Recombinant glycoprotein based vaccine for Chandipura virus infection

C.H. Venkateswarlu, V.A. Arankalle

Hepatitis Division, National Institute of Virology, Microbiological Containment Complex, Sus Road, Pashan, Pune 411021, India

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A B S T R A C T
Chandipura virus (CHPV) has emerged as an important pediatric encephalitis-causing pathogen with very high mortality in India. No specific vaccine or treatment is available till date. We attempted to prepare a candidate vaccine employing recombinant CHPV Glycoprotein (rGp). The Glycoprotein gene (G-gene) of CHPV was expressed using Baculovirus expression system. The rGp was purified by HPLC and used for mice immunization, 3 doses, and 4 weeks apart. One microgram rGp was found to be optimum. Seroconversion was observed as early as 2nd week by detecting anti-CHPV IgG antibodies. Antibody titres were immunogen-concentration dependent. Intracerebral challenge of the immunized mice with 100 LD₅₀ of the homologous strain demonstrated 90% protection. In vitro neutralization, antibodies from the immunized mice were able to neutralize heterologous viruses. There was 60% T cell proliferation observed against rGp in immunized mice. The study shows that rGp induces both arms of immune response and represents an ideal vaccine candidate for further evaluations.

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1. Introduction

Encephalitis caused by Chandipura virus (CHPV) has emerged as an important pediatric health problem in India as evidenced by the epidemics of the disease with high mortality in 2003 in the state of Andhra Pradesh (183/329, 55%) [1], in 2004 in the state of Gujarath (20/26, 78.4%) [2] and in 2007 in Maharashtra and Andhra Pradesh states (our unpublished observations). These fatalities occur within 48 h of appearance of clinical symptoms, majority being within 24 h. As a result, documentation of the presence of IgM antibodies to CHPV (IgM-anti-CHPV-IgM) remains infrequent and the diagnosis is mainly dependent on the detection of the viral RNA in the serum samples. Though the virus was first isolated from the sera of two patients during an outbreak of febrile illness in 1965 in India [3] and subsequently from a child with acute encephalitis in 1980 [4], the epidemic potential of CHPV causing encephalitis and high mortality in children was recognized only in 2003. CHPV is transmitted to humans by sandflies [5]. Retrospective studies revealed that the virus was highly prevalent at least since 1955 in India [3], Sri Lanka [6] and Africa (Nigeria, Senegal) [7,8]. During the epidemics of the disease [1,2] and one year study of sporadic encephalitis in children from endemic areas [9], it was clearly shown that CHPV infection is highly endemic in these areas. Detection of IgM-anti-CHPV among febrile cases indicate clinical spectrum of Chandipura infection. However, of the infected cases what proportion progresses to encephalitis is not clear. For obvious reasons, studies related to antiviral therapy or vaccine development were not vigorously pursued earlier. However, as CHPV has recently emerged as a serious localized problem in India, immunization of the children from the affected areas was considered to be helpful in preventing encephalitis associated rapid and high mortality.

CHPV is a member of the family Rhabdoviridae and genus Vesiculovirus. It is an enveloped, bullet shaped, single stranded RNA virus with genome size of approximately 11 kb [10]. Viral genome codes for five polypeptides, namely, Nucleocapsid protein N, Phosphoprotein P, Matrix protein M, Glycoprotein G and Large protein L. N protein encapsidates genomic RNA into a nuclease resistant form to protect it from cellular RNases. L with the help of P protein acts as a viral RNA dependent RNA polymerase (RdRp), Matrix protein glues the encapsidated genome RNA with the outer membrane envelope. G-protein spikes out of the membrane and acts as a major antigenic determinant [11–13]. Studies on Vesicular stomatitis virus (VSV) and Rabies virus (both belong to family Rhabdoviridae) revealed that the G-protein could be an excellent vaccine candidate [14–18]. Therefore, CHPV G protein was the immunogen of choice for vaccine development. We report here the utility of recombinant glycoprotein (rGp) expressed using Baculovirus expression system in protecting mice from the intracerebral challenge of the virus and propose this protein to be an ideal vaccine candidate for Chandipura encephalitis.

2. Materials and methods

2.1. Construction of recombinant plasmids

Complete G gene (1593 bp, position 2-1566, 524 aa) from supernatant of Vero E6 cell line infected with CHPV (isolate CIN034627, GenBank accession No: AY382603) representing a strain from

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* Corresponding author. Tel.: +91 20 26006323; fax: +91 20 25871895. E-mail address: varankalle@yahoo.com (V.A. Arankalle).
2003 epidemic in Andhra Pradesh was amplified by reverse transcription-PCR according to the protocol described earlier [1]. The primers used were: Forward primer 5′- ATG ACT TCA TGA ACA ATT AGT GTG ATCC 3′ and Reverse primer 5′- TCA TAC TCT GGC TCT CAT GTT GAA GGG CTT 3′. The resultant PCR product (~1.6 kbp) was TA-cloned in pGEM T Easy vector (Promega, Madison, USA) and sub-cloned in to pFAST Bac1 expression vector (Invitrogen, CA, USA) according to manufacturer’s instructions. The insert was confirmed by sequencing of both the strands using multiple primers and Big Dye Terminator cycle sequencing Ready Reaction Kit (version 3.1, Applied Biosystems, Foster city, CA) and an automated sequencer (ABI 3130xl, Applied Biosystems, Foster city, CA). In order to construct recombinant bacmid (rBac), recombinant pFAST Bac1 vector was transformed into maximum efficiency DH-10 Bac competent cells.

2.2. Expression of CHPV G-gene in Baculovirus expression system

rBac was transfected in Spodoptera Frugiperda (SF9) cells according to the standard protocol and after 72 h of post-transfection, pellet and supernatant were harvested. To determine the maximum yield and immunoreactivity of rGp at different time-points, the time course, fractions of both pellets and supernatants were collected at 12 h interval post-infection (PI) up to 96 h. All the fractions were subjected to ELISA for the detection of rGp as described later.

2.3. SDS-PAGE and Western blot

Supernatant and cell pellet of infected SF9 cells were harvested at 48 h post-infection. Cell pellets as well as supernatants were analyzed on 10% SDS-PAGE. Gels were either stained directly or blotted onto nitrocellulose membrane. Blots were blocked with phosphate-buffered saline (PBS), pH 7.4 containing 5% nonfat milk powder O/N. The blots were then probed with polyclonal IgG antibody conjugated to horseradish peroxidase (HRP). Blots were developed using diaminobenzidine and H2O2. 2.5% nonfat milk powder in PBS was used as diluent and PBS containing 0.05% Tween 20 was used for washing.

2.4. Purification of rGp

Fifteen milliliters of the rGp expressing SF9 cell supernatant was concentrated to 5 ml with low protein binding Amicon filters (Millipore Corporation, MA 01821, USA). 2.5 ml of rGp was loaded on HiPrep 16/60 sepharose Gel-filtration column (AKTA BASIC 100 HPLC system, Amersham Pharmacia, USA). Fractions of 0.5 ml were collected and coated at 1:10 dilution and screened for the presence of rGp by using anti-CHPV-IgG positive and negative sera for each fraction. The ELISA was carried out as described below. Fractions showing rGp activity were pooled and concentration of the purified rGp was measured by Lowry’s method.

2.5. Mice immunization

The purified rGp was used for immunization of mice (10 mice/group, Swiss albino, Female, 6–8 weeks old). Initially, four concentrations of rGp were used for immunization, i.e., 100 ng, 500 ng, 1 μg and 2 μg. One micrograms of rGp was adsorbed on 3.25 μg of AlPO4 (Sigma chemicals, St. Louis, MO, USA) by vortexing at a low speed for 1 h at RT followed by centrifugation at 5000 rpm for 5 min at RT. The pellet was re-suspended in 0.01 M PBS, pH 7.2 and diluted to the required rGp dose at the time of immunization. For further experiments, a total of 3 doses of rGp (1 μg/mice), 4 weeks apart, were used for immunization. Serum samples were collected periodically by retro-orbital plexus bleeding and subjected to ELISA for IgG-anti-CHP detection/quantitation as well as tissue culture based in vitro virus neutralization test (NT).

2.6. ELISA for the detection/quantitation of IgG-anti-rGp antibodies

Mice sera were screened for the presence of IgG-anti-rGp antibodies in ELISA employing rGp as a coating antigen. Infected SF9 supernatant containing rGp was diluted 1:10 in 50 mM carbonated buffer (pH 9.5) and coated as 100 μl/well. Following coating at 37 °C for 2 h, blocking solution (10% donor calf serum, 0.5% Tween 20, 0.5% gelatin) was added to each well and incubated at 37 °C for 30 min. After washing with wash solution (0.01 M PBS, 0.5% Tween 20), mice sera diluted 1:25 in blocking solution were added (100 μl/well) to different wells according to the protocol. A 1:25 dilution of pre-immune sera served as negative controls. Incubation was continued at the same temperature for 30 min. All the wells were washed three times and each well was added with horseradish peroxidase conjugated goat anti-mouse IgG (Sigma chemicals, St. Louis, MO, USA) at 1:10,000 dilution and incubated at 37 °C for 30 min. Unbound conjugate was removed by washing 4 times. The enzyme substrate (O-phenylenediamine and urea peroxide) was added (200 μl/well) and allowed to incubate at RT in dark for 8–10 min. The reaction was stopped by the addition of 4N H2SO4 and the absorbance was measured at 492 nm (Labsystems Multiskan MS, MTX Lab Systems Inc., Virginia, USA). A serum sample was considered to be reactive when the optical density (OD) value was ≥ cut-off value. The cut-off value for the anti-rGp IgG antibodies was calculated as mean OD values for the three negative controls multiplied by 3. The CHPV specific IgG antibody titers were determined for individual mouse from each group. The reciprocal of the highest dilution that had an absorbance ≥ than the cut-off value was taken as the IgG-anti-CHPV antibody titer. All the analyses were carried out on the geometric mean titers (GMTs) and log-transformed antibody titers with standard error.

2.7. Tissue culture based in vitro virus neutralization test (NT)

Initially, tissue culture infectious dose (TCID50) of CHPV stock (CIN034627 strain), propagated in Vero E6 cell line was determined. Equal volumes of mice serum and 100 TCID50 CHPV stock were mixed and incubated for 1 h at 37 °C. 100 μl of the above mixture was added on monolayer of Vero E6 cells incubated at 37 °C and observed periodically for 48 h for cytopathic effects (CPE). Antibody negative and positive controls and virus alone control were included in every test. When control wells demonstrated evidence of extensive replication of the virus, at 35–48 h, the wells were stained with amido-black. A sample was considered positive for neutralizing antibodies when no CPE observed in the corresponding well. The reciprocal of the highest dilution that showed no CPE in at least two wells out of four used was taken as the NT CHPV antibody titer.

2.8. Lymphocyte proliferation assay (LPA)

To assess the cell-mediated immunity, mice were sacrificed 2–3 weeks after the last dose by cervical dislocation and spleen cells were harvested. 1 × 106 cells/well were cultured in quadruplicates in 96-well flat bottom plate (Nunc, Denmark) in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% CO2. The positive and negative controls included cells stimulated with T cell mitogen phytohaemagglutinin (PHA) (Sigma chemicals, St. Louis, MO, USA) at the concentration of 10 μg/ml or with the medium alone respectively. Following dose–response experiment, 20 μg/ml rGp (optimum concentration) was added to the respective wells.
Fig. 1. A–C: (A) Depicts ELISA reactivity of the supernatant/pellet-derived rGp from the SF9 cells harvested at different time intervals. (B and C) Document SDS-PAGE of uninfected and recombinant baculovirus infected SF9 cells and supernatants collected at 48 h post-infection (B) and Immunoblot analysis of the same employing IgG-anti-CHPV antibody positive human serum (C). IgG-anti-CHPV antibody negative serum did not show any reactivity (photograph not shown).

for specific proliferation. Cells were pulsed at 96 h with 1 μCi of tritiated thymidine for 24 h. Cells were harvested onto GF/C filter (Whatman, UK) and the incorporated radioactivity was measured by β-counter (LKB Pharmacia, Sweden). The stimulation index (SI) was determined as ratio of counts per minutes (CPM) in the presence of rGp/CPM in absence of rGp. Mice with SI value ≥ 3 were considered to be the responders [19].

2.9. Statistical analysis

ELISA and NT titers were compared using t-test employing SPSS 9.0 software. Non-responders in each group were included in the analyses.

2.10. Intracerebral challenge of immunized mice with CHPV

The homologous strain of CHPV (CIN034627) was used as a challenge virus. The lethal dose 50 (LD50) of the stock was determined in 16–18 weeks old Swiss albino female mice by Reed and Munch method [20]. The immunized mice were bled periodically and challenged intracerebrally at 2 weeks after the 3rd dose with 100 LD50 of the virus. All the challenged mice were observed for 21 days.

3. Results

3.1. PCR amplification and cloning of G gene

The complete G-gene of CHPV was amplified by reverse transcription PCR and the product was TA cloned into pGEM T Easy vector and subsequently in the pFAST Bac1 expression vector. The orientation of the insert in both the vectors was checked with restriction enzymes and both the clones were sequenced completely. 100% identity was noted with the sequence of the same isolate deposited in the GenBank (accession No: AY382603).

3.2. rGp expression and characterization

SF9 cells were infected with CHPV-G-recombinant baculovirus and temporal expression of G protein was studied. ELISA was used to screen the presence of rGp in both cells and supernatants harvested at different time intervals of post-infection (12–96 h). The maximum expression of rGp was observed at 48 h PI and continued at significant levels thereafter (Fig. 1A). The majority of the rGp was detected in the supernatant. Both SDS-PAGE and Western blot showed the presence of a single band of expected size of approximately 60-kDa in the supernatant of the infected cells (Fig. 1B and C).

3.3. Purification of rGp

Fig. 2 depicts elution profile of the serum-free concentrate of the rGp positive SF9 cell culture supernatant loaded on gel filtration HPLC column. All the fractions were subjected to ELISA for rGp detection. Among these, only fractions corresponding to a single peak (Peak: 13.61) were scored as reactive. Similar to culture supernatant (Fig. 1), this rGp peak was confirmed by SDS-PAGE and Immunoblotting to be a single protein of approximately 60-kDa (expected size of rGp, data not shown). This protein was used for mice immunization.

3.4. Humoral immunity

Both pre- and post-immunization mice sera were subjected to ELISA and NT for the detection of anti-CHPV antibodies. Both assays
Fig. 2. Elution profile of the serum-free concentrate of rGp positive SF9 culture supernatant loaded on gel filtration HPLC column. Peak: 13.61 corresponded to rGp.

Table 1
Percent seroconversion in mice immunized with three doses of different concentrations of rGp at 4 weeks interval (0, 4 and 8 weeks). Antibody testing was done by both ELISA and NT prior to every immunogen dose and 2 weeks after the last dose.

<table>
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<tr>
<th>rGp concentration</th>
<th>Percent seroconversion</th>
<th>Pre-1st dose</th>
<th>Pre-2nd dose</th>
<th>Pre-3rd dose</th>
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detected antibodies as early as 2 weeks after 1st dose (Table 1). Pre-immunization sera were scored negative by both the tests. As compared to NT, ELISA detected seroconversion earlier when the dose of the immunogen was low (100 ng). Antibody response was immunogen concentration dependent, increasing gradually with dose from 100 ng to 1 μg. Both 1 and 2 μg gave comparable response. Percentage sero-conversion increased after each dose and the maximum (90%) sero-conversion was observed at 10th week of post-immunization, i.e., 2 weeks after the last dose, either 1 or 2 μg.

Fig. 3 compares anti-CHPV antibody titers by ELISA and NT at 2 weeks after the last dose. Similar to seroconversion rates, the antibody titers were function of the concentration of the immunogen used, 1 and 2-μg rGp producing comparable titers. The maximum titers in NT and ELISA were 1:320 and 1:1200 respectively. Neutralizing antibody titers were consistently lower than the ELISA titers. As evident from Fig. 4, irrespective of the dose of rGp used for immunization, the anti-CHP-antibody titers remained almost constant during the observation period of 6 months.

To assess the efficacy of the antibodies generated by immunizing with the 2003 isolate-derived rGp against the viruses isolated in 1965, 2004 and 2007 neutralization tests were performed employing different virus isolates (Table 2). Serum samples from five immunized mice were screened in NT with the homologus as well as heterologus CHPV isolates. An excellent cross protection was recorded; NT titers employing different isolates did not differ significantly (p = 0.423–0.510).

3.5. Cell mediated immunity

To assess the T cell-response, spleens from 10 mice from each group harvested at 2–3 week after the last immunization were used. The optimum concentration of rGp for the stimulation of spleen cell was found to be 20 μg/ml. Fig. 5 records stimulation indices for the
mice immunized with different doses of rGp. Similar to humoral immune response, the lymphocyte proliferative response increased with the dose of the immunogen, 60% mice immunized with 1 or 2 µg rGp responding with high SI values.

3.6. Intracerebral virus challenge experiments

An intracerebral challenge of 100LD₅₀ of the homologous strain yielded satisfactory results. Survival was directly proportional to the immunogen dose, 20 and 40% mice surviving with 100 and 500 ng doses respectively. Both 1 and 2 µg doses gave 90% protection. Mortality in control, unimmunized group infected with 100LD₅₀ virus was 100% (2–7 days). The surviving mice immunized with different doses of the rGp were healthy during the observation period of 21 days. The immunized mice succumbing to the challenge died at times similar to the controls. As evident from Table 3, an ELISA titer of 1:40 and NT titer of 1:20 was predicting protection against the intracerebral challenge. Overall, 1 µg purified recombinant G protein expressed employing Baculovirus Expression System was shown to be a promising candidate vaccine.

4. Discussion

This study reports utility of rGp of CHPV as the vaccine candidate for Chandipura encephalitis, a recently emerged disease with high mortality in children from three Indian states. As the disease progression is very rapid, 47% mortality within 24 h and 55% at 48 h of the appearance of clinical symptoms [1], development of a prophylactic vaccine was thought to be the priority over the evaluation of antivirals. Considering the documented immunogenicity of glycoproteins of two other rhabdoviruses, rabies [21] and VSV [22,23], glycoprotein of CHPV was the protein of choice. Our data demonstrates the efficacy of the recombinant glycoprotein produced employing the baculovirus expression system. It was pleasing to note that the protein was predominantly present in the supernatant of the SF9 cells. In the serum-free medium almost entire protein was the protein of our interest. The activity of rGp was present in a single peak of expected size when HPLC-based gel filtration was carried out. Thus, purification of protein was relatively easy and the yield was also high (Figs. 1A and 2).

Following confirmation of immunoreactivity of the rGp in ELISA and Western blot, mice were immunized with three doses of different concentrations of the protein (100 ng to 2 µg) and both humoral as well as cell-mediated responses were monitored. As low as 100 ng rGp was able to induce IgG-anti-CHPV antibodies and neutralizing antibodies in 50% each of the immunized mice, as evidenced by ELISA and NT respectively. Anti-CHPV antibody titers rose with increase in the immunogen dose. However, both 1 and 2 µg doses of rGp yielded comparable titers and 1 µg was considered the optimum concentration of the immunogen. We were able to demonstrate T cell responses in immunized mice (rGp concentration dependent) with a maximum of 60% response with 1 or 2 µg doses. Thus, the recombinant protein generated both arms of immunity. However, we need in-depth studies employing better tools to clarify the cell-mediated immune responses during vaccination and disease.

Based on the results of intracerebral challenge with 100LD₅₀ of the homologous virus strain, utility of rGp as vaccine candidate for CHPV infection was obvious. With both 1 and 2 µg dose schedule, 90% mice survived the challenge of the virus. The two mice not protected by the vaccine died on 4 (1 µg) and 7 (2 µg) days post-infection; with 500 ng rGp five mice died during an interval of 2–7 days post-infection, similar to unimmunized controls. It is pertinent to note here that rabies glycoprotein sub-unit vaccine at as low dose as 360 ng could offer protection to 87.5% immunized mice after intracerebral challenge [24].

When anti-CHPV antibody titers as determined by NT or ELISA were compared with the protection offered against the intracerebral challenge of CHPV in mice, it was estimated that an ELISA titer of 1:40 or NT titer of 1:20 could be considered protective. Though CHP viruses isolated so far are closely related to each other [25,2 and our unpublished observations], we used viruses isolated during 1965, 2004 and 2007 epidemics, i.e., all the viruses isolated so
far, for in vitro NT for estimating neutralizing antibody titers in mice immunized with rGp based on 2003 strain. The homologus strain (2003) was used as the standard virus. With all the different viruses used, similar neutralization titers were obtained, suggesting protection against all the known strains of CHP virus. Percent nucleotide and (amino acids) homologies with the 2003 strain were 99.0 (99.2, 2003 strain, 98.1 (99.2, 2007 strain) and 96.3 (98.0, 1963 strain) (our unpublished data). Thus the G gene was conserved among the viruses isolated during 1965–2007. Experiments using different strains of Chandipura viruses, though closely relate to each other are under progress.

These encouraging results make us believe that rGp expressed using baculovirus expression system will provide an excellent candidate vaccine for controlling Chandipura encephalitis with high mortality in children from the endemic areas from India and should be immediately subjected to the required evaluation protocols.

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