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Original Article

Recombinant Outer Capsid Glycoprotein (VP7) of Rotavirus Expressed in Insect Cells Induces Neutralizing Antibodies in Rabbits

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Abstract

Background: Rotaviruses cause diarrhea in infants and young children worldwide. Rotavirus outer capsid protein, VP7 is major neutralizing antigen that is important component of subunit vaccine to prevent rotavirus infection. Many efforts have been done to produce recombinant VP7 that maintain native characteristics. We used baculovirus expression system to produce rotavirus VP7 protein and to study its immunogenicity.

Methods: Simian rotavirus SA11 full-length VP7 ORF was cloned into a cloning plasmid and then the cloned gene was inserted into the linear DNA of baculovirus *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) downstream of the polyhedrin promoter by in vitro recombination reactions. The expressed VP7 in the insect cells was recognized by rabbit hyperimmune serum raised against SA11 rotavirus by Immunofluorescence and western blotting assays. Rabbits were immunized subcutaneously by cell extracts expressing VP7 protein.

Results: Reactivity with anti-rotavirus antibody suggested that expressed VP7 protein had native antigenic determinants. Injection of recombinant VP7 in rabbits elicited the production of serum antibodies, which were able to recognize VP7 protein from SA11 rotavirus by Western blotting test and neutralized SA11 rotavirus in cell culture.

Conclusion: Recombinant outer capsid glycoprotein (VP7) of rotavirus expressed in insect cells induces neutralizing antibodies in rabbits and may be a candidate of rotavirus vaccine.

Keywords: Rotavirus, VP7, Expression, Baculovirus, Insect cell

Introduction

Rotaviruses belong to the family *Reoviridae* and are common cause of diarrhea in infants and young children. Rotaviruses cause about 0.6 million deaths annually worldwide (1-3). Nearly all children are infected with rotaviruses by the age of 5 years regardless of their country or socioeconomic status. Rotavirus infections can be asymptomatic but in some can cause fever, vomiting, and diarrhea leading to intensive dehydration and electrolyte disturbances. However, most of the rotavirus related deaths occur in developing countries (4).

Effective rotavirus vaccine is needed in regions where mortality by rotavirus infection is high (5). A reassortant rhesus-human rotavirus vaccine, Rotashield, was developed and used in 1998. Because of a few cases of intussusceptions were

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identified, leading to the withdrawal of vaccine. There are important inquiries about these oral live vaccines related with safety, side effects and production costs, thus development of non-replicating rotavirus vaccine should be considered as an alternative to live vaccines. Other approaches to the development of rotavirus vaccines are rotavirus outer capsid proteins (VP4 and VP7) expressed in different vectors; virus-like particles produced by baculovirus, DNA vaccines, and killed viruses (5).

The rotavirus virion is a nonenveloped icosahedral particle, consisting of six structural proteins (VP1–VP4, VP6 and VP7) and six non-structural (NSP1–NSP6) proteins. Structural proteins are organized in three-layered capsid containing the genome of 11 segments of double-stranded RNA (1,6). The outer smooth layer of capsid is composed of the most abundant VP7, 37-kDa glycoprotein as 260 trimers and VP4 spikes, 88-kDa protein as 60 dimers, which, both induce the production of neutralizing antibodies and define virus G (VP7) or P (VP4) serotypes specificity. Group A rotavirus comprises at least 19 G serotypes and 27 P serotypes (1,7). Proteolytic cleavage of VP4 into two subunits, VP8* (28 kDa) and VP5* (60 kDa), is necessary for the virus to be infectious (1). Attachment and penetration of the virus particles to the cell is related to VP4; however, the role of VP7 in these events is unknown (8-10). Recently, it has been clear that VP4 and VP7 contain binding motifs for α/β integrins, which assumed to mediate rotavirus attachment and penetration into the cell (11,12). Epitope-specific antibodies to VP4 and VP7 associate with viral neutralization and protection from infection (13,14).

VP7 is a highly immunogenic glycoprotein (15) and it is a primary candidate for inclusion in a subunit vaccine. Expression of rotavirus VP7 has been reported for *E. coli* (16-19), herpes virus (19), vaccinia virus in mammalian cells (20,21) and baculovirus (22-24). However, most of them were not full-length VP7 protein. Advanced technique in anchoring the simian rotavirus SA11 VP7 to the surface of eukaryotic cells (VP7sc) has done using recombinant vaccinia virus and adenoviruses. The expressed VP7 protein appeared to be both antigenic and immunogenic and induced passive protection against rotavirus disease in mice (25,26).

Using the right system for viral gene expression is very important in producing biologically active recombinant protein. Baculovirus expression system has some unique features that made it the system of choice for many protein expressions, such as solubility, correctly folding, signal peptide cleavage, oligomerization, functional activity, phosphorylation, and glycosylation of recombinant proteins (27). Baculovirus has been used successfully as an expression system for the production of rotavirus proteins (22-24). The baculovirus system is a candidate for the expression of VP7 in that it offers the possibility of synthesis of a recombinant protein in high yield with the conformational requirements necessary to permit immunological and functional studies (24,28).

In this study, SA11 rotavirus VP7 gene was cloned and expressed in insect cells and its immunogenicity was assayed in rabbits. The ability of baculovirus-expressed VP7 to stimulate an antibody response that, recognize and neutralize SA11 rotavirus, suggested that, recombinant VP7 mediated native antigenic determinants in the absence of other rotavirus proteins.

Materials and Methods

Cells and viruses

African green monkey kidney epithelial cell line, BSC-1, was grown as a monolayer in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2mM L-glutamine, and 100U of penicillin and 100µg of streptomycin per ml at 37º C. Simian rotavirus SA11 was treated with 5 µg/ml trypsin for 60 min at room temperature to activate virus infectivity. The virus was propagated in serum free medium supplemented with 0.5 µg/ml trypsin. When infected cells showed 80% of CPE, cultured flasks were harvested by freeze thawing three times and clarified by low speed centrifugation at 4°C. Stock of SA11 rotavirus was used for virus titration and neutralization assay. Partially purified rotavirus prepared by ultracentrifugation on 40% sucrose cushion, at 22000 rpm for 2h at 4°C. *Autographica californica*

nuclear Polyhedrosis Virus (AcNPV) DNA, and recombinant baculoviruses expressing VP7 (AcNPV-VP7) DNA transfected into the *Spodoptera frugiperda* (SF9) cells and were grown on Grace's insect medium/TNM-FH (GIBCO) containing 10% fetal bovine serum (FBS) at 28°C (29).

RNA extraction

RNA was extracted from virus pellet or rotavirus infected BSC-1 cell culture using RNAfast RNA extraction reagent, manufactured in National Research Center for Genetic Engineering and Biotechnology (NRCGEB), according to manual. Finally, extracted RNA dissolved in 50 µl of diethyl pyrocarbonate-treated water and stored at -20ºC. dsRNAs of rotavirus were electrophoresed on 10% PAGE and bands were identified by staining with ethidium bromide.

Primer designing

Oligonucleotide primers, specific for rotavirus genome segments 9, which encodes VP7, were designed according to simian rotavirus SA11 sequence data in GeneBank (accession #: K02028) (30). To generate *att* (attachment site) flanked VP7 cDNA suitable for using as substrate in Gateway® BP recombination reaction with donor vector (pDONR™221) (invitrogen), incorporation of *attB* sites into our RT-PCR product was necessary (31). Therefore, in forward primer, four G residues at 5' end followed by 26 bases (*attB1*) and gene specific sequences (nucleotide no 47 to 70). In reverse primer, four G residues at 5' end followed by 25 bases (*attB2*) and gene specific sequences (nucleotide no 1003 to 1028). We removed stop codon from VP7 gene in the reverse primer for in frame C-terminal 6×His tagging to our recombinant VP7 in the BaculoDirect™ Linear DNA (Invitrogen). Thus, primers were 55 and 53 nucleotides long, with following sequences. *AttB1* and *attB2* sites are underlined. **VP7-F1**: 5´GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AAT GTA TGG TAT TGA ATA TAC CAC A3'; **VP7-R11**: 5'GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA CAC TCT GTA ATA AAA TGC TGC TG3'.

cDNA synthesis

Rotavirus dsRNA was used as template to synthesize cDNA copies from both viral strands. Reverse transcription was carried out using a mixture of 10µl dsRNA template and, 1µl of 100µM of each primers. The reaction tube was heated at 95ºC for 5 min and quickly cooled at 4ºC, then reverse transcription mixture consisting of 4 µl of 5X incubation buffer, 1µl of 2.5mM dNTPs mix (TAKARA), 20 U/µl RNase inhibitor (Fermentas), and 40U M-MuLVirus reverse transcriptase (Roche) were added in total volume of 20 µl. The tubes were mixed and incubated at 37ºC for 60 minutes in a thermocycler.

PCR amplification

PCR reaction was carried out in total volume of 50µl containing: 5µl of cDNA synthesis reaction mixture, 5µl of 10X pfu PCR buffer with $MgSO_4$, 1µl of 20µM each of primers, 4µl of 10mM dNTPs mix, 2.5 U/µl pfu DNA polymerase (Fermentas), PCR program was an initial denaturation at 95ºC 5 min; 95ºC 30 sec, 55ºC 30 sec 72ºC 2 min (35 cycles); Final extension 10 min at 72ºC and final hold at 4ºC. *attB1* and *attB2* flanked PCR product (called *VP7-attB-PCR* product), was electrophoresed on 1% agarose gel, bands were identified by ethidium bromide and was verified primarily by restriction enzyme DdeI digestion, then, for removing primers and primer-dimers the PCR product was purified with 30% PEG 8000/30 mM $MgCl_2$ in TE buffer pH 8.0 by centrifugation.

VP7 gene cloning

We transferred VP7 gene flanked by *attB1* and *attB2* sites from *VP7-attB-PCR product* to an *attP1* and *attP2*-containing donor vector (pDONR™221) by the BP recombination reaction to create an entry clone using BP clonase™ II enzyme mix (λ phage & E.coli recombination factors and enzymes) (31). Transformation of competent *E. coli* cells (One Shot® OmniMAX™ 2-T1) was performed as Mandel & Higa methods (32). Colonies were grown in LB agar, containing 50µg/ml kanamycin. Miniprep plasmid was extracted, and screened for desired clone by digestion with NcoI and EcoRV restriction enzymes.

We named the resulting clone as, *VP7-entry clone* and confirmed by bidirectional sequencing using universal M13F and M13R primers. Result was analyzed by BLAST software.

Recombinant baculovirus DNA construction

The construction and characterization of the recombinant baculovirus DNA containing VP7 gene were done according to manufacturer's instructions (33). Briefly, LR recombination reaction was performed to transfer the *attL1* and *attL2* flanked VP7 gene from *VP7-entry-clone* into *attR1* and *attR2* containing BaculoDirect™ Linear DNA by LR Clonase™ II enzyme mix (λ phage & E.coli recombination factors and enzymes). Thus, by this reaction HSV1 tk and lacZ genes were deleted from BaculoDirect™ Linear DNA as by products.

Insect cell culture (Sf9) and Transfection

The resulting recombinant DNA was transfected into Sf9 cells by lipofection. In the presence of ganciclovir in the Sf9 cell culture medium, non-recombinant baculovirus DNA or by products were negatively selected as follow. Sf9 cells were grown and maintained as monolayer in Grace's insect medium with supplements (lactalbumin hydrolysate, L-glutamine, and yeast extract) and 10% FBS. Cell density was calculated by cell counting with a hemocytometer, and cell viability was determined by trypan blue exclusion (29). Sf9 cells were seeded 8×10^5 per well in 2 ml of complete Grace's Insect medium in a six-well plate. The cells were incubated at 28°C for 1h to allow the cells fully attach to the bottom of the wells. Transfecting of the LR recombination reaction into Sf9 cells carried out using Cellfectin reagent (33). Briefly after preparing transfection mixture the medium was removed from the cells and washed with 2ml of fresh Grace's Insect medium (unsupplemented). The medium was removed from the cells and the entire transfection mixture was added onto the cells. The cells incubated at 28ºC for 5h. Then, the transfection mixture was removed and 2 ml of complete growth medium with antibiotics (100 U/ml penicillin and 100 mg streptomycin) and 100 µM ganciclovir was added to each well; and incubated at 28°C. For generating non-recombinant baculovirus as negative control we transfected Sf9 cells by BaculoDirect™ Linear DNA alone and Sf9 cells were cultured in the absence of ganciclovir. After 4-5 days post-infection, once the cells showed very late stage of infection, virus was harvested from the cell culture medium by centrifugation at 3000 rpm for 5 min. The supernatant was transferred to a fresh 15ml tube as P1 baculovirus stock and stored in a cool (4°C) and dark place. The P1 viral stock was used for preparing high titer viral stocks and their titers were determined by plaque assay. Working viral stock (1×10^7 pfu/ml) was prepared with Grace's medium (33).

Screening recombinant baculovirus DNA for VP7 gene

To verify the presence of VP7 gene in recombinant baculovirus, viral DNA was isolated from supernatant of infected Sf9 cells using 20% PEG 8000 (Fluka) in 1M NaCl, 0.1% Triton X-100, proteinase K, phenol, chloroform, isoamyl alcohol (25:25:1), sodium acetate, and precipitated by ethanol, then, was re-suspended in 10 µl of sterile water and used as template in PCR.

Immunofluorescence (IF) assay

Expression of recombinant VP7 in Sf9 cells was confirmed by immunofluorescent staining as described previously (34) with modifications. Briefly, Sf9 cells were infected with recombinant baculovirus expressing VP7 (AcNPV-VP7) approximately at 1 pfu/cell and incubated at 28°C for 4 days. The cells were washed two times with PBS and re-suspended in PBS. Cells were fixed on a slide by cold acetone for 10 min at -20°C. IF test, was carried out, using rabbit polyclonal anti-rotavirus SA11 and anti-rabbit FITC-conjugate antibodies. Sf9 cells infected with non-recombinant baculovirus (AcNPV) and non-infected cells used as negative controls.

SDS-PAGE

Protein electrophoresis was carried out by the method of Laemmli (35), using 13% separating and 5% stacking gels. Briefly, samples of Sf9 cells infected by AcNPV-VP7, AcNPV and uninfected

cells and cells supernatants or partially purified rotavirus were lysed in SDS-PAGE sample buffer and boiled at 95ºC for 5 min. Prepared samples subjected to electrophoresis and gels were stained with Coomassie blue.

Western blot analysis

SDS-PAGE resolved protein bands were transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membranes by liquid transfer system. Nonspecific sites were blocked by incubation for 2 h in 5% skim milk prepared in western washing buffer, and membranes were incubated 2 h in a 1:500 dilution of the appropriate antisera (mouse anti-HisTag (Roche) or SA11 rotavirus rabbit hyperimmune antiserum). After washing steps bound antibodies were detected by incubation in a 1:1000 dilution of HRP-conjugated anti-mouse antibody (Roche) or HRP-conjugated goat anti-rabbit antibody (KOMA) prepared in western washing buffer. Following additional washing steps, the membranes were developed by 0.06% 4-chloro-1-naphtol prepared in washing buffer-methanol (4:1; vol/vol) which contained 5 µM H_2O_2.

Immunization of rabbits

Three to six-month-old rotavirus negative rabbits as determined by dot blot and neutralization assays were immunized subcutaneously by injecting cell extracts expressing VP7 protein. The extracts were prepared from Sf9 cells by 3 times freeze and thawing of the cells followed by centrifugation at 500 rpm for 15 min. 0.5 ml of the clarified extract containing about 100 µg of recombinant VP7 protein was mixed with equal volumes of complete Freund`s adjuvant and were injected to rabbits. Rabbits were boosted 2 times at weekly intervals with the same amount of immunogen in incomplete Freund`s adjuvant. Animals were bled 10 days later. Antiserum against whole rotavirus prepared. Preimmunized sera were taken as control. Potency of sera was tested against VP7 by dot blot. Partially purified SA11 rotavirus proteins blotted on PVDF membrane and subjected to dot and western blot assays.

Neutralization assay

Neutralizing effect of antisera produced in rabbits immunized with recombinant VP7 against SA11 rotavirus was determined on monolayer of BSC-1 cells in a 24-well microplate. Serial dilutions of heat inactivated (56°C for 30 min) antisera prepared in DMEM medium were incubated with of 100 $TCID_{50}$ of activated SA11 rotavirus at 37ºC for 1h. Then inoculated into BSC-1 cells in microplate and incubated at 37°C for 1h in order to virus absorption. DMEM (without serum) was added into the wells and incubated at 37ºC for development of CPE. Final dilution of sera that could prevent CPE development with respect to the control wells was considered as neutralizing titer. Pre-immune sera were used as negative control and rabbit hyperimmune antiserum against native SA11 rotavirus used as positive control.

Results

RT-PCR amplification

The study was initiated by culturing SA11 rotavirus and extracting its dsRNA segments from partially purified rotavirus (Fig. 1). The cDNA of VP7 coding genome segment 9 from nucleotides 47 to 1028 (Complete VP7 ORF without 5' and 3' consensus sequences) flanked by *attB1* and *attB2* sites was synthesized and amplified by RT-PCR, thus *VP7-attB-PCR* product has a total length of 1039 nucleotides and confirmed by restriction digestion.

Cloning of VP7 gene

A transfer plasmid (*VP7-entry clone*), containing rotavirus VP7 gene, was constructed (Fig. 2). The *VP7-attB-PCR* product and its orientation in the *VP7-entry clone* was verified by restriction digestion (Fig. 3) and sequencing. Sequence analysis confirmed that *VP7-entry clone* contained a full-length copy of SA11 gene 9 ORF, and showed 100% homology with SA11 rotavirus genome segment no 9 compared to previously published sequence data.

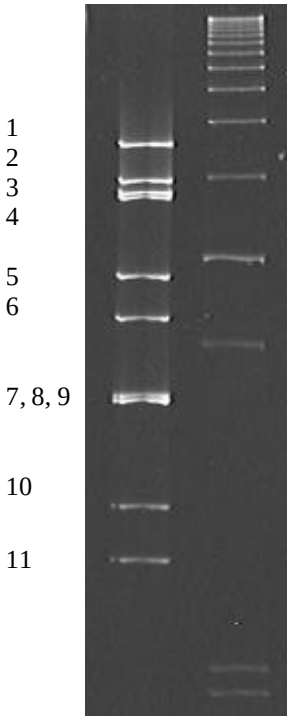

Fig. 1: Electropherogram of rotavirus RNA segments, Left: pattern of total dsRNAs extracted from partially purified SA11 rotavirus pellet, Right: DNA marker, 10% PAGE stained with ethidium bromide

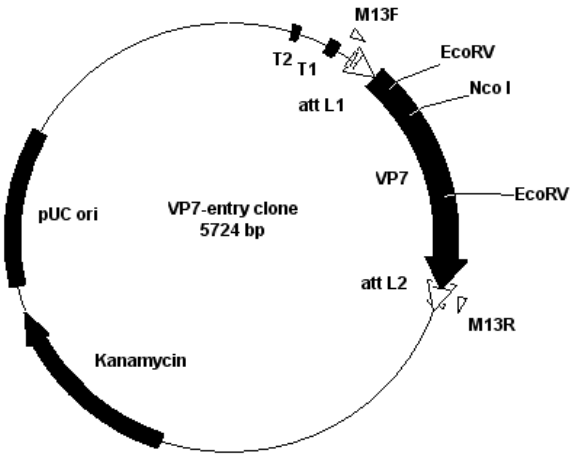

Fig. 2: Map of VP7-entry clone. Its genes and Restriction Enzyme digestion sites

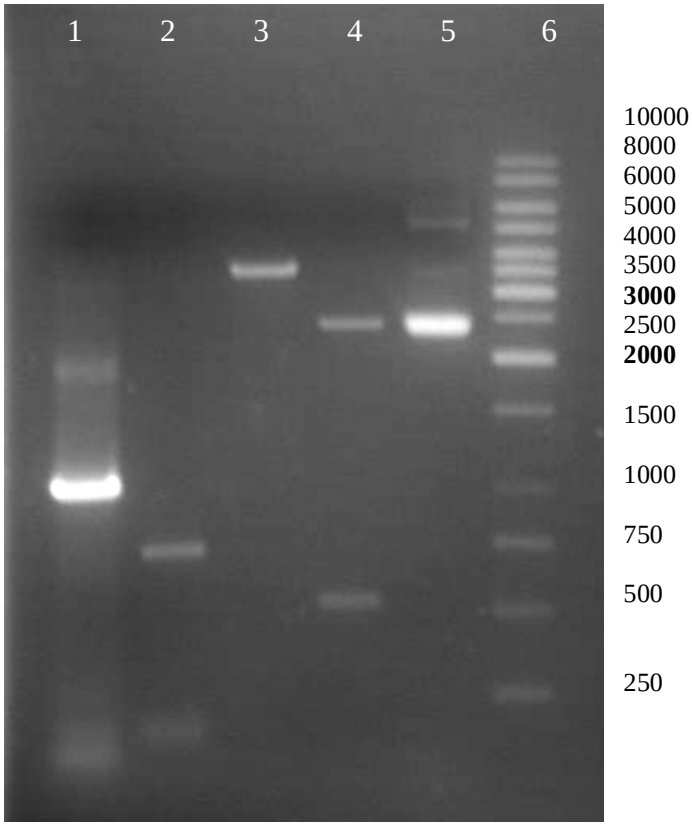

Fig. 3: One percent gel agarose electrophoresis of DNA molecules. lane 1**:** RT-PCR product (VP7-attB-PCR product) 1039 bp; lane 2**:** VP7-attB-PCR product digested by restriction enzyme, Dde I, because this DNA product has two sites for this enzyme, three segments (748, 173, 118 bp), resulted; lane 3: VP7-entry clone digested by NcoI, (~3386 bp); lane 4**:** VP7-entry clone digested by EcoRV, because this enzyme have two RE sites, two 2873 and 513 bp fragments were resulted; lane 5**:** VP7-entry clone undigested; lane 6: molecular weight DNA markers

Recombinant baculovirus construction

VP7 gene was transferred from *VP7-entry clone* into the Gateway® cassette of baculovirus™ linear DNA under the control of the polyhedrin promoter. The VP7 gene insert contains an intact translation start site and lacks its stop codon, instead it have an in frame C-terminal V5 epitope and 6×His tag and V5 epitope. We confirmed the presence of VP7 gene in baculovirus DNA by PCR analysis (Fig. 4).

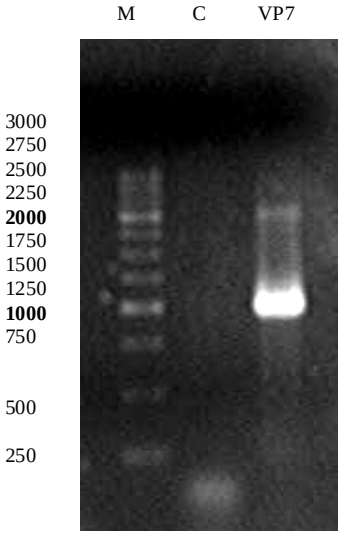

Fig. 4: PCR screening of recombinant baculovirus. One percent agarose gel electrophoresis showing PCR product of VP7 gene (1039 bp) cloned into the baculovirus, using VP7-F1 and VP7-R11 primers and baculovirus extracted DNA as template; C: non-recombinant baculovirus DNA as negative control; M: molecular weight DNA markers

Expression of VP7 protein

Recombinant baculovirus initially was screened for VP7 expression in the cytoplasm of infected Sf9 cells by the indirect immunoflurecent technique using SA11 specific antiserum (Fig. 5). Considering the size of the VP7 (38 kDa) and an in frame V5 epitope and 6×HisTag (4 kDa) in baculovirus DNA, in sum our VP7 fusion protein would be 42 kDa. Molecular weight of expressed protein in SDS-PAGE stained by Coomassie blue, and western immuno-blotting analysis using rotavirus antiserum confirmed the VP7 expression in Sf9 cells (Fig. 6 and 7). Although it appeared primarily to be localized within the cells, but low amount of VP7 protein was determined exteracellularly as early as 2 days post infection (data not shown). As VP7 is a glycosylated protein, it is possible that insect cells secrete it. Similar locations of VP7 have been observed by others (36,37). Yields of VP7 were greatest when cells were grown in complete Grace's insect media containing 10% FBS.

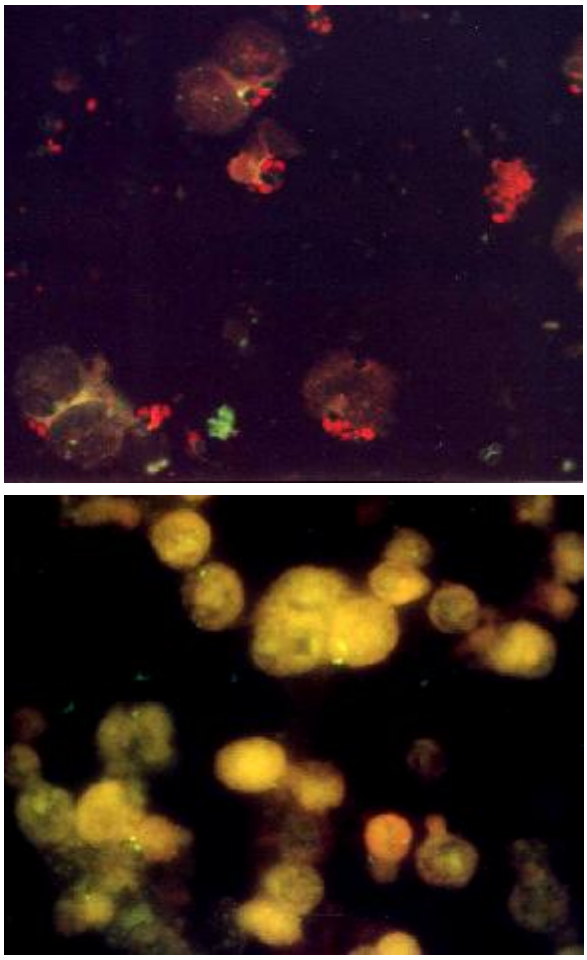

Fig. 5: Expression of rotavirus VP7 protein by recombinant baculovirus in Sf9 cells detected by indirect immunofluoresnce. Uninfected Sf9 cells (A); and Sf9 cells infected by recombinant baculovirus expressing VP7 (B), after staining with rabbit anti-rotavirus SA11 polyclonal serum and anti-rabbit FITC-conjugated antibody. Cells were examined and photographed under UV microscope at a magnification of 400x

Baculovirus construct produced sufficient protein to be visible after Coomassie blue staining of the gel including a viral protein, which appeared to migrate as a 42-kDa band on the gel. Western blotting of recombinant VP7 expressed in Sf9 cells using mouse anti-HisTag and anti-mouse HRP-conjugated antibodies showed several tagged protein bands on PVDF membrane, two strong

and one weak, about 42, 35, and 38 kDa respectively (Fig. 6). VP7 protein expression in the Sf9 cells is increased in various passages of recombinant VP7 baculovirus (Fig. 7). SA11 rotavirus specific antiserum recognized only one band on each sample that agrees with VP7 glycoprotein.

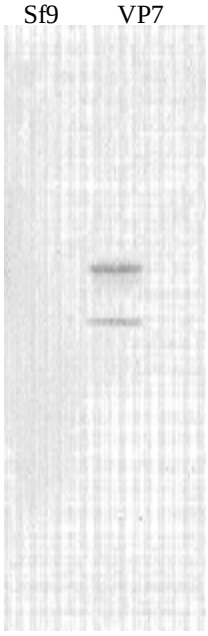

Fig. 6: Western blotting of VP7 expression in Sf9 cells. Un-infected Sf9 cells (Sf9), non-recombinant (NR), and recombinant baculovirus expressing VP7 (VP7). 13% SDS-PAGE, Western blotting of transferred proteins on PVDF membrane, developed by mouse anti-HisTag and anti-mouse HRP-conjugated antibodies. VP7-HisTag is a 42 kDa protein

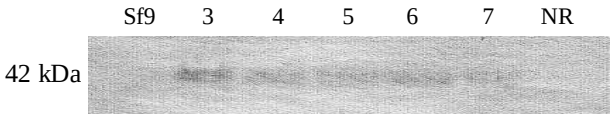

Fig. 7: Immunoreactivity of SA11 VP7 expressed in Sf9 cells. Western blotting analysis of recombinant VP7 expressed in Sf9 cells by baculovirus in various passages (3-7); uninfected Sf9 cells (Sf9), Sf9 cells infected by non-recombinant baculovirus (NR), proteins resolved by 10% SDS-PAGE and transferred on nitrocellulose membrane, then stained by rabbit anti-rotavirus and HRP-conjugated anti-rabbit antibodies; recombinant VP7-HisTag is a 42 kDa protein

Immune response against recombinant VP7

Rabbit's antisera after final step of inoculation could recognize rotavirus in dot blot and native VP7 protein band (38 kDa) in Western immunoblotting test (data not shown). In contrast, pre-immune serum could not recognize any rotavirus proteins. This result indicates that induced antibodies function specifically. Antiserum against baculovirus-infected cells containing recombinant VP7 reduced rotavirus infectivity with a neutralizing titer of approximately 160. By contrast, pre-immune serum from inoculated rabbits failed to elicit a neutralizing response. Rabbit antiserum against native rotavirus neutralized SA11 rotavirus infectivity with a titer of 640.

Discussion

A complete sequence of SA11 rotavirus VP7 ORF was cloned into the plasmid vector. We omitted 3´ and 5´ non-coding nucleotides and stop codon from cloned gene. Sequencing result confirms highly conserved nucleotide sequences of gene 9 after long and continuous passage of SA11 rotavirus in our laboratory.

A full-length VP7 ORF was placed under control of the polyhedrin promoter in baculovirus linear DNA cassette. This construction enabled the methionines at first or other positions of the amino acid sequence of VP7 to function as potential sites for initiation of protein synthesis. The second initiation codon has been shown to function in vivo during viral protein synthesis in mammalian cells (38) and insect cells (22). Smaller protein products of recombinant VP7 protein that are visible in western blot using mouse monoclonal anti-HisTag (Fig. 6); indicates probability of first and other initiation codon usage. Only one protein band (42 kDa) was detected with anti-rotavirus polyclonal antibody in western blot (Fig. 7), the reasons, for these differences maybe are sub-products of recombinant VP7s have not retained their antigenic epitopes, thus are not reactive with SA11 anti-rotavirus antibodies or disfolding of VP7 antigenic sites in truncated VP7 fragments. Thus, harvesting time should be based on VP7 synthesis, because

delay in harvesting, may cause unassembled VP7 to degrade over time in cells and cell supernatant.

In a study polyhydrin protein was expressed at a level of 15% of total cellular protein (39). Because we did not have polyhydrin gene in non-recombinant baculovirus control, thus we could not able to compare VP7 expression level to native polyhydrin protein (29 kDa) expression. Yields of VP7 were greatest when cells were grown in complete media containing 10% FBS. That is compatible with the result of VP6 expression in Sf9 cells by other study (40).

It have been shown that in group A strains the VP4 and VP7 proteins are antigenically and functionally intact when expressed by a recombinant baculovirus and immunization with the expressed VP4 and VP7 provides passive protection to a rotavirus challenge in an animal model (22,39,41,42). However, we have constructed a recombinant baculovirus that expressed VP7 protein (42kDa). The expressed polypeptide maintains viral antigenic sites in the absence of other rotavirus outer or inner capsid components and induced VP7 specific antibody in rabbits. This indicates that the expressed VP7 protein is antigenically similar to VP7 on the rotavirus SA11 outer capsid. Inoculation of recombinant VP7 contained in soluble insect cell lysate into rabbits resulted in a weaker immune response relative to that obtained by inoculation of whole virus. However, it should be considered that the antibodies induced by whole virus would direct against VP7 as well as VP4. The difference of our antibody titers from others maybe is immunization protocol, animal host variation, and immunogen amount that we used.

Many of the conformation-dependent epitopes present on VP4 and VP7 are well present in the Sf9 cells infected with recombinant baculovirus (28). Our result of immunofluresence using SA11 rotavirus specific antiserum is consistent with that (Fig. 5). In addition, sera from the immunized rabbits recognized native VP7 protein in Western blot using partially purified SA11 rotavirus, but not other SA11 proteins confirming that the produced antibody activity was specific. Pre-immune sera did not possess the ability of to recognize VP7 or other viral proteins of intact SA11 rotavirus.

Attempts have been made by other researchers to express VP7 or VP4 as a fusion protein in bacteria (5,16); rotavirus SA11 VP7 has been expressed in *E. coli* as a fusion with β-galactosidase. There the partially purified fusion protein induced weak neutralization antibody in mice when injected subcutaneously with adjuvant. In other studies, bovine UK strain VP7 fused to lacZ proved to be toxic when expressed in *E. coli* (43). Similarly, vaccinia virus constructs of the wild-type VP7 gene and a modified construct resulting in partial secretion of VP7 (20) induced only a small increase in neutralizing antibody when injected intradermally into rabbits. An investigator has reported that live *E. coli* carrying outer membrane protein A and part of porcine rotavirus VP7 induced a serum neutralizing antibody in mice, without using adjuvant (44). In other study a vaccinia virus designed to express VP7 on the cell surface highly enhanced the immunogenicity compared with other vaccinia virus-expressed VP7s (25). This surface expressed VP7 contained neutralizing epitopes detectable on rotavirus VP7 but not on other vaccinia virus-expressed VP7s (45). The VP7 reacts within itself, with other VP7 molecules and with VP4, or the inner capsid protein VP6 in capturing up its conformation on the surface of rotavirus. Furthermore, VP7 may be added to novel rotavirus particles during their transport across the endoplasmic reticulum membrane or in enveloped intermediate particles (46,47) and membrane interactions may assist in the folding of VP7 during rotavirus maturation. Interaction between the cell surface-anchored VP7 expressed by vaccinia virus and the plasma membrane of cells may stabilize the epitopes of VP7 (45). However, we found that, using baculovirus expression system, the VP7 protein yield was satisfactory.

Antiserum rose against baculovirus-expressed VP7 in guinea pigs neutralized SA11 rotavirus infectivity, with titer of 1000 in fluorescent reduction neutralization (FRN) assay. Under similar conditions, the neutralizing antibody titer with whole rotavirus was higher (22). Antiserum produced in mice against VP7 expressed in baculovi-

rus, neutralized rhesus rotavirus (RRV) infectivity in FRN assay (24). However, in our study, neutralization titers induced by VP7 (titer: 160) were lower than that induced by whole virus (titer: 640). It may simply be because of the relative amount of antigen presented. In addition, it should be noticed that the neutralizing antibodies induced by whole virus would include antibodies against VP4. Considering that, the neutralization technique used in our study was different. However, in comparison to other studies, proportion of neutralizing antibody produced against recombinant VP7 (titer: 160) to whole virus (titer: 640) is remarkable.

In conclusion, a full-length VP7 protein of SA11 rotavirus containing 6×HisTag expressed by baculovirus in insect cells can form a structure resembling to its native form. Thus, the VP7 protein from simian rortavirus, which is antigenically similar to human rotavirus serotype 3, can induce neutralizing antibody in rabbits may be used for the development of vaccine against rotavirus-induced diarrhea in infants. Rotavirus genes of other serotypes can also be expressed by the baculovirus expression system for vaccine or other purposes.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

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