Virus Research 167 (2012) 236–246

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Subunit vaccine formulations based on recombinant envelope proteins of Chikungunya virus elicit balanced Th1/Th2 response and virus-neutralizing antibodies in mice

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ARTICLE INFO

Article history:
Received 24 February 2012
Received in revised form 8 May 2012
Accepted 9 May 2012
Available online 17 May 2012

Keywords:
Chikungunya vaccine
Envelope protein
Humoral response
Cell mediated response
Cytokine profiling

ABSTRACT

The recent resurgence of Chikungunya virus in India and Indian Ocean Islands with unusual clinical severity is a matter of great public health concern. Despite the fact that CHIKV resurgence is associated with epidemic of unprecedented magnitude, none of the vaccine candidate has been approved so far. The envelope protein E1 and E2 being the major immunodominant structural proteins with crucial role in virus attachment and entry, can prove to be potential vaccine candidates. In the present study, the immunogenic potential of bacterially expressed CHIKE1 and CHIKE2 recombinant proteins along with various adjuvants is reported. Assessment of the protective efficacy of both the vaccine formulations was further confirmed by both in vitro and in vivo neutralisation tests. Splenocytes from immunized mice, cultured in vitro when stimulated with the vaccine antigens revealed induction of very high levels of both pro- and anti- inflammatory cytokines indicating a balance of Th1 and Th2 response.

1. Introduction

Chikungunya is a debilitating viral illness that is becoming a disease of global concern due to its escalating outbreaks in different parts of the world particularly in Africa and South East Asia. Chikungunya disease was first recognized in the form of an epidemic in East Africa during 1952–53 (Jupp and McIntosh, 1988). Since then, a large number of outbreaks have been reported (Dash et al., 2007; Schuffenecker et al., 2006). The disease is caused by Chikungunya Virus (CHIKV), which is classified in the family *Togaviridae*, genus *Alphavirus*. CHIKV is transmitted by the bite of infected *Aedes aegypti* and *Aedes albopictus* (Strauss and Strauss, 1994). The emergence and sustained circulation of Chikungunya has driven the interest of the scientific community to this long neglected tropical disease (Enserink, 2006; Charell et al., 2007).

Chikungunya infection is characterized by a triad of fever, rash and arthritis and has an approximate incubation period of 1–2 weeks (Johnson and Peters, 1996). Other symptoms include myalgia, headache, muscle aches and retro-orbital pains (Dash et al., 2007). In 2006 outbreak of La Reunion, severe forms of Chikungunya infection were observed in adults like encephalopathy and haemorrhagic fever as well as mother-to-child transmission of CHIKV (Couderc and Lecuit, 2009; Gerardin et al., 2008). Chikungunya disease is rarely fatal but is associated with significant morbidity. While the acute febrile phase of the illness resolves within a few days, the joint pain may persists for months to years causing serious economic and social impact on both the individual and the affected communities. Unfortunately, there is no specific treatment, approved vaccine or drug available for CHIKV infection, despite the improvements of vaccine trials for other viruses that co-circulate with Chikungunya such as Dengue. This could be due to either poor understanding of pathogenesis of CHIKV or lack of proper animal model to study immune response and protection that has hindered the CHIKV vaccine development.

Many vaccines effective against CHIKV infection have been reported previously. Formalin based preparation of CHIKV vaccine was found to be immunogenic in human, monkey and mice (Edelman et al., 1979; Harrison et al., 1971; Nakao and Hotta, 1973; Tiwari et al., 2009). However the growth and production of large quantity of CHIKV antigen is a major constrain as it requires appropriate BSL-3 containment facility. A live attenuated CHIKV vaccine (TSI-GSD-218) was also reported to be effective but caused side effects in clinical trials (Levitt et al., 1986; Edelman et al., 2000). DNA based CHIKV vaccine encoding viral structural protein was shown to be immunogenic in mice (Muthumani et al., 2008) but in general the DNA vaccines are not effective in generating the strong humoral response and neutralizing antibodies which is crucial for virus clearance. Chimeric CHIKV vaccines (Wang et al., 2008) comprises various safety issues as the Alphaviruses can recombine and have ability to generate replication competent virions (Strauss and Strauss, 1997).

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0168-1702/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2012.05.004

However, protein subunit vaccines that employ only a portion of virus are safe, easy to produce, easy to scale up and economically useful than live vaccines. Moreover, production of such vaccines involves low cost when compared to inactivated or virus-like particle (VLPs) based vaccines. To date no report is available about the immunogenicity of characterized envelope proteins of CHIKV. Since the efficacy of the protein/peptide based vaccines is determined by the strength of the humoral response in association with Th1/Th2 response (as there is no CTL activation), the present study was undertaken to evaluate the immunogenic potential of recombinant envelope proteins of CHIKV with special emphasis on induction of neutralizing antibodies that are reported to play an effective role in CHIKV infection (Couderc et al., 2009). This study not only elucidated the suitability of above proteins as attractive vaccine candidates but also explained the immuno-modulatory potential of CHIKV surface proteins that has never been evaluated before. The study demonstrated that the subunit vaccine formulations based on envelope proteins of CHIKV are capable of eliciting strong humoral and cell mediated response that was confirmed through high neutralizing antibody titres, elevated level of IgG1, IgG2a, IgG2b and upregulation of Th1/Th2 cytokines (TNF-α, IL-10, IL-12, IL-6, etc.).

2. Materials and methods

2.1. Cells and viruses

An Indian strain of CHIKV DRDE-06 (Genbank accession no: EF-210157) of ECSA genotype was used in the present study (Dash et al., 2007). Other CHIKV strains A226V (DRDE-07) mutant (Santhosh et al., 2008) and African prototype strain of CHIKV S-27 (AF369024) were used for cross neutralization study. The virus was isolated during 2006 epidemic from a confirmed human patient in BHK-21 cells and was further passaged in Vero cell line (up to 5 passages) to increase the adaptability and the viral titre. The titre was analyzed by plaque assay and virus was maintained at −80 °C for further use. The cells were obtained from a certified master cell bank at National Centre for Cell Sciences, Pune, India, and were maintained in Eagle's Minimal Essential Medium (Sigma, St. Louis, MO, USA) supplemented with 2.1 g sodium bicarbonate/l, 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine (Sigma, St. Louis, MO, USA).

2.2. Recombinant protein

The E1 and E2 gene fragment from ECSA strain (DRDE-06) were cloned into a pET28b$^+$ vector bearing a 6x histidine tag and expressed in *E. coli BL21(DE3)* strain. The gene fragments were amplified by using primers 5′-***aaccatggcc***ccgccctttggc-3′ (rCHIKE1 forward, position 10,561–10,572) and 5′-***aactcgag***gtgcctgctgaacgacac-3′ (rCHIKE1 reverse position 11,293–11,310) that amplified 764 bp long C-terminal region of E1 gene. For E2, 5′-***aaccatggcc***agcaccaaggaca-3′ (rCHIKE2 forward, position 8542–8554) 5′-***aactcgag***cgctttagctgttc-3′ (rCHIKE2 reverse, position 9796–9810) were used that amplified 1254 bp of the full E2 gene. Primers were designed against the CHIKV genome sequence (EF210157). Bold and italicized segment shows nonspecific 5′-overhang added to incorporate Nco I and Xho I site in the forward and reverse primers respectively.

The recombinant proteins were purified under denaturing conditions from inclusion bodies by immobilized affinity chromatography and were recognized through western blotting using monoclonal antibody specific for the 6x histidine tag as well as the antibody raised against native CHIKV antigen in rabbit as described earlier (Tiwari et al., 2009). Briefly, the native CHIKV antigen was purified by density gradient centrifugation and immunized in rabbit with multiple boosters. The sera harvested was analyzed for the titre, IgG fraction was purified and used for further experiments. The endotoxin levels were measured by Limulus amoebocyte lysate (QCL-1000, Cambrex Bioscience Walkersville Inc., MD, USA) kit using chromogenic end point method as recommended by the manufacturer. ActiClean Etox endotoxin removal column (Sterogene Bioseparations Inc., Carlsbad, CA) was used to remove the endotoxin from the proteins. This column effectively removed endotoxin from purified proteins. The endotoxin content of purified proteins was adjusted to <10 EU/mg of protein, as determined using standard LAL assay. Additionally, native CHIKV antigen was prepared by previously described protocol (Gould and Clegg, 1991) and inactivated by Tween–Ether extraction protocol (Eckels et al., 1970).

2.3. Formulation of rCHIKE1 and rCHIKE2 antigen with adjuvants

The antigens (rCHIKE1 and rCHIKE2) were formulated with three adjuvants: Freunds complete adjuvant (FCA, Sigma, St. Louis, MO, USA), Alum (Sigma, St. Louis, MO, USA) and Montanide ISA720 (Seppic, Puteaux, France) according to manufacturers' instructions. Though the FCA and montanide have not been licensed for human use but in this study these two adjuvants are included in order to validate and authenticate the immune response of viral proteins. In case of FCA one part of adjuvant was mixed with one part of antigen (v/v). For montanide ISA720, 2.3 parts of adjuvant was mixed with one part of antigen (70:30, w/w). The contents were mixed by Leur-lock syringes till an emulsion was formed. The pH of alum (13 mg/ml) was first adjusted to 7.0 with NaOH and then mixed with antigen in the ratio of 1:1 (v/v). The formulation was incubated for 6 h at 4 °C on a gently rocking platform, and centrifuged at 3000 rpm for 10 min. The supernatant was checked for the presence of any un-adsorbed protein. More than 90% of the antigen was found adsorbed to alum. The pellet was resuspended in normal saline and used for immunization.

2.4. Immunization of BALB/c mice

Immunogenicity of all the formulations with FCA, montanide ISA720 and alum was tested in BALB/c mice. Three groups of BALB/c mice ($n = 6$) were immunized subcutaneously with 40 μg of antigen formulated with different adjuvants mentioned above, three groups were administered with adjuvants alone (as adjuvant control) and one group was immunized with antigen alone with out any adjuvant. The total volume used per mouse at each immunization was 100 μl. Priming was followed by two booster immunizations on days 21 and 35. The animals were handled as per the guidelines of the institutional animal ethical committee. Blood was collected from retro-orbital plexus with heparinized capillaries on days 0, 14, 28, 42 and 56. Serum was separated from all the samples and was stored at −80 °C until used.

2.5. Evaluation of humoral immune response

2.5.1. Antibody titre

Sera collected at various time points from mice (test and control groups) were tested for the recognition of their respective antigen by ELISA. Microtitre ELISA plates (Nunc, Roskilde, Denmark) were coated with rCHIKE1 or rCHIKE2 (200 ng/well) in coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plates were washed three times with phosphate buffered saline containing 0.05% Tween 20 (PBS-T) and blocked with 200 μl of 5% bovine serum albumin in PBS-T for 2 h at 37 °C. Serially two fold diluted test sera in PBS-T starting with 1:100 were incubated in triplicate wells (100 μl/well) at 37 °C for 1 h. The wells were washed three times with PBS-T. Goat anti-mouse (IgG) antibody conjugated to horseradish peroxidase (HRP, Sigma,