately 1.98% of total splenocytes produced IFN-γ in response to M6 peptide stimulation, while approximately 0.51% in response to N1 peptide (Fig. 1c).

These results indicated that HTNV infection elicited the strong NP-specific $CD8^+$ T cell response. Furthermore, several different methods to check the NP-specific $CD8^+$ T cell response showed a perfect correlation among assays. These results also showed that M6 epitope is more specific than N1 epitope for the detection of NP-specific $CD8^+$ T cell response.

We also performed an anti-NP antibody ELISA to check the NP-specific humoral immune response. HTNV infection elicited the NP-specific humoral immune response beginning 4 ~ 5 days after infection (Fig. 1e).

***Construction of plasmid encoding nucleocapsid gene and confirmation of NP expression in vitro***

The HTNV nucleocapsid gene was cloned from HTNV-infected cells by RT-PCR and cloned into pcDNA3.1 (Fig. 2a).

To determine whether the plasmid encoding N gene can express the immunologically relevant NP, pcDNA3-N and empty pcDNA3 were transfected into BHK cell, and Western blot analysis was performed with the lysates of transfected cells using an anti-NP monoclonal antibody. The plasmid encoding nucleocapsid gene was confirmed to lead the efficient expression of HTNV NP in transfected cells (Fig. 2b).

***Immune response after DNA vaccination***

Mice were injected with pcDNA3-N into both quadriceps muscles with 50 μg DNA each. Splenocytes were isolated every week after immunization for cellular immune response tests. DNA vaccination with pcDNA3-N elicited the strong NP-specific $CD8^+$ T cell response reaching peak three weeks after immunization. The amount of splenocytes that produced IFN-γ in response to M6 peptide stimulation was 0.55%, while approximately 0.20% were produced in response to N1 peptide by intracellular cytokine staining assay (Fig. 3a). The frequency of specific cells determined by the tetramer binding assay was 0.44% of $CD8^+$ T cells in M6 peptide and 0.06% of $CD8^+$ T cells in N1 peptide (Fig. 3b). In the ELISPOT assay, IFN-γ secreting cells stimulated with the M6 peptide were rapidly increased at three weeks (Fig. 3. c). Even though the NP-specific $CD8^+$ T cell response after DNA vaccination was relatively lower compared than HTNV infection, the patterns of reaction were similar to the HTNV infection. Also, the M6 epitope showed better specificity than the N1 epitope for the NP-specific $CD8^+$ T cell responses after DNA vaccination.

The NP-specific antibody was detected at four weeks after DNA vaccination and maximized at 6 ~ 8 weeks. Humoral immune response was sustained for over 14 weeks (Fig. 3d).

***Protective immune response after DNA vaccination***

To examine the efficacy of NP-specific $CD8^+$ T cell response to clear viruses and protect against virus infection, at 4 weeks after immunization, we challenged the mice with $1 \times 10^6$ PFU of rVV-HTNV-N. rVV-HTNV-N was confirmed to express NP by immunoblotting of

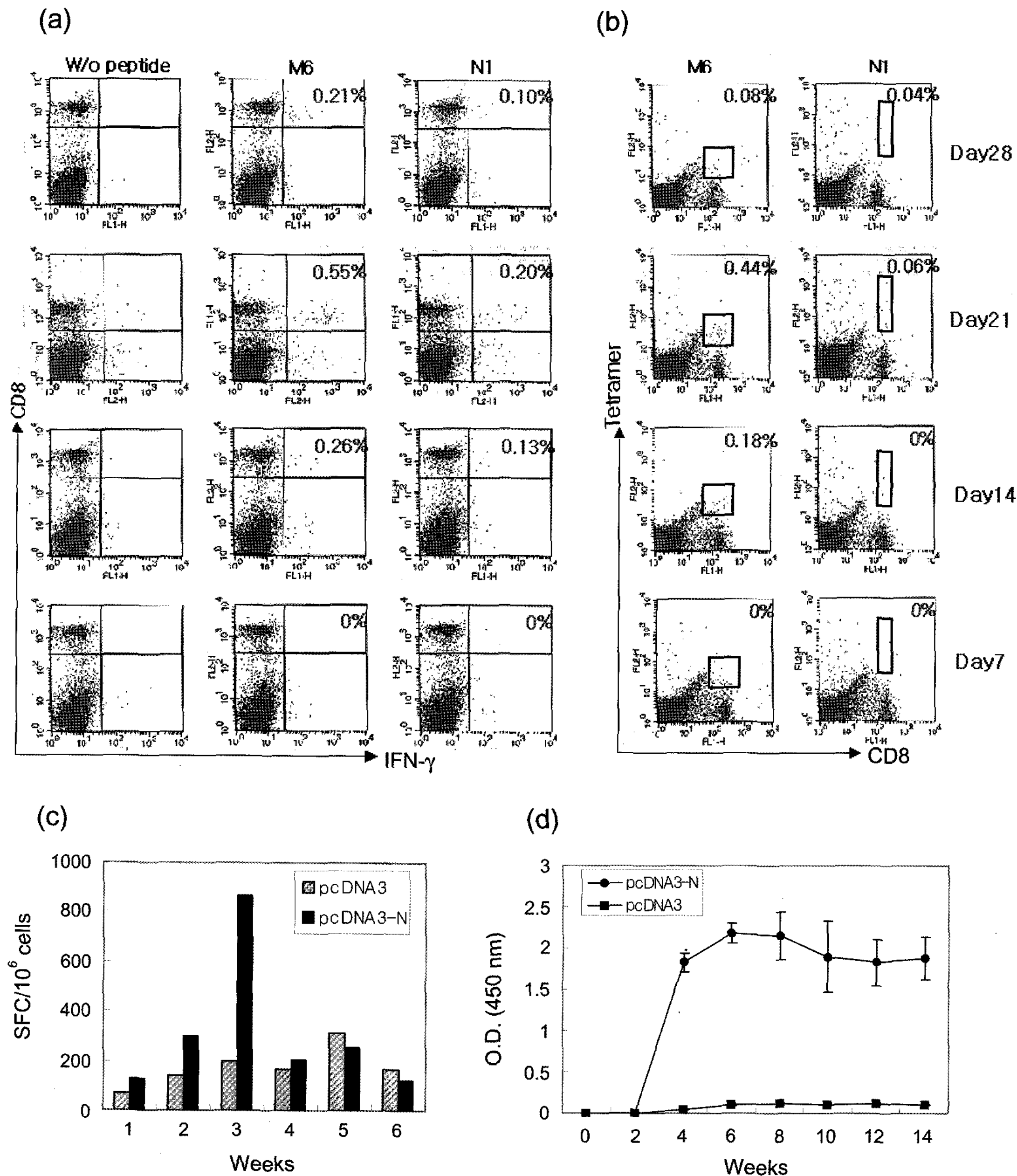


**Fig. 3. Immune responses after DNA vaccination in C57BL/6 mice.**
Mice were immunized with DNA vaccine into both quadriceps muscles with 50 μg DNA each. Splenocytes were isolated at every week after immunization for the cellular immune response tests, and sera were taken at every 2 weeks for the humoral immune response test. **(a)** Intracellular cytokine staining assay. Percentages of IFN-$\gamma^+$ $CD8^+$ cells were shown in the right upper quadrants. Splenocytes were stimulated with M6 or N1 peptide. W/o Pep is no peptide treated group for control. The day of splenocytes isolation was indicated at right side. **(b)** $K^b$-Tetramer (M6, N1) binding assay. Percentages of splenocytes bound with $K^b$-tetramers in the presence of 25 μg/ml each peptide are shown in the right upper side. **(c)** ELISPOT Assay for HTNV-specific $CD8^+$ T cell producing IFN-γ. Splenocytes at $10^6$ cells/well were tested in the presence of 5 μg/ml M6 peptide according to time after immunization. **(d)** Analysis of the NP-specific antibody response in ELISA using the homologous antigen. Sera were taken at indicated time after DNA vaccination and tested after 50 fold dilution.

lysates of cells infected with rVV-HTNV-N (Fig. 4b). Five days later, mice were sacrificed and the ovaries were harvested for the titration of rVV-HTNV-N, because this is the organ where the vaccinia preferentially replicates (Alexander-Miller *et al.*, 1996; Belyakov *et al.*, 1998).

The titer of challenged virus in the pcDNA3-N vaccinated group was decreased about 100 fold compared to that of the mock-immunized group (Fig. 5a). We also harvested the spleens when mice were sacrificed for evaluation of the relationship between IFN-γ producing $CD8^+$ T cell response and rVV-HTNV-N infection. After challenge, NP-specific $CD8^+$ T cell response was rapidly

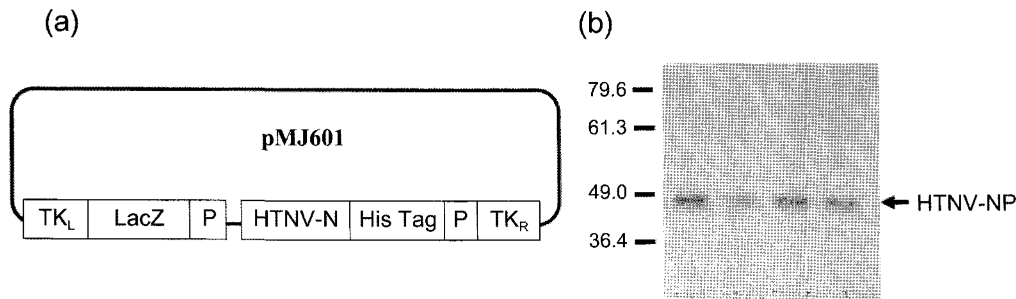


**Fig. 4. Cloning of HTNV nucleocapsid gene into vaccinia transfer vector and confirmed recombinant vaccinia virus expressing HTNV NP.** **(a)** Construction of HTNV nucleocapsid gene cloned into vaccinia transfer vector. Nucleocapsid gene was cloned into *SmaI* site of pMJ601 vector under the control of the synthetic late promoter. TK, *LacZ*, P and His Tag indicate Thymidine kinase, β-galactosidase, Promoter and tagged Histidine, respectively. **(b)** Confirmation of recombinant vaccinia virus expressing HTNV NP by immunoblotting of the recombinant vaccinia virus infected cells. Maker size (Kd) was indicated at left side.

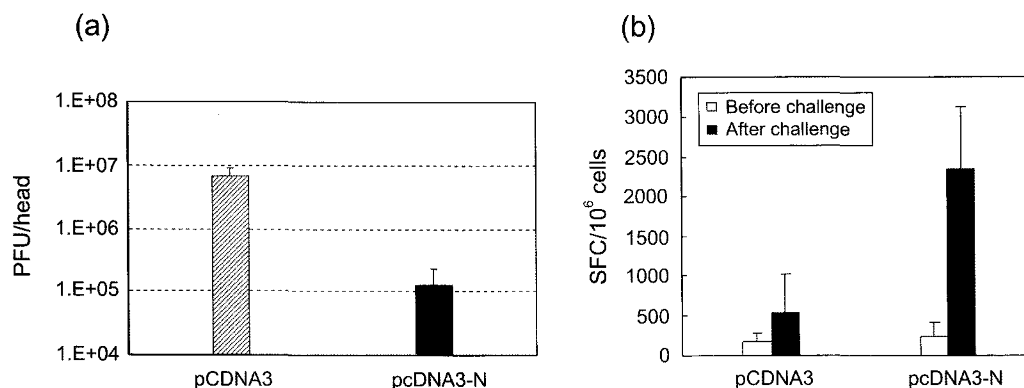


**Fig. 5. Protective immune response against rVV-HTNV-N challenge after DNA vaccination.** Five C57BL/6 mice per group were challenged with $1 \times 10^6$ PFU/Head of the recombinant vaccinia viruses expressing HTNV NP (rVV-HTNV-N) via intra-peritoneal administration at 4 weeks after DNA vaccination. After 5 days, the ovaries were harvested and homogenized for virus titration. **(a)** Titers of rVV-HTNV-N recovered from the ovaries of challenged mice. The titers of rVV-HTNV-N were analyzed by plaque assay using human 143B TK(-) cell. Data are shown for the mean recombinant vaccinia virus titer and standard deviation. **(b)** Comparison of IFN-γ secreted splenocytes number before and after rVV-HTNV-N challenge by ELISPOT assay. Before challenge was at 4 weeks after DNA vaccination, and after challenge was 5 days after rVV-HTNV-N challenge in DNA vaccinated group.

increased in the pcDNA3-N vaccinated group (Fig. 5b). These results indicated that NP-specific memory $CD8^+$ T cells were proliferated and activated to function as effector cells after challenge.

## Discussion

CTLs are an important host defense mechanism against many viral infections especially in the clearance of virus infected cells. In mice, adoptive transfer experiments have demonstrated that CTLs mediate protective immunity against LCMV, herpes simplex virus, and influenza virus (Zinkernagel and Doherty, 1979; Sethi *et al.*, 1983). In the case of HTNV, CTLs are also considered to be important for *in vivo* HTNV clearance. The adoptive transfer of immune T cells helped suckling mice survive following infection with HTNV (Nakamura *et al.*, 1985a). Others reported that T cell-mediated immunity plays an important role in the resistance of mice to HTNV infection (Nakamura *et al.*, 1985a; Asada *et al.*, 1988; Yoshimatsu *et al.*, 1993). It was also discovered that by transferring T-cell subsets into HTNV-infected nude mice, T cells expressing $CD4^-$ $CD8^+$ markers on their surface were especially important for the elimination of infectious viruses *in vivo* (Asada *et al.*, 1987).

In this study, we have identified two H-$2K^b$ restricted T-cell epitopes (N1 and M6) of HTNV NP with several $CD8^+$ T cell assay systems, such as ELISPOT assay, $^{51}$Cr release assay, tetramer binding assay, and ICCS assay. We have compared the cellular immune response between the cases of infected with HTNV and immunized with DNA vaccine. Several different methods to check the NP-specific $CD8^+$ T cell response showed a very high correlation among assays. ELISPOT assay and $^{51}$Cr release assay were more sensitive than the other assay systems. $^{51}$Cr release assay has traditionally been used to investigate effector cell cytotoxic function against labeled targets, but this method has inherent problems that include hazards

associated with radioactive cell labeling and high spontaneous releases. That's why we have tested $CD8^+$ T cell responses with the ELISPOT assay in a DNA vaccine efficacy test.

Our results showed that M6 peptide (221-228) was more specific than the N1 peptide (328-335) for the NP-specific CTL assays. These results were correlated with Maeda *et al.* (Maeda *et al.*, 2004).

Even though it is known that the HTNV NP-specific antibody plays no role in virus clearance (Arikawa *et al.*, 1989), we have tested the NP-specific antibody response in order to monitor the HTNV infection or DNA vaccination. HTNV infection elicited the NP-specific humoral immune response, which was beginning 4 ~ 5 days after infection, but a DNA vaccine elicited it 4 weeks after vaccination and maximized at 6 ~ 8 weeks. The Ab response was sustained for over 14 weeks.

A single dose injection of 50 μg of plasmid DNA into each quadriceps muscle of C57BL/6 mice induced a high level of humoral and cellular immune responses. In challenge tests with the recombinant vaccinia virus expressing NP (rVV-HTNV-N), the titers of challenged virus in pcDNA3-N vaccinated mice were decreased about 100 fold compared to that of the mock-immunized group. NP-specific $CD8^+$ T cell response was rapidly increased after being challenged in the pcDNA3-N vaccinated group. These results clearly demonstrated that NP-specific memory $CD8^+$ T cells proliferated and activated to function as effector cells after challenge.

Even though challenge with rVV-HTNV-N in HTNV infected mice were perfectly protected (data not shown) but DNA vaccination showed the partial protection. We assume the reason for partial protection against the challenge is poor efficacy of the DNA vaccine. If we can improve the efficiency of a vector by adding the co-stimulatory molecule gene or develop the more efficient vaccine delivery method, we could improve the high protection efficacy.

In conclusion, we showed that (i) HTNV infection in C57BL/6 mice elicited the strong NP specific $CD8^+$ T cell response at eight days after infection, (ii) DNA vaccination elicited the strong NP-specific humoral and cellular immune responses, and (iii) DNA vaccination elicited partial protection against challenge with recombinant vaccinia virus.

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