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Development of a serotyping enzyme-linked immunosorbent assay system based on recombinant truncated hantavirus nucleocapsid proteins for New World hantavirus infection

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A B S T R A C T

New World hantaviruses were divided into five groups based on the amino acid sequence variability of the internal variable region (around 230–302 amino acids) of hantavirus nucleocapsid protein (NP). Sin Nombre virus (SNV), Andes virus, Black Creek Canal virus (BCCV), Carrizal virus (CARV) and Cano Delgadito virus belong to groups 1, 2, 3, 4 and 5, respectively. Patient and rodent sera were serotyped successfully by an enzyme-linked immunosorbent assay (ELISA) with recombinant truncated NP lacking 99 N-terminal amino acids (trNP100) of SNV, CARV and BCCV. The trNP100 of BCCV showed lower reactivity to heterologous sera. In contrast, whole recombinant NP antigens detected both homologous and heterologous antibodies equally. The results together with results of a previous study suggest that trNP100 can distinguish infections among viruses in groups 1, 2, 3 and 4 of New World hantaviruses. The serotyping ELISA with trNP100 is useful for epidemiological surveillance in humans and rodents.

1. Introduction

Hantaviruses belong to the family *Bunyaviridae* and are maintained in rodents and other small mammals that are infected persistently (Schmaljohn and Hjelle, 1997). Hantaviruses cause two febrile illnesses in humans, hemorrhagic fever with renal syndrome (HFRS) in the Old World and hantavirus pulmonary syndrome (HPS) in the New World (Kariwa et al., 2007; Schmaljohn and Hjelle, 1997). Transmission of the viruses to humans occurs through inhalation of aerosolized animal excreta or rodent bites (Lee and van der Groen, 1989; Meyer and Schmaljohn, 2000). Hantaviruses appear to have co-evolved with the rodent reservoir host species over many thousands of years (Hughes and Friedman, 2000; Schmaljohn and Hjelle, 1997). The difference in epidemic areas of HFRS and HPS depends on the rodent habitat (Zeier et al., 2005).

Hantavirus virions contain three segmented negative-sense RNAs designated S, M, L; they encode a nucleocapsid protein (NP), enveloped glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (L protein), respectively (Elliott, 1990; Schmaljohn, 1996). Hantavirus NP is the most abundant viral component in both virions and infected cells and can form a stable trimer (Elliott et al., 2000; Kaukinen et al., 2001, 2004). The NP of hantaviruses possesses immunodominant, linear, cross-reactive epitopes around the first 100 amino acids (aa) of the N-terminus (Elgh et al., 1996; Gott et al., 1997; Vapalahti et al., 1995; Yamada et al., 1995). On the other hand, the variable region around 230–302 aa forms serotype-specific epitopes after multimerization of NP (Tischler et al., 2008; Yoshimatsu et al., 2003).

Recombinant antigens were expressed with multimerization-dependent serotype-specific epitopes after truncation of the N-terminal 49 aa in NP (trNP50) by a baculovirus (Araki et al., 2001; Nakamura et al., 2008; Yasuda et al., 2012). Enzyme-linked immunosorbent assay (ELISA) using trNP50 differentiated successfully infections with four different serotypes of Old World hantavirus: Hantaan, Seoul, Dobrava, and Thailand viruses in HFRS patient and rodent sera (Araki et al., 2001; Nakamura et al., 2008). ELISA using trNP lacking 99 aa of the N-terminal end of the NP (trNP100) differentiated successfully infections with three different serotypes of New World hantaviruses: Sin Nombre virus (SNV), Andes virus (ANDV) and Laguna Negra virus (LANV) in HPS patient and rodent sera (Koma et al., 2010). Therefore, the serotyping ELISA using trNPs is a more rapid, safe and simple method as a substitute

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for the neutralization test, which has been the only serological assay for determining the serotype (Araki et al., 2001; Koma et al., 2010; Nakamura et al., 2008).

Since the first recognition of HPS in the United States in 1993, more than 30 new hantaviral strains or genetic lineages have been identified from patients with HPS or various rodent species throughout the Americas (Jonsson et al., 2010; Peters and Khan, 2002; Schmaljohn and Hjelle, 1997). However, the antigenic relationship among the New World hantaviruses has not been studied in detail.

Since serotyping with trNP depended on the antigenic difference of serotype-specific epitopes within the internal region of trNP, it was expected that variability of the aa sequences in the region also correlated to the hantavirus serotype. In the present study, therefore, amino acid sequences of the internal variable regions of NP of many New World hantaviruses were compared. The results showed that they were divided into 5 groups. Therefore, SNV (group 1) and Black Creek Canal virus (BCCV) (group 3), which were associated with HPS in the United States (Hjelle et al., 1994; Ravkov et al., 1995), and Carrizal virus (CARV) (group 4), which was recognized recently as a New World hantavirus isolated from *Reithrodontomys sumichrasti* in Mexico (Kariwa et al., 2012), were selected, and the applicability of their trNPs for a serotyping antigen was examined.

2. Materials and methods

2.1. cDNAs

cDNAs containing coding information for the S segment of SNV strain SN 77734 (Botten et al., 2000), CARV strain 2/2006 (Kariwa et al., 2012) and BCCV (GenBank ID: AB689163) were used. CARV was recognized recently from *R. sumichrasti* in Mexico (Kariwa et al., 2012).

2.2. Monoclonal antibodies and human and rodent sera

Monoclonal antibodies (MAbs) to the NP of HTNV and PUUV were used for antigenic characterization of the NP by an indirect immunofluorescence assay (IFA). The MAbs 2E12, 4C3, 4E5, GBO4, ECO2 and ECO1 recognize the N-terminal epitope of the NP. The MAbs F23A1 and E5/G6 recognize aa 291–402 and aa 165–173 of the NP, respectively (Lundkvist et al., 1991; Ruo et al., 1991; Yoshimatsu et al., 1996). The epitope for MAb C16D11 is unknown. MAbs except for GBO4 and ECO1 were obtained from the cell culture supernatant. The MAbs GBO4 and ECO1 were obtained from ascitic fluid. Sera from three patients infected with SNV were supplied kindly by Brian Hjelle of the University of New Mexico Health Sciences Center, New Mexico, USA. Negative control human sera were obtained from healthy volunteers. This study was approved by the ethics committee of Hokkaido University Graduate School of Medicine, and informed consent was obtained from all human subjects, including healthy volunteers. Three sera from *Peromyscus maniculatus* infected with SNV and one serum from hantavirus-uninfected *P. maniculatus* were supplied kindly by David Safronetz of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Montana, USA. Several species of *Reithrodontomys* were captured in Guerrero, Mexico. Three *R. sumichrasti* were infected with CARV, and one *R. megalotis* was infected with Huitzilac virus (HUIV), which showed 96.7% amino acid identity to NP of CARV (Kariwa et al., 2012). Sera from hantavirus-uninfected *P. maniculatus*, *R. sumichrasti* and *R. megalotis* were used as negative controls. These viral types in the patients and rodents were determined by detection of the virus genome by reverse transcriptase (RT)-PCR.

2.3. Amino acid and nucleotide sequence comparison and phylogenetic analysis

Amino acid and nucleotide sequences of the variable region in NP (230–302 aa and 690–906 nucleotides) of New World hantavirus in North America and South America were aligned and compared with sequences determined previously using GenetyxMac Ver.13 (Genetyx Corporation, Tokyo, Japan). Phylogenetic relationships among the hantavirus sequences of the variable region of NP were evaluated using the Neighbor-Joining program with the Kimura 2 parameter distance in CLUSTALW version 1.83 (European Bioinformatics Institute, Cambridge, UK). The phylogenetic tree was visualized using the NJ plot program (Perriere and Gouy, 1996). Bootstrap resampling analysis was performed using 1000 replicates.

2.4. Construction of recombinant baculoviruses expressing whole rNPs and trNPs

The gene encoding whole NP (aa 1–428) and truncated genes encoding truncated NP (aa 50–428 and aa 100–428) were PCR-amplified from cDNA of the S segment. The primers listed below were used for amplification of whole and truncated S segments. A 5′ SpeI site and a 3′ XhoI site were introduced into the primers (both sites shown in italics). Primer sequences (forward and reverse) were as follows: SNV whole rNP, 5′-gac*actagt*atgagcaccctcaaagaa-3′ and 5′-tac*ctcgag*ttaaagtttaagtttaagtggttc-3′; CARV whole rNP, 5′-aaa*actagt*atgagcaacctcaaagaa-3′ and 5′-gat*ctcgag*ttatagttttagagg-3′; BCCV whole rNP, 5′-gaa*actagt*atgagcaacctcaaagaa-3′ and 5′-gatt*ctcgag*tcatatctttaaaggctc-3′; SNV trNP50, 5′-tcg*actagt***atg**gctgtgtctgcattggag-3′ and 5′-tac*ctcgag*ttaaagtttaagtttaagtggttc-3′; CARV trNP50, 5′-aga*actagt***atg**gctgtgtctgcattggaa-3′ and 5′-gat*ctcgag*ttatagttttagagg-3′; BCCV trNP50, 5′-aac*actagt***atg**gctgtgtctgcattggag-3′ and 5′-gatt*ctcgag*tcatatctttaaaggctc-3′; SNV trNP100, 5′-tcg*actagt***atg**gctgtgtctgcattggag-3′ and 5′-tac*ctcgag*ttaaagtttaagtttaagtggttc-3′; CARV trNP100, 5′-aga*actagt***atg**gctgtgtctgcattggaa-3′ and 5′-gat*ctcgag*ttatagttttagagg-3′; BCCV trNP100, 5′-ctt*actagt***atg**aatgtgcttgacgtcaat-3′ and 5′-gatt*ctcgag*tcatatctttaaaggctc-3′. Boldface indicates an added start codon. After amplification, the DNA fractions were subcloned into pFastBac1 (Invitrogen, Groningen, The Netherlands). The recombinant baculoviruses were expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer's instructions. Mock baculovirus was made from original pFastBac1. The titers of recombinant baculoviruses in the culture supernatant were determined by 50% tissue culture infective dose ($TCID_{50}$) with High Five cells.

2.5. Preparation of whole rNPs and trNPs expressed by baculoviruses

High Five cells (Invitrogen) were grown in Grace's insect cell culture medium (Invitrogen) supplemented with 10% fetal bovine serum as described previously (Araki et al., 2001). High Five cells were infected with recombinant baculoviruses at a multiplicity of infection of 1 for 3 days. Collection and lysis of infected cells were performed using methods described previously (Araki et al., 2001). Briefly, infected High Five cells were collected in phosphate-buffered saline (PBS) of 2.5×10^6 cells/mL and sonicated. The cell lysate containing recombinant NP (rNP) was used as ELISA antigen. The lysate of cells infected with mock baculovirus was used as a negative control. The expression of rNPs of SNV, CARV and BCCV was confirmed by Western blotting (data not shown) using methods described previously (Yoshimatsu et al., 1995). High Five cells expressing whole recombinant NPs (whole rNPs) of PUUV