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A neutralization assay for chikungunya virus infections in a multiplex format

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A B S T R A C T

Chikungunya virus (CHIKV) is a mosquito-transmitted Alphavirus that causes chikungunya fever and has infected millions of people mainly in developing countries. The associated disease is characterized by rash, high fever and severe arthritis that can persist for years. Since the epidemic on La Réunion in 2006, CHIKV has adapted to *Aedes albopictus*, which also inhabits temperate regions of the eastern and western hemispheres, including Europe and the United States. *A. albopictus* might continue migrating north with continuing climate change and CHIKV would then no longer be confined to the developing nations. No treatment or licensed CHIKV vaccine exists. A CHIKV neutralization assay in a 384-well format by using CHIKV-pseudotyped lentiviral vectors was established. This assay system can be used for entry inhibitor screening under a reduced safety level (S2). Production of CHIKV-pseudotyped lentiviral vectors and the reaction volume are optimized. A dose dependent, specific neutralization of CHIKV-pseudotyped vectors with sera of CHIKV-infected individuals could be measured in a 384-well format. A safe and simple multiplex assay for the analysis of CHIKV neutralizing activities was developed and will be able to improve drug and vaccine development as well as it would improve the understanding of CHIKV epidemics regarding antibody responses.

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1. Introduction

Chikungunya virus (CHIKV) is a mosquito-transmitted Alphavirus that causes chikungunya fever in humans. Most CHIKV infections are symptomatic, with an incubation period of 2–4 days. The disease is characterized by a sudden onset of fever, headache, malaise, arthralgias or arthritis, myalgias, and lower back pain. The term 'chikungunya' means 'that which bends up' in the Kimakonde language of Mozambique and describes the disease phenotype. After the acute phase, polyarthritis can be recurrent and may persist for several years after infection. These factors raise a serious public health concern and contribute to a significant economic cost during large outbreaks.

CHIKV was first isolated from the blood of a febrile patient in Tanzania in 1953, and has since been identified as the cause of numerous human epidemics in many areas of Africa, Southeast Asia and on the Indian subcontinent (Thiboutot et al., 2010; Schwartz and Albert, 2010). *Aedes aegypti* has been the primary CHIKV vector in Asia, but in La Réunion, *Aedes albopictus* (the Asian tiger mosquito) was the primary vector (Solignat et al., 2009). During this epidemic, CHIKV adapted effectively to *A. albopictus*, which also inhabits temperate and even cold temperate regions of the eastern and western hemispheres, including Europe and the United States (Medlock et al., 2012; Rochlin et al., 2013). *A. albopictus* might continue migrating north with continuing climate change and CHIKV would then no longer be confined to the developing nations. A further risk of introducing CHIKV into previously non-endemic areas exists from travelers with viremia, leading to local transmission of the virus, especially in tropical or subtropical areas of the United States and also in southern Europe (Rezza et al., 2007). As a result, the NIAID has designated CHIKV as a Category C pathogen alongside the influenza and SARS-CoV viruses. There is no specific treatment

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for chikungunya fever and care is only supportive, based on the symptoms. No licensed CHIKV vaccine exists.

CHIKV is a (+) single stranded (ss)RNA virus and belongs to the Alphavirus genus in the *Togaviridae* family. Alphaviruses enter cells by receptor mediated endocytosis and a subsequent pH-dependent fusion step. CHIKV has three surface proteins: the two transmembrane glycoproteins E2 and E1, and the mainly secreted E3 protein, which presumably facilitates secretion of the E2 and E1 glycoproteins (Mayne et al., 1984; Snyder and Mukhopadhyay, 2012). E1 is a class II viral fusion protein and E2 most likely mediates cell attachment, however the cellular receptor is still unknown. E2 and E1 associate as trimers of heterodimers (E2–E1) on the particle surface (Voss et al., 2010).

The early steps of infection, such as receptor binding and membrane fusion, are carried out solely by the viral glycoproteins. Additionally, the glycoproteins are the major target of neutralizing antibody responses. A tool used to investigate the glycoproteins of viruses is the pseudotyping of vectors with desired glycoproteins. With this strategy, the vectors incorporate a heterologous viral glycoprotein and thereby acquire the host range of the virus the glycoprotein is derived of. They enable studies without the need of using the native virus, which usually requires a higher safety level. Pseudotyping of retro- or lentiviral vectors is frequently used to study viral entry and to evaluate entry inhibitors (Siegert et al., 2005), or to harvest neutralizing antibodies at reduced safety levels (Schnierle et al., 1997; Seaman et al., 2010; Dervillez et al., 2010). It has been previously described that lentiviral vectors can be pseudotyped with CHIKV E1/E2 and transfer the CHIKV host range to these vectors (Salvador et al., 2009; Akahata et al., 2010; Kishishita et al., 2013). This vector system was used to create a reduced safety level assay to analyze sera of CHIKV-infected individuals for their neutralizing activity, and thereby established a multiplexed assay in 384-well format for the analysis of compounds able to inhibit CHIKV entry.

2. Materials and methods

2.1. Cell culture

All cells used in this study were cultured at 37 °C under 5% CO_2. HeLa (ATCC: CCL-2), BSC-1 (CCL-26), HEK 293T (CRL-1573), NIH 3T3 (CCL-92), HT 1080 (CCL-121), Huh7 (CCL-185), HepG2 (HB-8065) and A549 (CCL-185) cells were grown in Dulbecco's modified Eagle medium (DMEM; Lonza, Verviers, Belgium). Renca (CRL-2947), HaCat (Boukamp et al., 1988), MCF7 (HTB-22), BHK 21 (CCL-10), Jurkat (TIB-152), HuT78 (TIB-161), Bjab (Kruger et al., 1996) and PM-1 (derived from HuT78 by Lusso et al. (1995)) cells were incubated in Roswell Park Memorial Institute medium (RPMI; Biowest, Nuaille, France). RK13 (CCL-121) and MRC5 (CCL-171) cells were cultured in Eagle's minimal essential medium (EMEM; Biochrom, Berlin, Germany). All media were supplemented with 10% FBS (v/v; PAA, Pasching, Austria) and 5% L-glutamine (200 mM; Lonza, Verviers, Belgium).

2.2. Human sera

Human sera CHIKV 1–3 and DENV 1–3 were obtained from the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany. The serum CHIKV 4 was obtained from the Robert-Koch-Institute, Berlin, Germany. The human naïve serum (Nr. 032) was obtained from a healthy volunteer. All human sera used were taken with consent of the patient for diagnostic purposes according to ethical regulations in Germany.

2.3. Plasmids and DNA

The gene for the CHIKV E3-E1 envelope polyprotein was synthesized by GeneArt (Life Technologies, Darmstadt, Germany) on the basis of the "S27-African prototype" sequence (codon-optimized for the expression in mammalian cells). The gene was cloned *via* blunted ends (*Pac*I and *Asc*I) into the plasmid pIRES2-eGFP (digested with *Sma*I; Clontech/Takara, 78100 Saint-Germain-en-Laye, France). Furthermore, the plasmids pMDLg/pRRE, pRSVrev, pRRLsinCMV-GFPpre (Dull et al., 1998), pCSII-Luc (Agarwal et al., 2006) (kind gift of N. Somia to R. König), pHIT-G (encoding VSV-G; Soneoka et al., 1995) and pGaLV TM (encoding a modified GaLV Env; Stitz et al., 2000) were used for the production of vector particles.

2.4. Polyethylenimine solution (PEI)

For preparation of PEI transfection solution, 5 g of polyethylenimine were mixed with 5 ml purified water. Afterwards, another 10 ml of purified water were added. Following shaking and total dissolving of the PEI, 0.69 ml of this mixture were added to 39.5 ml of purified water. Then, 9.5 ml of this solution were mixed with another, 35 ml purified water. The pH was adjusted to 7.0 with 1 N HCl. Subsequently, the PEI solution was filled with purified water up to a total weight of 50 g, mixed well, sterile filtered (0.22 μm filters, Sartorius), aliquoted and stored at −20 °C (Boussif et al., 1995).

2.5. Lentiviral vector particle production

HEK 293T cells were seeded in 10 cm dishes in 10 ml DMEM. After 16 h, the subconfluent cells (~80% density) were cotransfected with the plasmids pRRLsinCMV-GFPpre or pCSII-Luc (5.5 μg), pMDLg/pRRE (2.4 μg), pRSVrev (1.2 μg), and pHIT-G or pIRES2-eGFP-CHIKV E3-E1 (1.9 and 3.0 μg, respectively) using 25 μl PEI solution. PEI was mixed with plasmid DNA in a volume of 1.4 ml serum-free DMEM, incubated for 30 min at room temperature and subsequently added to the cells. For transfection with Lipofectamine® 2000 (according to the manufacturer's protocol; Life Technologies), the following amounts of plasmid DNA were used: pRRLsinCMV-GFPpre or pCSII-Luc (10.0 μg), pMDLg/pRRE (6.5 μg), pRSVrev (2.5 μg), pHIT-G or pIRES2-eGFP-CHIKV E3-E1 (3.5 and 5.3 μg, respectively). After 24 h of incubation, the medium was replaced by 5 ml fresh DMEM per dish. Another 24 h later, the vector particle containing supernatant was harvested, sterile filtered with 0.45 μm filters (Sartorius, Göttingen, Germany), ultracentrifuged (1 h at 50,000 rpm, rotor TLA 100.3; Optima TLX Ultracentrifuge, Beckman Coulter, Krefeld, Germany) and the particles were resuspended in DMEM and/or frozen at −80 °C.

2.6. Transduction of cells with lentiviral vector particles

For the transduction of cells with vector particles produced by the GFP encoding plasmid, 1.25×10^5 cells of the respective cell line per well were seeded in a 1 ml volume in 24-well plates one day prior to transduction. For the human hematopoietic cells, transduction was carried out directly after counting the cells. For all adherent cell lines, 16 h after seeding, the medium was removed and replaced by DMEM containing lentiviral vector particles (eitherVSV-G, GaLV TM or CHIKV Env pseudotyped) up to a volume of 500 μl. After 6 h of incubation at 37 °C, a medium change was performed, substituting the supernatant with 1 ml fresh DMEM. Cells were incubated another 72 h at 37 °C and analyzed by flow cytometry. For this, cells were trypsinated (Trypsin EDTA, Lonza), washed twice with 1 ml PBS + 2% FBS and fixed in 100 μl PBS + 2% paraformaldehyde. Subsequently, the GFP signal was detected with a LSR II SORP flow cytometer (Becton, Dickinson and Company, Heidelberg, Germany).

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