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TLR3 mediated innate immune response in mice brain following infection with Chikungunya virus

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ABSTRACT

Chikungunya virus (CHIKV) has received global attention due to the series of large-scale outbreaks in different parts of the world. Many unusual clinical severities including neurological complications and death were reported in recent outbreaks. The mechanism underlying the host immune response to CHIKV in the brain is poorly characterized. In this study, the neuropathogenesis of CHIKV with E1:A226 V mutation was elucidated in 1 week old BALB/c mice. The virus was found to replicate in mice brain with peak titer of 10^4 on 6th day post infection. Immunohistochemical analysis revealed preferential virus localization in neuronal cells of cerebellum. The expression profiling of TLR, antiviral genes and cytokines in mice brain revealed significant up regulation of TLR3, TRAF-6, TICAM-1, MCP-1, CXCL-10, IL-6, IL-4, ISG-15, MX-2, IFN-β, OAS-3 genes that ultimately resulted in virus clearance from brain by day 9–10 suggesting activation of innate immune pathway. Further the effect of poly I: C (Polyinosinic: Polycytidylic acid), a TLR-3 agonist and potent IFN inducer on CHIKV neuropathogenesis was studied. Pretreatment of mice with Poly I: C caused reduction of CHIKV titer in brain and offered 100% protection of animals. The protection was mediated by an increased induction of TLR3, IFN-β and antiviral genes in mice brain. Our result demonstrates that pre immune stimulation of animals by Poly I: C is effective inhibitor of CHIKV replication and might be a promising prevention agent against this virus.

1. Introduction

Chikungunya virus (CHIKV) has been responsible for large scale epidemics with serious economic and social impact during the last 6 years. Transmitted by *Aedes* mosquitoes it causes Chikungunya fever, an acute illness in patients with a stooped posture often associated with chronic and incapacitating arthralgia (Powers and Logue, 2007). Several outbreaks of disease have been reported in Asia and Africa over the last 60 years. In the recent outbreaks of Indian Ocean islands that took place in the year 2005–2007, this virus attained special attention in the global scientific community (Chandak et al., 2009; Economopoulou et al., 2009). These outbreaks came up with the adaptability of the virus to a new vector that caused exceptional forms of disease as well as severe complications (Hoarau et al., 2010) such as persistent arthralgia, destructive arthritis, cardiologic manifestations, hepatitis, neurological complications (characterized by meningitis, parenchyma meningitis, Guillian–Barre syndrome, external opthalmoplegia, optic neuritis and neuroinflammation) and deaths (Economopoulou et al., 2009; Lemant et al., 2008; Nouranifar et al., 2003; Pialoux et al., 2007; Robin et al., 2008; Sissoko et al., 2009; Tournebize et al., 2009). For the first time mother to child transmission was also reported during these outbreaks (Gerardin et al., 2008; Ramful et al., 2007). The reason for the neurological complications is still not known but might be due to the occurrence of several novel genetic changes in newer strain as compared to prototype strain.

CHIKV mediated neurological disorders have been reported in many recent studies (Ernould et al., 2008; Rampal and Meena, 2007; Robin et al., 2008). Clinical evidences suggest that CHIKV spreads to the CNS in the case of severe infection, especially in neonates, young children or in old aged people with poor immunity.CHIKV reaches the CNS exclusively via the choroid plexus route and

Abbreviations: CPE, Cytopathic effect; M.O.I, multiplicity of infection; TPB, tryptose phosphate buffer; MEM, minimal essential media; PFU, plaque forming unit; RIPA, radioimmunoprecipitation assay; TLR, Toll like receptors; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; TRAF, TNF receptor associated factor; TICAM, Toll-IL-1 receptor containing adapter molecule; IRF, interferon response factor; ISG15, interferon stimulated gene; MX-2, myxovirus resistance; OAS-3, 2′-5′-oligoadenylate synthetase; qPCR, real time PCR; Poly I:C, polyinosinic: polycytidylic acid; MCP, monocyte chemoattractant factor.

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undergoes amplification at the ependyma and leptomeningeal (Couderc et al., 2008). CHIKV has been detected in CSF in patients affected by severe CHIKV disease associated with CNS disease (Grivard et al., 2007). CHIKV infects mouse brain cells, replicates in primary brain cell mix cultures (Chatterjee and Sarkar, 1965; Precious et al., 1974) and infects neuronal (Dhanwani et al., 2012; Priya et al., 2013) and Glial cell line (Abere et al., 2012; Abraham et al., 2012). Inspite of these evidences this virus has never been considered as a 'true' neurotropic virus. Subcutaneous inoculation of newborn mice with CHIKV leads to invasion and replication of virus in brain and further clearance by 9–10 dpi (Ziegler et al., 2008) confirming involvement of immune response. The role of innate immune defense has been explored in case of CHIKV and other alphaviruses infection (Her et al., 2010; Morrison et al., 2006; Ng et al., 2009; Priya et al., 2013; Schilte et al., 2010; Sharma and Maheshwari, 2009; Wauquier et al., 2011) however, its role in neuropathogenesis of this virus has not yet studied. The neonatal mice serve as an established animal model for the study of the CHIKV pathogenesis. In this study, the upregulation of TLR3 and other associated genes in brain of 1 week old mice in response to CHIKV infection have been reported. It is also elucidated that Poly I:C (Polyinosinic: polycytidylic acid) treatment before CHIKV infection resulted in enhanced adaptive and innate immune response without exacerbating the disease resulting in reduction of virus titer and more rapid clearance from brain.

2. Materials and methods

2.1. Virus propagation

The CHIKV strain (DRDE-07) used in this study was originally isolated from the serum of patient from Kerala (India) during 2006–2007 outbreaks (GenBank Accession No. – EU372006; Santhosh et al., 2008). In addition to E1:A226V mutation, the virus was having four unique amino acid substitutions, two from nonstructural (Nsp1: T128K and T376M) and two from structural proteins (Capsid: P23S and V27I) as compared to S-27 prototype Chikungunya virus strain. The virus was isolated in BHK-21 cell line as per published protocol (Yamada et al., 2002). Briefly delta tubes (Nunc, Denmark) containing monolayer of BHK-21 were adsorbed with 0.2 ml of plasma samples (diluted 1:10). The inoculum was then replenished with 2 ml of maintenance media after 2 hpi (hour post infection). The cells and supernatant was harvested on appearance of CPE (cytopathic effect) and virus was confirmed by RT-PCR. The aliquots of supernatant were used for 3 passage in BHK-21 cells and stored at −80 °C. For the present study the virus at m.o.i (multiplicity of infection)-1 was passaged twice in C6/36 in MEM (Minimal essential media) plus TPB (tryptose phosphate broth) media supplemented with 2% fetal calf serum at 37 °C in 5% CO_2 incubator and aliquots of supernatant were stored at −80 °C. For mock infection culture supernatant from healthy C6/36 cells (without virus) was used.

2.2. Ethic statement

Inbred BALB/c mice of 1 week old of either sex along with mother were taken from the animal house facility of DRDE, Gwalior, India. They were housed in polypropylene cages with sterilized paddy husk as the bedding material. The paddy husk was changed at alternate day. The animals were kept in environmentally controlled room (25 ± 2 °C; relative humidity 40–60%). Animals were provided with pellet diet (M/s Ashirwad brand, Chandigarh, India) and water ad libitum. All animal experiments were carried out strictly following the approved study protocol of Institutional Animal Ethics Committee (IAEC) of Defence Research Development Establishment (DRDE), Gwalior, India that is registered with committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forestry, Government of India (Regd. No. – 37/GO/c/1999/CPCSEA).

2.3. Virus infection

The study protocol was approved by Institutional Animal Ethics Committee (IAEC) vide approval no. Viro/07/50/MMP, dated 16 July, 2012. Animals were handled in strict accordance with good animal practice as defined by CPCSEA. Inbred 1 week old BALB/c mice were used in this study. Mice of either sex were inoculated with 10^3 PFU of CHIKV in 30 μl of PBS (Phosphate Buffered saline) through subcutaneous route on the loose skin at back. For mock infection culture supernatant of C6/36 cells without virus diluted in 30 μl of PBS was injected in animals. Mean body weight, mortality rate and clinical symptoms like hair loss, hind limb paralysis, lethargicity and in coordination in movement were observed and recorded daily for 10 days. In some experiments, mice were inoculated intracranially with 10^3 PFU (plaque forming unit) of CHIKV in 30 μl of PBS in right cranium.

2.4. Viral titer determination by plaque assay

To determine the virus titer in mice brain, animals were perfused by cold PBS and sacrificed at 3, 6 and 9 dpi to harvest brain tissue. The brain tissue was homogenized in MEM to produce 10% (wt/vol.) homogenate, centrifuged at 10,000 × *g* for 30 min at 4 °C and filtered through 0.22 μm sterile filters. The lysate was serially diluted and assayed by plaque formation on Vero cells. Virus titer was calculated as the number of plaques forming unit/ml of brain lysate.

2.5. Detection of CHIKV RNA copy number in brain by Real time PCR (qPCR)

The viral RNA was extracted from brain tissue homogenate from control and infected mice by using QIAamp viral RNA minikit (Qiagen) according to the manufacturer's protocols. The viral RNA was eluted from the QIAspin columns in a volume of 30 μl of elution buffer and was stored at −80 °C until it was used. The RNA was reverse transcribed, amplified by Real time PCR assay and Ct value was calculated by using the primers (F3-B3) targeting 205 bp of E1 gene of the CHIKV genome. The RNA copy number was determined by the Ct value by using the standard curve given earlier (Santosh et al., 2007) and calculated as the RNA copy number/ml of brain lysate.

2.6. Detection of CHIKV protein by western blotting

Total protein was extracted from brain tissues of CHIKV and mock infected group. For this brain tissues were homogenized in RIPA (Radioimmunoprecipitation assay) buffer (50 mM Tris HCl pH-7.4, 150 mM NaCl, 1%Triton X 100, 0.5% Sodium deoxycholate, 0.1%SDS, 1 mM EDTA, protease inhibitor cocktail) and centrifuged at 12,000 × *g* for 20 min at 4 °C. Protein concentration was quantified in tissue supernatant by Bradford Protein Assay kit (Bio-Rad, USA) as per manufacturer's instructions. Equivalent amounts of the tissue lysate (50 μg) were subjected to 12% SDS-PAGE and then transferred to PVDF – immobile membrane (Millipore, Bedford, MA, USA). The membrane was incubated with E2 monoclonal antibody (Kumar et al., 2012) in dilution of 1:500 at 4 °C overnight. The membrane was thoroughly washed 6 times with PBST and incubated with Anti mice HRP conjugated antibody (Sigma, St. Louis, USA) for 60 min at room temperature (R/T). After washing 6 times with PBST bound antibody was detected by chemiluminescence using an ECL

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