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Research Paper

Preventative Vaccines for Zika Virus Outbreak: Preliminary Evaluation

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ABSTRACT

Since it emerged in Brazil in May 2015, the mosquito-borne Zika virus (ZIKV) has raised global concern due to its association with a significant rise in the number of infants born with microcephaly and neurological disorders such as Guillain-Barré syndrome. We developed prototype subunit and adenoviral-based Zika vaccines encoding the extracellular portion of the ZIKV envelope gene (E) fused to the T4 fibritin foldon trimerization domain (Efl). The subunit vaccine was delivered intradermally through carboxymethyl cellulose microneedle array (MNA). The immunogenicity of these two vaccines, named Ad5.ZIKV-Efl and ZIKV-rEfl, was tested in C57BL/6 mice. Prime/boost immunization regimen was associated with induction of a ZIKV-specific antibody response, which provided neutralizing immunity. Moreover, protection was evaluated in seven-day-old pups after virulent ZIKV intraperitoneal challenge. Pups born to mice immunized with Ad5.ZIKV-Efl were all protected against lethal challenge infection without weight loss or neurological signs, while pups born to dams immunized with MNA-ZIKV-rEfl were partially protected (50%). No protection was seen in pups born to phosphate buffered saline-immunized mice. This study illustrates the preliminary efficacy of the E ZIKV antigen vaccination in controlling ZIKV infectivity, providing a promising candidate vaccine and antigen format for the prevention of Zika virus disease.
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1. Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus of the Flaviviridae family that was first identified in Uganda in 1947. The virus has recently attracted global attention due to its rapid spread from Brazil to other countries in the Americas (Dick et al., 1952; Zanluca et al., 2015). The ZIKV outbreak in Brazil has been associated with a significant rise in the number of babies born with microcephaly (Zanluca et al., 2015) and neurological disorders such as Guillain-Barré syndrome and has been declared a "Global Emergency" by the World Health Organization (WHO 2016 http://www.who.int/mediacentre/factsheets/zika/en/; WHO 2016 http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/; CDC 2016 http://www.cdc.gov/zika/). Concern over the spread of ZIKV to the Northern Hemisphere with its concomitant morbidity is spurring the search for an effective vaccine. ZIKV is related to dengue, yellow fever, Japanese encephalitis, and West Nile viruses (WNV), all of which are arthropod-borne flaviviruses. Like other flaviviruses, ZIKV contains a positive, single-stranded, genomic RNA encoding a polyprotein that is proteolytically processed to yield three structural proteins: the capsid (C), the precursor of membrane (prM), and the envelope (E), and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (Dick et al., 1952).

The succesful development of flavivirus vaccines began 80 years ago in 1937 with the yellow fever YFV17D live-attenuated vaccine (Monath, 2008). Since then, >600 million people have been vaccinated, with 98% protection and a >10 year persistence of vaccine–induced immunity (Barrett and Teuwen, 2009). However, several severe adverse events associated with vaccine administration have been observed over the last 20 years. Thus, a purified, inactivated vaccine has been recently developed and its testing results suggest good immunogenicity and tolerability (Monath et al., 2011). A few weeks ago, two studies showing immunogenicity of a plasmid DNA or adenovirus (serotype 52) expressing virus-like particles in mice and non-human primates were published (Larocca et al., 2016; Abbink et al., 2016). Here, to build on these initial findings to develop an effective ZIKV vaccine, we describe the development of a recombinant adenoviral vector expressing codon-optimized ZIKV E antigen and a subunit recombinant ZIKV E vaccine delivered transcutaneously by carboxymethyl cellulose (CMC) microneedle arrays (MNAs) (Bediz et al., 2014; Korkmaz et al., 2015), investigate their ability to induce neutralizing immune responses, and

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assess their ability to passively protect against ZIKV challenge in a novel neonatal ZIKV infection mouse model.

2. Materials & Methods

2.1. Adenoviral Construction and Purification of Recombinant Protein

For construction of pAd/ZIKV-Efl, the gene encoding human secretory signal peptide hidden Markov model (SP-HMM, MWWRLWW LLLLLLLLWPMVWA), the extracellular portion of the ZIKV strain BeH815744 envelope gene (GenBank KU365780, defined as amino acids 216–794 of the polyprotein), *Bam*H I-linked T4 fibritin foldon trimerization domain (GSGYIPEAPRDGQAYVRKDGEWVLLSTFL), Tobacco Etch Virus Protease (Tp) (ENLYFEG), and six histidine tag were codon-optimized for optimal expression in mammalian cells using the UpGene codon optimization algorithm (Gao et al., 2004). pAd/ZIKV-Efl was generated by subcloning the codon-optimized ZIKV-Efl gene into the shuttle vector, pAd (GenBank U62024) at *Sal*I/*Not*I sites. Subsequently, replication-defective adenovirus 5, designated as Ad5.ZIKV-Efl, was generated by loxP homologous recombination. Moreover, we also purified recombinant proteins named ZIKV-rEfl from the supernatant using His60 Ni Superflow Resin (Clontech) under native conditions to be used as a subunit vaccine. Briefly, the supernatant of Human Embryonic Kidney (HEK) 293 cells infected with Ad5.ZIKV-Efl was heat-inactivated at 65 °C for 30 min and mixed with the same volume of binding buffer (40 mM imidazole, 900 mM NaCl, 100 mM sodium phosphate, pH 7.4). His60 Ni Superflow Resin (Clontech) previously equilibrated with equilibration buffer (20 mM imidazole, 500 mM NaCl, 50 mM sodium phosphate, pH 7.4) was added and the mixture was incubated overnight at 4 °C with rotation. The next day, the settled resin mix was packed into an empty column. The column was washed with 10 ml of equilibration buffer three times followed by 10 ml of wash buffer (40 mM imidazole, 500 mM NaCl, 50 mM sodium phosphate, pH 7.4) three times and eluted in 10 ml of elution buffer (500 mM imidazole, 500 mM NaCl, 50 mM sodium phosphate, pH 7.4). The eluate was concentrated and desalted with phosphate buffered saline (PBS) in an Amicon Ultra-15 filter (Millipore). This desalting step was repeated three times. The concentrations of the purified recombinant ZIKV-Efl were determined by the Bradford assay using bovine serum albumin (BSA) as a protein standard.

2.2. Virus Stock

ZIKV stocks were provided by Dr. Rober Tesh of University of Texas Medical Branch. Vero cells were infected with ZIKV DAKAR41542 at MOI of 0.01 and incubated until the monolayer showed significant cytopathic effect. Culture supernatant was clarified by centrifugation at 3000*g* for 15 min. Virus was precipitated overnight by addition of NaCl (0.4 M) and 6% polyethylene glycol. After centrifugation at 10,000*g* for 30 min, the viral pellet was re-dissolved to 1/100 of the original volume in PBS and centrifuged on a 5 to 50% sucrose gradient at 90,000*g* for 3 h, followed by dialysis with PBS buffer. The virus was diluted to a proper concentration with 5% Trehalose Buffer (20 mM Tris, pH 7.8, 75 mM NaCl, 2 mM $MgCl_2$, 5% Trehalose, 0.0025% Tween 80) and kept at −80 °C. For the virus titer, vero cells were seeded in a six-well plate at 1×10^5 cells per well. The next day, cells were infected with log dilutions of ZIKV for 1 h and overlayed with 1% methyl cellulose media containing 5% fetal bovine serum. After three days of infection, cells were stained with 1% crystal violet. Plaques were counted and titers were calculated by multiplying the number of plaques by the dilution and dividing by the infection volume.

2.3. Animal Experiments

Six- to eight-week-old C57BL/6 female mice (five animals per group) were inoculated subcutaneously (s.c.) with 1×10^{11} viral particles (v.p.) of Ad5.ZIKV-Efl or PBS as a negative control, and intradermally (i.d.) with MNA coated with 20 μg of ZIKV-rEfl. Two weeks after the primary immunization, mice were boosted intranasally (i.n.) or i.d. with the same dose of the respective immunogens. Mice were bled from the retro-orbital sinus at week 0, 2, 4, and 6, and serum samples were evaluated for ZIKV antibody by enzyme-linked immunosorbent assay (ELISA) and plaque reduction neutralization assay (PRNT). For the immunization study, a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee was followed.

To evaluate passive protection by maternal antibody, pups were obtained by mating non-immunized males with immunized females at three weeks following booster vaccination. Pups were challenged intraperitoneally (i.p.) with ZIKV DAKAR41542 (10^5 pfu/50 μl) at seven days after birth. Two non-challenged pups from each litter were used as a control and bled at 28 days after birth to determine passive maternal antibodies. The physical condition of the pups was observed and their body weights were measured daily for 15 days. Exhibiting >10% loss of body weight was defined as onset of disease. In addition to mice that were found dead, mice with weight loss exceeding 25% of their highest body weight were euthanized and recorded as dead. Severity of neurological signs was scored as described previously (Yoshii et al., 2014). Signs of paralysis and loss of balance associated with viral infection were scored as 0 (absent), 1 (present), or 2 (severe). Scoring for paralysis was assigned as follows: 0, normal; 1, dragging limbs or inversion of dorsum pedis; and 2, complete paralysis and no spontaneous movement. Scoring for loss of balance was assigned as follows: 0, normal; 1, leaning of head or trunk posture to one side; and 2, inability to retain posture and falling to one side or a circling movement to one side. Total scores were quantified and were expressed as means ± the standard errors of the mean.

2.4. ELISA Assay

Sera from the animals were collected every two weeks and tested for ZIKV-specific IgG by conventional ELISA. Briefly, ELISA plates were coated with 2×10^5 pfu of heat-inactivated ZIKV DAKAR4542 at 60 °C for 20 min per well overnight at 4 °C in carbonate coating buffer (100 mM, pH 9.5) and then blocked with PBS containing 0.05% Tween 20 (PBS-T) and 2% BSA for 1 h. Mouse sera were diluted 1:200 or 1:20 for pups sera in PBS-T with 1% BSA and incubated for 2 h. After the plates were washed, HRP-conjugated anti-mouse IgG (1:2000, Santacruz) was added to each well and incubated for 1 h. The plates were washed three times and developed with 3,3′5,5′-tetramethylbenzidine, and the reaction was stopped with 1 M H_2SO_4 and absorbance at 450 nm was determined using an ELISA reader (BIO-TEK instruments).

2.5. Plaque Reduction Neutralization Assay (PRNT)

To determine the plaque reduction neutralizing titer at week 6, 60 μl of the pooled sera or 30 μl of each mouse sera was diluted in twofold serial dilutions (from 1/16 to 1/516 or from 1/32 to 1/1024) and incubated with 100 pfu of ZIKV DAKAR41542 in 100 μl of serum-free media at 37 °C for 1 h and subsequently added to a Vero cell monolayer at a density of 5×10^4 cells grown in six-well tissue culture plates and further incubated at 37 °C for 1 h. After incubation, the inoculant was removed, the semisolid media was added, and the plates were incubated for an additional five days. Titers were expressed as the reciprocal of the highest serum dilution still giving a 50% reduction in plaque number ($PRNT_{50}$) relative to samples incubated with pre-immunized control pooled sera.

2.6. Statistical Analysis

In vitro experiments in this paper were repeated at least twice and data shown are means of those replicates ± standard error. For the statistical analysis, the Student's *t*-test, one-way analysis of variance and

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