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Original Article

Peptide based polyclonal antibody production against bovine rotavirus non structure protein4 (NSP4)

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ABSTRACT

The rotavirus nonstructural protein 4 (NSP4), is a multi functional protein that play key role in both viral morphogenesis and cytopathic effect associated with cell death. However, the complete biological effect of NSP4 remains to be clarified. Since to obtain further knowledge about this protein there is a need for recognizing antibody and there is no commercial antibody against this protein, this study was designed to produce polyclonal antibodies against a synthetic immunogenic peptide of RF rotavirus NSP4 protein in order to be used as diagnostic and research tool. In the present study a peptide sequences corresponding to NSP4 114-135 conjugated to Bovine Serum Albumin (BSA) by glutaraldehyde through a single-step coupling protocol. The conjugated peptides were extensively dialyzed and injected to New Zealand white rabbits. The NSP4 114-135 peptide-specific antiserum was confirmed by both IF and Western blot analysis. The result indicated successful production of polyclonal antibody raised against native NSP4 protein. In conclusion, since NSP4 is a toxic protein for the cells, it is impracticable to express full length of NSP4 protein in expression systems. Therefore, the introducing immunogenic peptide in appropriate animal may be the best approach to produce its specific antibody.

Keywords: Rotavirus, NSP4, protein, peptide, polyclonal antibody

INTRODUCTION

Rotavirus is one of the most common causes of severe diarrhea in infants and young children throughout the world and more than 600,000 children under five years of age still die from rotavirus (Lorrot & Vasseur 2007, Malek *et al* 2010, Mukherjee & Chawla-Sarkar 2011). Rotaviruses are non-enveloped and belonging to the Reoviridae family, consist of three concentric layers of proteins and a core containing 11 genes that code for 6 structural proteins (VP1–VP4, VP6, and VP7) and 5 nonstructural proteins (NSPs) (Estes & Cohen 1989). It has been established that the rotavirus pathogenesis is related to the NSP4, which is encoded by gene segment 10 (Didsbury *et al* 2011, Lee *et al* 2000). NSP4 is a glycoprotein of 175 amino acids that has been proposed as the first explained viral enterotoxin. Intra-peritoneal delivery of either purified NSP4 or peptide derived from its cytoplasmic domain (amino acids 114–135) causes an age-dependent

diarrhea in a mouse model (Browne, Bellamy, & Taylor 2000, Didsbury *et al* 2011, Guzman & McCrae 2005). NSP4 has also been implicated in the cytopathic effect of rotaviruses that is associated with the inherent ability of NSP4, to disturb the calcium homeostasis of host cells (Newton, Meyer, Bellamy & Taylor 1997). Moreover, NSP4 has a role in assembly of the virus particle. It acts as an intracellular receptor on the Endoplasmic Reticulum (ER) membrane for newly synthesized double layer particles and mediates the budding of these particles with a transient envelope into the ER (Didsbury *et al* 2011). On the other hand, the induction of cell-mediated and humoral responses to NSP4 has been demonstrated in both humans and in animal models. Furthermore, it has been shown that antibodies to NSP4, reduced severity of diarrhea caused by rotavirus infection in newborn mice (Ball, Tian, Zeng, Morris & Estes 1996). Altogether, NSP4 is a complex protein that its biological effect is associated to viral pathogenesis and cell death. The mechanism of action for this protein has not yet fully understood. Therefore, there is a need for further studies to identify its functions. One of the most important tools for studying a protein is specific antibody, but there is no commercially antibody available against NSP4. In this regard, we designed this study to produce a polyclonal antibody against a synthetic peptide derived from amino acids 114-135 of the NSP4 protein as a tool for diagnostic and research applications.

MATERIALS AND METHODS

Peptide design and conjugation. A 22-mer synthetic peptide, with sequence composition of DKLTTREIEQVELLKRIYDKLT corresponding to NSP4 amino acids 114-135 of RF rotavirus was selected as an immunogen. The selected peptide was synthesized by GenScript Company and cross-linked to the carrier protein, Bovine Serum Albumin (BSA), by glutaraldehyde using a single-step coupling protocol (Reichlin 1980). Briefly, the peptide immunogen was coupled to BSA at a ratio of 100 nmol peptide: 1 nmol BSA by the addition of glutaraldehyde to a final concentration of 0.4%. The reaction was quenched by the addition of 1M glycine. The cross-linked peptides were extensively dialyzed against 10 mM phosphate buffer, pH 7.4 for 24h (The dialysis buffer was changed 3 times during the total dialysis process).

Immunization protocol. Two New Zealand White rabbits were immunized 4 times with two-week intervals for each injection. The rabbits were injected subcutaneously in 4-6 sites. In primary injection, rabbits were immunized with 0.50 mg of BSA-peptide conjugated in an equal volume of Freund's complete adjutant's emulsions. For the subsequent immunizations, 0.25 mg of BSA-peptide conjugates in an equal volume of Incomplete Freund's Adjuvant (IFA). The animals were bled 2 weeks after the last injection. Sera were collected from the rabbits and tested for reactivity with NSP4 protein by immunoflurecene (IF) and Western blotting assay.

Cell and virus. MA-104 cells (Embryonic rhesus monkey kidney cells) were grown as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine in the presence of penicillin G (100 U/mL), streptomycin (100 µg/mL) at 37°C in a 5% CO2 controlled atmosphere. To examine NSP4 expression in the context of a viral infection, MA-104 cells were plated at a density of 105 cells/cm2 on sterile glass coverslips, incubated at 37 °C in 5% CO2 for 24 h. Bovine rotavirus strain RF was activated with 5 µg/ml of trypsin at 37 °C for 1h. MA104 cell monolayer were prewashed with PBS and inoculated with activated rotaviros at a Multiplicity of Infection (MOI) of 10. The inocula were removed 1 h later and replaced with medium without FBS.

Immunofluorescence staining of rotavirus NSP4 protein. Subconfluent MA104 cells in 24-well tissue culture plates were infected with RF virus at a MOI of 10. At 8-10 h post-infection (hpi), cells were fixed with cold methanol for 10 min at -20 °C. Cells were permeabilized with 0.2% Triton X-100 for 10 min and then blocked with 1% gelatin for 30 min at room temperature. Duplicate wells were incubated with

rabbit serum raised against NSP4 peptide diluted at 1: 20, 1: 50, 1:80, and 1:100 in phosphate-buffered saline with Tween 20 (PBST) for 1h at room temperature. The wells were washed three times with PBST buffer and were incubated with FITC-conjugated goat anti-rabbit (AbCam, USA) diluted at 1: 1000 for 1 h at 37 °C, followed by rinsing three times with PBST and the stained cells then were analyzed by a fluorescent microscope.

Western blotting assays. At about 8 hpi, infected cells were recovered by scraping, washed twice with PBS and lysed in Tris-HCl buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (12.5% gel) and transferred into nitrocellulose (Newton *et al* 1997). The membrane was blocked in 3% non-fat skimmed milk in TBS plus 0.05% Tween 20 (TBS-T) for 1 h at room temperature, and was cut into strips. Strips were incubated with rabbit polyclonal antibody raised against NSP4(amino acids 114-135) diluted 1:200, 1:500 and 1:1000 in TBS-T at 4 °C overnight. The membranes were rinsed with TBS-T, reacted with goat anti -rabbit IgG conjugated with HRP (Sigma) diluted 1: 10,000 in TBS-T for 1 h at 27 °C, washed again and developed with diaminobenzidine (Roche, Germany).

RESULTS

Rabbit immune response against NSP4 peptide (114-135) NSP4 peptide was conjugated to BSA protein and the conjugated peptide was injected into rabbits. The production of rabbit polyclonal antiserum against native NSP4 protein and its titer was measured by direct Immunofluorescence and Western blot analysis. Immunofluorescence staining of RF rotavirus infected MA104 cells.To analyze the production of anti-body against RF NSP4 protein and for determination optimized titer of antiserum, IF assay was performed using different titer of antiserum against RF infected MA104 cells. Fluorescent stained cells indicated presence of antibody to NSP4 protein (Figure 1).

Western blot analysis of anti-NSP4 antibody. The reactivity of NSP4 amino acids 114-135 rabbit polyclonal antibody to its corresponding native protein was determined by Western blot analysis. The produced antibody recognized a sharp band of 26 kD represents the NSP4 protein of RF rotavirus (Figure 2).

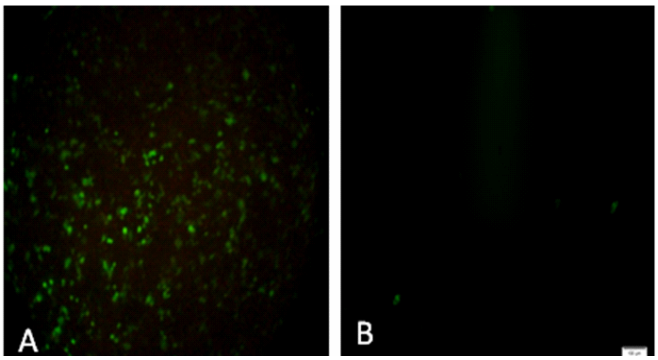

Figure 1. IF staining of RF rotavirus infected MA104 cells incubated with rabbit anti-NSP4 antibody diluted at 1:50 by florescent microscopy (× 100). A; MA104 cells were infected with RF rotavirus (moi = 10), B; uninfected MA104 cells (negative control).

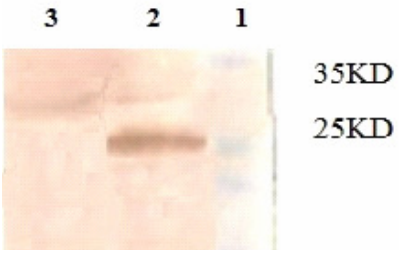

Figure 2. Western blot analysis of rabbit anti-NSP4 antibody by RF NSP4 expressed in MA104 cells. Western blotting carried out using rabbit anti peptide serum diluted 1:200 and HRP-conjugated goat anti-rabbit antibodies. Lane 1; molecular weight protein markers (Sinaclon, Iran), lane2; NSP4 protein (~ 26 KD), lane 3; uninfected MA104 cells as negative control.

DISCUSSION

Several cytopathic and enterotoxigenic properties of rotavirus infection has been associated with the NSP4. NSP4 dramatically increase cytosolic calcium in two different mechanisms. First, it is due to viroporin properties of this protein that leading to ER disruption and followed by increasing in cytoplasmic Ca+ concentration (Browne *et al* 2000, Diaz *et al* 2008, Hyser, Collinson-Pautz, Utama & Estes 2010). The second mechanism is related to enterotoxine properties

of this protein. Increasing evidence indicates that a phospholipase C (PLC)-mediated signaling cascade is activated when NSP4 or NSP4 (114–135) synthetic peptide is added to cells exogenously (Tian, Ball, Zeng & Estes 1996, Zhang, Zeng, Morris & Estes 2000). However, despite numerous studies, many aspects of NSP4 function, its role in viral pathogenesis, morphogensis and its- cell interactions remains to be elusive. Therefore, to obtain further knowledge about the NSP4 proprtice, its-protein interactions and the events leading to pathogenesis, production of antibody against it would be necessary. Because there is not any commercial antibody against NSP4 available in the market, in the present study, production of polyclonal antibody against a synthetic peptide of RF NSP4 protein was done. Generally, there are three conventional approaches to produce polyclonal antibodies against a protein. The first approach is to purify the protein and use it as an immunogen. However, this approach is not possible for minor proteins such as viral non structure proteins. The second approach is to express the cDNA in an expression vector with subsequent purification of the expression product. It has been reported that the membrane association properties of NSP4 make purification of this protein difficult (Sharifi 2005). Moreover it has been shown that expression of the rotavirus NSP4 has toxic effect on E.coli and leads to a decrease in the growth of the bacterium. Because of the lipid composition of the bacterial inner membrane is similar to the membrane of the ER. So that full length expression of NSP4 in *E. coli* expression system is impossible. The third approach is to synthesize selected oligopeptide sequences in the protein and then conjugate them to a carrier protein for immunization. The suitability of the use of synthetic peptides for the induction of antibodies has been demonstrated previously for several bacterial and viral proteins (Harari, Donohue-Rolfe, Keusch & Arnon 1988). Ball et al. (1996) reported the immunogenicity properties of the NSP4 114-135 peptide for the first time. They cross-linked this peptide to the carrier protein, keyhole limpet hemocyanin (KLH), by glutaraldehyde then inoculated it to rabbit. Afterward polyclonal antibody was raised against native NSP4 protein (Ball *et al* 1996). In the present study we used of BSA as a carrier protein for conjugating of NSP4 114-135 peptide and the same results was achieved. KLH and BSA have been most commonly used as carrier proteins. KLH is a protein derived from the mollusk Megathura crenulata, it is therefore a good carrier to use in mammals such as rabbits and mice (Vona-Davis *et al* 2004). However, KLH is more expensive than BSA, for this reason we preferred to use of BSA as a carrier protein. Because when an animal is immunized with a peptide/carrier conjugate, antibodies are also generated to the carrier protein, we used of gelatin and skim milk as blocking agent in IF and Western blotting assays respectively.

In conclusion, production of peptide based - polyclonal anti-body will be an effective approach, if immunogenic peptide be selected carefully.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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