Genotype-specific neutralization determinants in envelope protein: implications for the improvement of Japanese encephalitis vaccine

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Japanese encephalitis remains the leading cause of viral encephalitis in children in Asia and is expanding its geographical range to larger areas in Asia and Australasia. Five genotypes of Japanese encephalitis virus (JEV) co-circulate in the geographically affected areas. In particular, the emergence of genotype I (GI) JEV has displaced genotype III (GIII) as the dominant circulating genotype in many Asian regions. However, all approved vaccine products are derived from GIII strains. In the present study, bioinformatic analysis revealed that GI and GIII JEV strains shared two distinct amino acid residues within the envelope (E) protein (E222 and E327). By using reverse genetics approaches, A222S and S327T mutations were demonstrated to decrease live-attenuated vaccine (LAV) SA14-14-2-induced neutralizing antibodies in humans, without altering viral replication. A222S or S327T mutations were then rationally engineered into the infectious clone of SA14-14-2, and the resulting mutant strains retained the same genetic stability and attenuation characteristics as the parent strain. More importantly, immunization of mice with LAV-A222S or LAV-S327T elicited increased neutralizing antibodies against GI strains. Our findings will aid in the rational design of the next generation of Japanese encephalitis LAVs capable of providing broad protection against all JEV strains belonging to different genotypes.

INTRODUCTION

Japanese encephalitis, a typical mosquito-borne viral disease, is one of the most important causes of viral encephalitis, resulting in ∼68 000 cases and 10 000–15 000 deaths worldwide annually (Solomon et al., 2003; van den Hurk et al., 2009). Japanese encephalitis virus (JEV) is transmitted by Culex mosquitoes between vertebrate hosts, particularly pigs and birds, and humans are occasional dead-end hosts. Clinical manifestation of JEV infection ranges from undifferentiated febrile illness and aseptic meningitis to acute encephalitis and death. About half of survivors have severe neurological sequelae (Solomon et al., 2000). The geographical range of Japanese encephalitis has expanded to larger areas in Asia and Australia over recent decades (Hanna et al., 1996; Mackenzie et al., 2004). To date, Japanese encephalitis affects >25 countries, where ∼60% of the global population lives at risk of JEV infection.

JEV is a member of the family Flaviviridae, genus Flavivirus, together with other important human pathogens, including dengue virus (DENV), West Nile virus, yellow fever virus and tick-borne encephalitis virus. JEV has an ∼11 kb positive-sense, ssRNA genome, which contains a single ORF that encodes three structural proteins [capsid (C), pre-membrane (prM) and envelope (E)] and seven
non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), flanked by the 5’ and 3’ UTRs. Based on the E gene sequence or the complete genome, JEV can be further divided into five genotypes (GI–GV) (Uchil & Satchidanandam, 2001) and each genotype has a specific geographical distribution pattern. Currently, GI and GIII strains represent the most prevalent genotypes in many epidemic countries. In the first half of the 20th century, the majority of JEV isolates from humans belonged to GIII; however, over the last two decades, GI has gradually replaced GIII as the dominant circulating genotype in many areas of Asia, including China, Vietnam, Japan, India and Thailand (Ma et al., 2003; Nga et al., 2004; Nitapat et al., 2008; Pan et al., 2011; Parida et al., 2006; Sarkar et al., 2012). The mechanism underlying the genotype displacement as well as the impact of the genotype shift on transmission/outbreak control strategies remain largely unknown (Han et al., 2014; Schuh et al., 2013, 2014).

Vaccination has proven to be the most effective method to prevent and control Japanese encephalitis, and the incidence has decreased significantly in many Asian countries. Currently, several inactivated vaccines and live-attenuated vaccines (LAVs) have been licensed for clinical use (Beasley et al., 2008; Halstead & Thomas, 2010). The inactivated vaccines are cultivated in mouse brain, primary hamster kidney cells or Vero cells. The LAV SA14-14-2 was first licensed in 1989 in China, and has been widely used in JEV-endemic countries in Asia, including China, South Korea, Thailand, India, Nepal and Sri Lanka (Gatchalian et al., 2008; Yu, 2010). More than 300 million children have been immunized with LAV SA14-14-2, and the safety and efficacy profile has been well established (Bista et al., 2001; Hennessy et al., 1996; Kumar et al., 2009; Liu et al., 1997; Tsai et al., 1998). Very recently, a recombinant chimeric LAV based on the yellow fever vaccine strain (17D) backbone carrying the prM and E gene of JEV SA14-14-2 (JE-CV) was licensed in multiple countries, including Australia, Thailand, Malaysia and Philippines (Bonaparte et al., 2014; Feroldi et al., 2014; Monath et al., 2015). However, all the licensed Japanese encephalitis vaccines, including SA14-14-2, are derived from GIII viruses. Previous clinical trials with GIII vaccines in areas where heterogeneous genotypes are circulating have shown effectiveness. It is also expected that immunity induced by GIII vaccines would provide cross-protection against infection by other JEV genotypes. However, various immunological assays with antisera or mAbs have demonstrated the existence of antigenic differences amongst genotypes of JEV strains (Hashgawa et al., 1994, 1995; Kimura-Kuroda & Yasui, 1983; Kobayashi et al., 1985; Wills et al., 1993). Data from inactivated GIII vaccines demonstrated reduced neutralization capability against heterologous GI viruses (Beasley et al., 2004; Erra et al., 2013; Fan et al., 2012; Ferguson et al., 2008; Kuranoe & Takasaki, 2000). Mice immunized with SA14-14-2 showed decreased neutralization antibody titres against GI viruses (Liu et al., 2011). Recently, we also demonstrated that LAV vaccination induced reduced levels of neutralizing antibody against GI viruses in humans (Ye et al., 2014). In particular, several groups reported Japanese encephalitis cases in individuals who had been vaccinated with LAV SA14-14-2 (Hu et al., 2013; Sarkar et al., 2013; Zhang et al., 2011). Therefore, the rapid spread and replacement of GI viruses might depress the protective efficacy induced by current Japanese encephalitis vaccines and an improved Japanese encephalitis vaccine that would confer broad protection against all circulating genotypes, especially GI viruses, is needed.

Flavivirus E glycoprotein is the principal antigen in eliciting neutralizing antibodies and plays a critical role in inducing protective immunity against virus infection. The E protein contains three discernible domains (DI–DIII) (Luca et al., 2012). Numerous neutralizing epitopes have been identified at the tip of DII (Oliphant et al., 2006), in the hinge region between DI and DII (de Alwis et al., 2012; Teoh et al., 2012), and on DIII (Austin et al., 2012; Gromowski & Barrett, 2007; Oliphant et al., 2005). Previous investigations on DENV revealed genotype- and strain-dependent epitopes that impact neutralization and protection (Shrestha et al., 2010; Sukupolvi-Petty et al., 2013). In the present study, by combining bioinformatic analysis and reverse genetics technology, we identified two JEV genotype-specific neutralization determinants within E protein (E222 and E327). The corresponding mutations (A222S and S327T) were rationally engineered into the LAV strain SA14-14-2 to generate an improved version of Japanese encephalitis LAV with enhanced neutralization capability against circulating GI strains.

RESULTS

E222 and E327 are distinct in JEV GI and GIII strains

A total of 40 E gene sequences of JEV strains were retrieved from GenBank; detailed information about the origin, collection date and hosts of the JEV strains used in this study is shown in Table S1 (available in the online Supplementary Material). Phylogenetic analysis and multiple sequence alignment of E protein identified two genotype-specific amino acid residues (Fig. 1a, b). Aa 222 and 327 of E protein (E222 and E327) were alanine (A) and serine (S), respectively, in all GIII strains, including LAV SA14-14-2, whilst in all GI strains contained serine (S) and threonine (T) at the same positions, respectively. In addition, E222 was serine (S) in GII viruses or alanine (A) in GIV and GV viruses; E327 was threonine (T), leucine (L) or glutamine (Q) in GII, GIV and GV viruses, respectively. All available sequences of complete E genes of JEV were retrieved from GenBank, and E222 and E327 were confirmed as GI- and GIII-specific in all 570 E protein sequences (Fig. S1).

To determine the three-dimensional location of E222 and E327, the two residues were mapped to the crystal structure of JEV E protein (Fig. 1c) and the reconstructed virion
Fig. 1. E222 and E327 are distinct in JEV GI and GIII strains. (a) A neighbour-joining tree was reconstructed by the Tamura–Nei model with an among-site F distribution with 40 envelope gene sequences of JEV isolates. (b) Sequence alignment of representative GI and GIII strains. The genotype-specific residues 222 and 327 are highlighted in different colours. (c) Structural model of JEV monomer E protein. DI, DII and DIII are coloured in pale cyan, yellow and blue, respectively. All the identified neutralizing epitopes are coloured in green, and the genotype-specific neutralization epitopes E222 and E327 in E protein are coloured in red and purple, respectively. (d) Pseudo-virion model of JEV based on the reconstruction of the DENV-2 cryo-electron microscopy density map.
(Fig. 1d). It was found that E222 and E327 located in DII and DIII, respectively. E327 was clustered with other epitopes recognized by known neutralizing antibodies (Fig. 1c). The reconstructed virion model revealed that E327 was largely exposed on the surface of mature virions, whilst E222 was buried inside (Fig. 1d), suggesting that these two amino acids may function in different mechanisms.

**A222S and S327T mutations decrease LAV-induced neutralization**

To clarify the potential role of E222 and E327 in neutralization, single and double mutations of these two residues were introduced into a full-length infectious clone of GIII JEV (pAJE70). After standard *in vitro* transcription and transfection procedures, all three mutant viruses (A222S, S327T and A222S/S327T) were recovered in BHK-21 cells. Reverse transcription-PCR and sequence analysis confirmed that the corresponding mutations were successfully engineered into the JEV genome. Immunofluorescence staining using anti-JEV mouse sera showed that A222S, S327T or A222S/S327T generated similar levels of viral protein expression as the WT virus (Fig. 2a). There was no significant difference in plaque morphology amongst the four viruses (Fig. 2b). Growth curves in BHK-21 cells showed that WT and the mutant viruses

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**Fig. 2.** Recovery and characterization of A222S and S327T mutants. (a) Indirect immunofluorescence assay of WT, A222S, S327T and A222S/S327T virus-infected BHK-21 cells. The expression of E protein was detected using anti-JEV mouse sera at 24 and 48 h post-infection (p.i.). (b) Plaque morphology of WT, A222S, S327T and A222S/S327T in BHK-21 cells. (c) Growth curves of WT, A222S, S327T and A222S/S327T in BHK-21 cells. BHK-21 cells were infected with viruses at m.o.i. 0.01. Culture supernatants were harvested at 24, 48 and 72 h p.i., and virus titres were determined using standard plaque assays in BHK-21 cells. Results are shown as mean ± SD from two independent experiments.
A222S, S327T and A222S/S327T replicated efficiently, and peaked at 48 h post-infection (p.i.) with titres of $10^{6.94}$, $10^{6.40}$, $10^{6.79}$ and $10^{6.69}$ p.f.u. ml$^{-1}$, respectively (Fig. 2c). These results demonstrated that A222S and S327T mutations in E protein did not significantly impact viral replication.

Then, to clarify whether these mutations affected JEV neutralization, a panel of human sera from volunteers immunized with LAV SA14-14-2 was used to assess the neutralizing antibody titres against WT and each mutant virus. As shown in Fig. 3, all human sera could efficiently neutralize the three mutant viruses together with the WT virus (Fig. 3). However, the 50% plaque reduction neutralization test (PRNT<sub>50</sub>) titres against each mutant virus significantly decreased in comparison with that against the WT virus for selected sera (sera 1, 2 and 5). These results showed that a single mutation in either E222 or E327 affected the neutralization capability against GI and GIII JEV.

**LAV-A222S and LAV-S327T induce higher neutralizing antibody titres against GI strains**

Furthermore, to clarify whether these genotype-specific mutations would enhance the neutralization capability of LAV SA14-14-2 against GI viruses, A222S and S327T mutations were introduced individually into the infectious clone of LAV SA14-14-2 by a standard reverse genetics approach. As expected, the resulting two mutant viruses, named LAV-A222S and LAV-S327T, respectively, were successfully rescued with similar viral protein expression efficiency (Fig. 4a) and plaque morphology (Fig. 4b) in BHK-21 cells in comparison with LAV. Growth kinetics in BHK-21 and C6/36 cells demonstrated that LAV-A222S and LAV-S327T replicated similarly to LAV SA14-14-2 in mammalian cells, but exhibited a slightly decreased multiplication capacity in mosquito C6/36 cells (Fig. 4c).

The immunogenicity of LAV-A222S and LAV-S327T was further tested in mice in comparison with LAV SA14-14-2. Groups of 3-week-old mice were immunized with equal doses of LAV, LAV-A222S or LAV-S327T, and neutralizing antibody responses against representative circulating GI strains SX06 and SH53 were determined. The results showed that both LAV-A222S and LAV-S327T could elicit higher titres of neutralization antibodies against GI strains SX06 and SH53 than that by SA14-14-2 (Fig. 5a, b). These results further demonstrated that the introduction of the A222S or S327T mutation into the LAV SA14-14-2 enhanced the neutralization capacity against GI strains.

**DISCUSSION**

In our study, phylogenetic analysis and sequence alignment of all available JEV E protein sequences revealed that E222
Fig. 4. Recovery and characterization of LAV-A222S and LAV-S327T. (a) Indirect immunofluorescence assay of LAV-, LAV-A222S- and LAV-S327T-infected BHK-21 cells. The expression of E protein was detected using anti-JEV mouse sera at 24 and 48 h p.i. (b) Plaque morphology of LAV, LAV-A222S and LAV-S327T in BHK-21 cells. (c) Growth curves of LAV, LAV-A222S and LAV-S327T in BHK-21 (left) and C6/36 (right) cells. Cells were infected with LAV, LAV-A222S or LAV-S327T viruses at m.o.i. 0.01. Culture supernatants were harvested at 24, 48 and 72 h p.i., and virus titres were determined using standard plaque assays in BHK-21 cells. Results are shown as mean ± sd from two independent experiments.
and E327 are conserved within, but distinct between, GI and GIII viruses. The introduction of mutations into the two residues significantly changed the neutralization capability of human sera, demonstrating the critical role of E222 and E327 in JEV genotype-specific neutralization. For the first time, to the best of our knowledge, E222 and E327 are reported as the only known genotype-specific neutralization determinants of JEV. Structural modelling revealed that E327 was in the lateral ridge in DIII and largely exposed on the mature virion (Fig. 1c, d), clustered with the residues (G302, E306, S331, D332, G333, I337, F360 and R387) recognized by JEV-specific neutralizing mAbs (Cecilia & Gould, 1991; Goncalvez et al., 2008; Lin & Wu, 2003; Wu et al., 1997, 2003), indicating that E327 probably directly influences neutralization by interacting with other identified neutralizing epitopes nearby. Structure modelling indicated that E222 is not exposed on the surface of mature JEV virion (Fig. 1d), although whether there is any conformational interaction with the identified neutralizing epitopes remains unknown. In addition, structural analysis revealed the diversification of flavivirus surface structure in the virus life cycle and the hidden epitopes may be exposed on the surface through the conformational changes in viral glycoprotein arrangement (Lok et al., 2008; Zhang et al., 2004).

These genotype-dependent epitopes are JEV-specific and completely different from those of DENV (Bernardo et al., 2009; Brien et al., 2010; Kochel et al., 2002; Shrestha et al., 2010; Wong et al., 2007). Previous studies using mAbs indicated that the potent neutralizing capacity was impacted by the genotype-dependent conformational change/exposure of the CC’ loop epitope in DIII (Austin et al., 2012). The natural occurring genotype- or strain-dependent amino acid variations in DIII have been demonstrated to affect the binding and neutralization of type-specific mAbs of DENV (Pitcher et al., 2012; Sukupolvi-Petty et al., 2013; Wahala et al., 2010).

The LAV SA14-14-2 possesses advantages of satisfactory immunogenicity, low vaccination dose and low cost of production compared with the inactivated vaccine. It has the largest Japanese encephalitis vaccine market share worldwide and further improvement in vaccine efficacy would be meaningful. Based on the infectious cDNA clone of LAV SA14-14-2, the newly identified genotype-specific neutralization determinants were rationally engineered. The mutant viruses LAV-A222S and LAV-S327T showed similar plaque morphology and replicated efficiently in Vero cells with peak titres of $10^{5.9}$ and $10^{6.5}$ p.f.u. ml$^{-1}$ (data not shown), indicating the potential for large-scale production and industrialization at low cost. Both mutants exhibited potential genetic stability after serial passages in Vero cells (data not shown). In the immunogenicity tests, immunization with LAV-A222S and LAV-S327T was capable of eliciting protective neutralizing antibody against the circulating GI viruses SX06 and SH53, with increased antibody titres compared with those induced by native LAV SA14-14-2. These properties support LAV-A222S and LAV-S327T as improved versions of JEV LAV.

The mechanisms of circulating JEV genotype replacement are not fully understood. E protein is the phylogenetic basis for JEV genotype division and the major component on the virion surface, as well as the critical factor of virulence and immunity; therefore, it is considered as one of the most important areas of investigation. Phyletic and adaptive evolutionary analyses predicted the positively selected sites within the GI and GIII alignment as potential genetic determinants affecting the genotype distribution. In addition, it has been suggested that increased viral multiplication in avians and mosquitoes as well as the more efficient replication and transmission cycle may be responsible for the genotype replacement (Han et al., 2014; Schuh et al., 2014). In this study, the recovered Japanese encephalitis viruses LAV-A222S and LAV-S327T with GI mutations showed a slight decrease in replication efficiency in C6/36

**Fig. 5.** Immunogenicity of LAV-A222S and LAV-S327T. BALB/c mice were immunized twice subcutaneously with $10^6$ p.f.u. LAV, LAV-A222S or LAV-S327T. Neutralizing antibodies against circulating JEV isolates (a) SX06 and (b) SH53 were determined using PRNT$_{50}$. Statistical significance was analysed by one-way ANOVA; *P<0.05; ***P<0.001.
cells, and the possible role of host adaptations remains to be determined. Moreover, naturally occurring sequence variation within or between genotypes may influence the antibody-binding and neutralization capacity (Austin et al., 2012; Sukupolvi-Petty et al., 2013; Wahala et al., 2010). More data from antigen variation surveillance and evolution analysis are required for the explanation of the genotype shift.

Our results have important implications for the evaluation and design of the next generation of Japanese encephalitis vaccine candidates. Currently, the Japanese encephalitis chimeric LAV JE-CV is used in Asian countries as well as SA14-14-2 (Bonaparte et al., 2014; Feroldi et al., 2014) and several flavivirus chimeric LAVs carrying JEV E protein are under development (Gromovski et al., 2014; Sjatha et al., 2014). The two amino acid mutations A222S and S327T should be taken into consideration for the rational design and improvement of a new JEV vaccine with potential protection capability against all circulating JEV strains.

**METHODS**

**Cells and viruses.** BHK-21 and Vero cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10 % FBS in growth medium at 37 °C in 5 % CO₂. C6/36 cells were grown in RPMI 1640 (Invitrogen) medium supplemented with 10 % FBS at 37 °C in 5 % CO₂. LAV strain SA14-14-2 (GIII) was from Chengdu Institute of Biological Products and its parental strain SA14 was from the Chinese National Institute for Food and Drug Safety. GI JEV strains SX06 and SH53 were isolated in Shanxi, in 2006, and Shanghai, in 2001, respectively (Ye et al., 2014). All JEV strains were propagated in BHK-21 cells cultured in DMEM with 2 % FBS. Virus stocks were stored in aliquots at −80 °C until use. Virus titres were determined by standard plaque-forming assay in BHK-21 cells (Li et al., 2013a).

**Phylogenetic analysis and sequence alignment.** A total of 40 E protein gene sequences of JEV strains (Table S1), which were isolated from a variety of geographical regions in Asia, were collected from GenBank.

Multiple sequence alignment and phylogenetic analysis were performed by CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and MEGA version 5.0 software (http://www.megasoftware.net). The phylogenetic tree was reconstructed by the neighbour-joining method and the maximum composite likelihood model.

**Structure modelling.** The crystal structure of JEV SA14-14-2 E protein [Protein Data Bank (PDB) ID: 3PS4] was used to show the modelled structure of the E protein. Considering the similarity between mosquito-borne flaviviruses, the E protein monomer was superimposed onto the DENV-2 virion model (PDB ID: 1THD) based on cryo-electron microscopy reconstruction to generate a rational model for the JEV virion. All the identified JEV neutralizing epitopes were mapped onto the JEV E protein and the modelled virion (Luca et al., 2012). Structure demonstration and graphic rendering were performed using PyMOL (https://www.pymol.org/).

**Construction and characterization of recombinant JEV.** All plasmids were constructed using standard molecular cloning protocols and confirmed by DNA sequencing. Single mutations (A222S or S327T) or both mutations (A222S and S327T) in E protein were introduced into the infectious full-length JEV (GIII) cDNA clone pAJE70 (Ye et al., 2012) by site-directed mutagenesis based on overlapping PCR. The constructed plasmids were named pAJE70-A222S, pAJE70-S327T and pAJE70-A222S/S327T, respectively. The mutations mentioned above were introduced into the infectious full-length cDNA clone of LAV SA14-14-2 (named LAV) in the same fashion.

Xhol-linearized plasmids were purified and used as DNA templates for in vitro transcription as described previously (Li et al., 2013a) in the presence of a m⁴GppA cap analogue with a RibMax Large Scale RNA Production system (Promega). RNA transcripts were transfected into BHK-21 cells with Lipofectamine 2000 (Invitrogen). Four days after transfection, culture supernatants were harvested and seeded into BHK-21 monolayers to prepare virus stocks. Recovered viruses derived from pAJE70, pAJE70-A222S, pAJE70-S327T and pAJE70-A222S/S327T were named WT, A222S, S327T and A222S/S327T, respectively. Mutant viruses based on LAV SA14-14-2 were named LAV-A222S and LAV-S327T, respectively.

Indirect immunofluorescence assays were performed in BHK-21 cells using a JEV mouse sera as described previously (Li et al., 2013b). Growth curves of all recombinant viruses were generated by infecting confluent BHK-21 and C6/36 cells in a 24-well plate. Cell supernatants were harvested at 24, 48 and 72 h p.i. and virus titres were determined by standard plaque assay in BHK-21 cells.

**Neutralization assay.** A panel of human serum samples was collected from volunteers vaccinated with LAV and subjected to the 50 % plaque reduction neutralization test (PRNT<sub>50</sub> (Ye et al., 2014). Briefly, twofold serial dilutions of serum sample were inactivated at 56 °C for 30 min, and then mixed with equivalent JEV suspension and incubated at 37 °C for 90 min. The PRNT<sub>50</sub> titre were calculated by the method of Karber (Traggiai et al., 2004). PRNT<sub>50</sub> >1:10 was considered protective (Hombach et al., 2005).

**Animal immunization.** To evaluate the immunogenicity of mutant vaccines, groups of 3-week-old female BALB/c mice were injected subcutaneously with equal doses (10⁵ p.f.u. per mouse) of LAV-A222S or LAV-S327T; the LAV group was set as control. A booster dose was given 30 days after primary immunization and mice sera were collected 30 days after the second immunization. Sera samples were inactivated and stored at −20 °C, and subjected to neutralization assay by PRNT<sub>50</sub>.

**Statistic analysis.** The significant difference of the neutralization titre was analysed by one-way ANOVA using GraphPad Prism 5.0.

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