Expression of Recombinant Rotavirus Proteins Harboring Antigenic Epitopes of the Hepatitis A Virus Polyprotein in Insect Cells

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Abstract
Rotavirus and hepatitis A virus (HAV) spread by the fecal-oral route and infections are important in public health, especially in developing countries. Here, two antigenic epitopes of the HAV polyprotein, domain 2 (D2) and domain 3 (D3), were recombined with rotavirus VP7, generating D2/VP7 and D3/VP7, cloned in a baculovirus expression system, and expressed in Spodoptera frugiperda 9 (Sf9) insect cells. All were highly expressed, with peak expression 2 days post-infection. Western blotting and ELISA revealed that two chimeric proteins were antigenic, but only D2/VP7 was immunogenic and elicited neutralizing antibody responses against rotavirus and HAV by neutralization assay, implicating D2/VP7 as a multivalent subunit-vaccine Candidate for preventing both rotavirus and HAV infections.

Key Words: Rotavirus, Hepatitis A virus, Recombinant chimera protein

INTRODUCTION

Human infections by rotavirus and hepatitis A virus (HAV) are important public health problems worldwide, especially in developing countries. Although their clinical manifestations are different, both viruses are transmitted by a fecal-oral route. The viruses shed by a susceptible individual’s feces can spread to contaminate food or water (Nwachuku and Gerba, 2006). As a major cause of diarrhea-related illness and death, rotavirus is responsible for 2 million hospitalizations and more than 600,000 deaths worldwide among children younger than 5 years of age (Parashar et al., 2003). Rotavirus, a member of the family Reoviridae, consists of 11 segments of a double-stranded RNA genome that encodes for 6 structural and 6 non-structural proteins (Ofit and Blavat, 1986; Estes and Cohen, 1989). The outer capsid is composed of 2 glycoproteins, VP7 and VP4, are involved in protective immunity. Initially, VP7 was thought to be the most important antigen in stimulating the synthesis of neutralizing antibodies (Ofit and Blavat, 1986; Estes and Cohen, 1989).

HAV belongs to the picornavirus family and causes hepatitis A in humans. Children are primarily affected by HAV infection, but its severity increases with age (Gust, 1992). The HAV genome is a 7.5-kb positive-stranded RNA, coding for a single 250 kD polyprotein that is further processed into structural and non-structural proteins (Totsuka and Moritsugu, 1999). Like all picornaviral genomes, the HAV genome can be divided into three regions; P1, P2, and P3. The P1 region encodes structural proteins, such as 1A (also referred as to VP4), 1B (VP2), 1C (VP3), and 1D (VP1), whereas the others encode nonstructural proteins, i.e. 2A-2C in the P2 region and 3A-3D in the P3 region (Totsuka and Moritsugu, 1999). Previously, 42 antigenic peptides were identified across the HAV polyprotein, using a total of 237 synthetic peptides spanning the entire polyprotein and convalescent antisera from HAV-infected patients (Khudyakov et al., 1999). Among them, two antigenic epitopes, including the second (D2) and third (D3) domains, are known to be able to induce a virus-neutralizing antibody (Ab) response (Khudyakov et al., 1999). The D2 domain at position 767-842 amino acids (aa) contains the C-terminal part of the VP1 protein and the entire P2A protein. The D3 domain at position 1403-1456 aa contains the C-terminal part of the...
P2C and the N-terminal half of the P3A protein (Khudyakov et al., 1999).

In this study, two defined antigenic epitopes of the HAV polyprotein, D2 and D3 were recombinated with rotavirus structural protein VP7. The resultant recombinant viral proteins were designated D2/VP7 and D3/VP7, respectively, and expressed in *Spodoptera frugiperda* 9 (Sf9) insect cells using a baculovirus expression system.

**MATERIALS AND METHODS**

**Ethics statement**

The study was performed according to protocol (number 2009-13) approved by the Human Subjects Institutional Review Board (IRB) of Chung-Ang University College of Medicine, Seoul, Korea.

**Viruses and cells**

Human rotavirus Wa G1P[8] and HAV CAU-H3 strains (isolated from a fecal specimen of a patient diagnosed with an acute form of hepatitis A at Kangnam St. Mary’s Hospital, The Catholic University of Korea School of Medicine, Korea, in 2002) were used as a viral genomic template for cDNA synthesis. Rotavirus Wa and cytopathic variant HM175 HAV were separately used for the infection of rotavirus and HAV and neutralization assay. MA104 cells and FRHK-4 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and used for cultivation of rotavirus and HAV, respectively. MA104 cells were grown in Minimum Essential Medium alpha (MEM-alpha; Gibco BRL Life Technologies, Grand Island, NY, USA) containing 5% fetal bovine serum (FBS; Gibco BRL Life Technologies) at 37°C in present of 5% CO2. FRHK-4 cells were grown in DMEM (DMEM; Gibco BRL Life Technologies) containing 10% fetal bovine serum (FBS; Gibco BRL Life Technologies) at 37°C in present of 5% CO2. Sf9 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and used for the production of baculoviruses. In general, the cells were grown and maintained in TNE-FH medium (Gibco BRL Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco BRL) at 37°C in a 250 ml spinner flask with shaking at 90 rpm. Cellular density and viability were determined by standard microscopic observations, using a hemocytometer after trypan blue staining.

**Reagents and animals**

All enzymes used in this study were purchased from New England Biolabs (Beverly, MA, USA). For the expression of recombinant rotavirus proteins, both a baculovirus transfer vector (pBlueBac4.5/V5-His) and a baculovirus expression system (Bac-N-Blue™) were employed (Invitrogen, Carlsbad, CA, USA). Plasmids were isolated using a plasmid miniprep kit (INtRON Biotechnology, Seoul, Korea). Rabbit serum against the rotavirus Wa strain was produced by mixing both Bac-N-Blue linear baculovirus DNAs and the baculovirus transfer vectors with the Cellfectin® II reagent in serum-free medium as per the manufacturer’s instructions (Invitrogen). Each recombinant virus was purified using a standard plaque assay (King et al., 2007).

**Construction of the recombinant rotavirus proteins carrying the HAV antigenic epitopes**

Complementary DNAs (cDNAs) corresponding to rotavirus structural protein VP7 was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using viral RNAs from the rotavirus Wa strain. Similarly, the 231-bp D2 and 165-bp D3 cDNA fragments were amplified using viral RNAs from the HAV CAU-H3 strain. Recombination events between D2 and VP7 and between D3 and VP7 were introduced via a Xhol restriction site (Fig. 1A). The resultant recombinant proteins were designated D2/VP7 and D3/VP7, respectively. They were cloned into the pBlueBac4.5 baculovirus transfer vector to produce the recombinant baculoviruses according to the manufacturer’s description (Invitrogen). All recombinant cassettes were confirmed by DNA nucleotide sequencing using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an automatic DNA sequencer (model 3730; Applied Biosystems). Two recombinant baculoviruses were produced by mixing both Bac-N-Blue linear baculovirus DNAs and the baculovirus transfer vectors with the Cellfectin® II reagent in serum-free medium as per the manufacturer’s instructions (Invitrogen). Each recombinant virus was purified using a standard plaque assay (King et al., 2007).

**Protein separation and Western blot analysis**

Protein separation was carried out as previously described by Laemmli (Laemmli, 1970). Briefly, each recombinant protein sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA). For Western blot, the resolved proteins were blotted onto Immun-Blot™ polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using a wet transfer system (Bio-Rad). Membranes were blocked in 5% (w/v) non-fat dried milk solution overnight at 4°C. The primary antibodies (Abs) were prepared in TBS-T (20 mM Tris-HCl, 500 mM NaCl, 0.1% Tween20, pH 7.9) at the following dilutions: anti-V5 Ab, 1:10,000; rabbit antiserum against the rotavirus Wa strain, 1:5,000; anti-HAV sera, 1:5,000. The blocked membranes were incubated with the primary Ab for 1 hr and washed 4 times with TBS-T. The appropriate secondary Abs conjugated with peroxidase were used after dilution by 1:10,000. Protein bands were visualized by an enhanced chemiluminescence (ECL) method, as previously described (Penna and Cahalan, 2007).

**Kinetic of the recombinant baculovirus synthesis in Sf9 cells**

Sf9 cells at a density of 1×10^6 cells/ml were seeded in a 6-well plate. The cultures were simultaneously infected with the recombinant baculoviruses of D2/VP7 at a multiplicity of infection (MOI) of 10 plaque forming units per cell (pfu/cell) (Jiang et al., 1998; Roldão et al., 2007) and harvested every 24 hrs during the experimental period of 10 days after the initial virus infection. Cells were clarified by centrifugation at 2,500×g for 5 min and lysed using RIPA buffer (RIPA cell lysis buffer; AMRESCO Inc., Solon, OH, USA). The supernatants were harvested after centrifugation at 14,000×g for 15 min at 4°C and stored frozen at 20°C until use.

**Rabbit immunization**

New Zealand white female rabbits were immunized intra-