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Characterization of human bocavirus-like particles generated by recombinant baculoviruses

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A B S T R A C T

Human bocavirus (HBoV) is a nonenveloped, single-stranded DNA virus, classified recently into the genus *Bocavirus* in the family *Parvoviridae*. A recombinant baculovirus expression system was used to express the major capsid protein VP2 of HBoV1, HBoV2, HBoV3 and HBoV4 in insect cells. A large amount of the 61-kDa VP2 capsid protein (p61) of HBoVs was generated and efficiently released into the supernatant. The capsid protein was self-assembled into 22-nm-dia. virus-like particles (VLPs) with a buoyant density of 1.30 g/cm^3. The morphology of HBoVs-LPs was similar to that of the native HBoV particles, and immunogenic studies demonstrated the cross-reactivity among HBoV1, HBoV2, HBoV3 and HBoV4. When VP1 and VP2 protein of HBoV1 were co-expressed in insect cells, both proteins were detected in the same fraction after CsCl gradient centrifugation, suggesting that the VP1 protein is a minor structural protein of HBoVs. We developed an ELISA using purified VLPs as the antigen and used it to detect antibodies against HBoV1, HBoV2, HBoV3 and HBoV4. A high prevalence of antibodies against HBoVs was found in a general population of healthy Japanese, indicating that HBoVs have spread throughout Japan.

1. Introduction

Human bocavirus type 1 (HBoV1), HBoV2, HBoV3 and HBoV4 are members of the genus *Bocavirus* in the subfamily *Parvovirinae* of the family *Parvoviridae* (Jartti et al., 2012). Following the first identification of HBoV1 in the nasopharyngeal secretion of a child with respiratory manifestations in 2005 (Allander et al., 2005), HBoV2, HBoV3 and HBoV4 were identified in fecal samples from children with non-polio acute flaccid paralysis and diarrhea in 2009 and 2010 (Arthur et al., 2009; Kapoor et al., 2009, 2010). HBoV is a small, nonenveloped icosahedral virus with a linear, single-stranded DNA genome of approx. 5.3 kilobases (kb). Sequence analyses of the HBoV genome revealed that it contains three open reading frames (ORFs) encoding four proteins in the following order: two nonstructural proteins (NS1 and NP1) and two viral capsid proteins (VP1 and VP2) (Allander et al., 2005). As in other members of the family *Parvoviridae*, the two capsid proteins, VP1 and VP2, have identical nucleotide and amino acid sequences except for an extra unique phospholipase-A motif (VP1u) at the amino-terminal end of the VP1 protein (Dijkman et al., 2009; Chen et al., 2010). The capsid protein exhibits $T = 1$ symmetry with 60 copies of the coat protein (Gurda et al., 2010). The functions of NS1 and NP1 are unknown.

HBoV infection is associated mainly with pediatric respiratory diseases and gastrointestinal diseases (Jartti et al., 2012). HBoV DNAs have been detected in 5% to 15% of patients with respiratory illness or gastrointestinal symptoms in North America, South America, Europe, Asia, Australia and Africa, indicating that HBoV infection is a global health concern (Longtin et al., 2008; Arthur et al., 2009; Fabbiani et al., 2009; Moreno et al., 2009; Soderlund-Venermo et al., 2009; Tozer et al., 2009; Vallet et al., 2009; Chow et al., 2010; Huang et al., 2010; Kapoor et al., 2009, 2010; Santos et al., 2010). The lack of an efficient cell culture system for HBoV has hampered the preparation of antigens for the serology assay. It is generally accepted that noninfectious virus-like particles (VLPs)

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assembled from the viral structural proteins are similar to native virions in size, shape and antigenicity, and thus that VLPs are useful as antigens for immunoassays.

To understand the antigenic properties of HBoVs, we established an efficient production system for HBoV1–4 VLPs using a recombinant baculovirus expression system, and we characterized the antigenicity of the purified VLPs. We performed seroepidemiological studies on HBoVs with sera from healthy populations in Japan using an enzyme-linked immunosorbent assay (ELISA) that was developed based on VLPs as the antigen.

2. Materials and methods

2.1. Construction of recombinant baculoviruses and expression of HBoV capsid proteins

We synthesized the viral genes encoding VP1 of HBoV1, VP2 of HBoV1, VP2 of HBoV2, VP2 of HBoV3 and VP2 of HBoV4, each of which contains a *Bam*HI site before the start codon and an *Xba*I site after the stop codon, based on published sequences (GenBank accession nos.: AB481080, FJ170279, FJ948861, and NC_012729), and cloned them into vector pUC57 (GeneScript, Piscataway, NJ) to generate plasmids pUC57-HBoV1-VP1, pUC57-HBoV1-VP2, pUC57-HBoV2-VP2, pUC57-HBoV3-VP2, and pUC57-HBoV4-VP2. These plasmids were digested with *Bam*HI and *Xba*I, and the purified VP1 or VP2 gene fragment was ligated to the transfer vector pVL1393 (Pharmingen, San Diego, CA), yielding plasmids pVL1393-HBoV1-VP1, pVL1393-HBoV1-VP2, pVL1393-HBoV2-VP2, pVL1393-HBoV3-VP2, and pVL1393-HBoV4-VP2.

An insect cell line, Sf9, derived from the armyworm *Spodoptera frugiperda* (Riken Cell Bank, Tsukuba, Japan), was co-transfected with a linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold 21100D; Pharmingen) and the transfer plasmid by a lipofectin-mediated method as specified by the manufacturer (GIBCO-BRL, Gaithersburg, MD). The cells were incubated at 26.5 °C in TC-100 medium (GIBCO-BRL) supplemented with 8% fetal bovine serum (FBS) and 0.26% bacto tryptose phosphate broth (Difco Laboratories, Detroit, MI). Each recombinant virus was plaque-purified three times in Sf9 cells. The baculovirus recombinants thus obtained were designated as AcHBoV1-VP1, AcHBoV1-VP2, AcHBoV2-VP2, AcHBoV3-VP2 and AcHBoV4-VP2, respectively. For large-scale expression, we used an insect cell line from the cabbage looper *Trichoplusia ni*, BTL-Tn 5B1-4 (Tn5) (Invitrogen, San Diego, CA). Tn5 cells were infected with the recombinant baculoviruses at a multiplicity of infection (m.o.i.) of 10 and cultured in EX-CELL 405 medium (JRH Biosciences, Lenexa, KS) at 26.5 °C as described (Li et al., 1997, 2011).

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

The proteins in the cell lysate and culture medium were separated by 5–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. For Western blotting, the proteins in the SDS-PAGE gel were electrophoretically transferred onto a nitrocellulose membrane. The membrane was then blocked with 50 mM Tris–HCl (pH 7.4) containing 5% skim milk and 150 mM NaCl, and incubated with a human serum positive for anti-HBoV IgG. Detection of human IgG antibody was achieved using alkaline phosphatase conjugate goat anti-human IgG (1:1000 dilution; Dako, Copenhagen, Denmark). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate P-toluidine were used as coloring agents (Bio-Rad Laboratories, Hercules, CA).

2.3. Purification of VLPs

The culture medium of the recombinant baculovirus-infected Tn5 cells was harvested on day 7 post-infection (p.i.). The intact cells, cell debris, and progeny baculoviruses were removed by centrifugation at 10,000 × *g* for 60 min. The supernatant was then spun at 126,000 × *g* for 3 h in a rotor (SW32Ti, Beckman Coulter, Indianapolis, IN), and the resulting pellet was resuspended in EX-CELL™ 405 medium at 4 °C overnight. For the CsCl gradient centrifugation, 4.5 mL of the samples were mixed with 2.1 g of CsCl, and then centrifuged at 116,000 × *g* for 24 h at 10 °C in the SW55Ti rotor. The gradient was fractionated into 250-μL aliquots, and each fraction was weighed in order to estimate the buoyant density and isopycnic point. Each fraction was diluted with EX-CELL™ 405 medium and centrifuged for 2 h at 237,000 × *g* in a Beckman TLA55 rotor to sediment the VLPs.

2.4. Electron microscopy (EM)

The purified VLPs were placed on a carbon-coated grid for 45 s, rinsed with distilled water, stained with a 2% uranyl acetate, and examined with an electron microscope (TEM-1400, JEOL, Tokyo) at 80 kV.

2.5. N-terminal amino acid sequence analysis

VLPs were purified by CsCl gradient centrifugation. N-terminal amino acid (aa) microsequencing was carried out using 100 pmol of the protein by Edman automated degradation on a protein sequencer (model 477, Applied Biosystems, Foster City, CA).

2.6. Healthy human serum samples

The specimens tested for HBoV serology consisted of 372 serum samples from healthy Japanese subjects collected in 1993. Serum samples were obtained from the Serum Bank of the National Institute of Infectious Diseases, Japan. The ages of the subjects ranged from 1 to 80 years old. Sixty-four percent (238/372) of the subjects were female, and 36% (134/372) were male.

2.7. Hyperimmune sera

Rabbits (Japanese White, 8 weeks old, female) were immunized with VLPs. The immunization was performed by a thigh muscle injection of purified VLPs at a dose of 500 μg per animal, and the booster injections were carried out at 4 and 6 weeks after the first injection with half doses of VLPs. All of the injections including the booster injections were carried out without any adjuvant. Immunized animals were bled 1 week after the last injection. The rabbit experiments were reviewed by the National Institute of Infectious Diseases (NIID) Ethics Committee and carried out according to the "Guides for Animal Experiments Performed at NIID" under code 111054.

2.8. Enzyme-linked immunosorbent assay (ELISA)

We developed an ELISA to detect anti-HBoV antibodies in human sera. Flat-bottom 96-well polystyrene microplates (Immulon 2; Dynex Technologies, Chantilly, VA) were coated with the purified VLPs (1 μg/mL, 100 μL/well) and incubated overnight at 4 °C. Unbound VLPs were removed, and the plates were washed twice with 10-mM phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and then blocked with 200 μL of 5% skim milk (Difco Laboratories) dissolved in PBS-T for 1 h at 37 °C. After three washes with PBS-T, diluted rabbit or human serum (100 μL/well) was added in duplicate. The plates were incubated