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Recombinant lipidated dengue-4 envelope protein domain III elicits protective immunity☆

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ABSTRACT

The combination of recombinant protein antigens with an immunostimulator has the potential to greatly increase the immunogenicity of recombinant protein antigens. In the present study, we selected the dengue-4 envelope protein domain III as a dengue vaccine candidate and expressed the protein in lipidated form using an *Escherichia coli*-based system. The recombinant lipidated dengue-4 envelope protein domain III folded into the proper conformation and competed with the dengue-4 virus for cellular binding sites. Mice immunized with lipidated dengue-4 envelope protein domain III without exogenous adjuvant had higher frequencies of dengue-4 envelope protein domain III-specific B cells secreting antibodies than mice immunized with the nonlipidated form. Importantly, lipidated dengue-4 envelope protein domain III-immunized mice demonstrated a durable neutralizing antibody response and had reduced viremia levels after challenge. The study demonstrates that lipidated dengue-4 envelope protein domain III is immunogenic and may be a potential dengue vaccine candidate. Furthermore, the lipidation strategy can be applied to other serotypes of dengue virus.

1. Introduction

Dengue virus is transmitted by mosquitoes, and infection causes dengue fever or severe dengue hemorrhagic fever and dengue shock syndrome. These diseases are important arthropod-borne viral diseases [1]. Dengue occurs in more than 120 countries throughout tropical and subtropical areas [2]. The growing public health threat of dengue is supported by its wide-spread presence and increasing number of cases. According to estimates, there are 390 million dengue infections per year [3]. It is generally accepted that vaccination is a cost-effective strategy to fight infectious diseases. However, the complex interaction of 4 serotypes of dengue virus with the immune system has complicated the development of an effective vaccine. Consequently, a licensed dengue vaccine is not currently available. Several vaccine candidates using different approaches are being assessed in clinical studies [4]. These approaches include using live attenuated virus [5,6], live chimeric virus [7,8], subunit vaccines [9], and DNA vaccines [10,11]. The most advanced dengue vaccine candidate is Sanofi Pasteur's live chimeric virus vaccine [12]. A phase 2b study of this tetravalent dengue vaccine in Thai schoolchildren has been completed [8]. The results obtained from this study were disappointing because the overall efficacy of the vaccine candidate was low, at 30.2%. Thus, further efforts are required to develop dengue vaccines.

Dengue envelope protein domain III (ED III) folds independently and is accessible and exposed on the virion surface. It has been demonstrated that ED III is the critical region for viral attachment to cellular receptors [13,14]. Several neutralizing epitopes have been mapped to ED III [15–17], indicating that ED III is a suitable target for dengue vaccine development [18]. Recently, ED III subunit vaccine candidates have been evaluated in mice [19–24] and nonhuman primates [25–29]. In general, the immunogenicities of recombinant subunit vaccines are poor, and adjuvants are necessary to enhance immune responses. Unfortunately, aluminum-containing adjuvants, which are the most widely used adjuvants in human vaccines, may not be suitable for complete protection against dengue viral infection [22,27,29].

Abbreviations: D4ED III, dengue-4 envelope protein domain III; ED III, envelope protein domain III; FFUs, focus-forming units; FRNT, focus reduction neutralization tests; LD4ED III, lipidated D4ED III.

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To overcome the obstacles associated with the low immunogenicity of recombinant proteins, we expressed high levels of recombinant protein in lipidated form to enhance the immunogenicity of the recombinant protein [30]. We found that the lipid moiety of the recombinant lipoprotein provided a danger signal that triggered antigen-presenting cell activation via toll-like receptor 2 [31]. Such activation further enhanced immune responses in the absence of exogenous adjuvants [23,24,32]. Herein, we describe the production of the recombinant lipidated dengue-4 envelope protein domain III (LD4ED III) and demonstrate its vaccine potential.

2. Materials and methods

2.1. Virus

Dengue-4/H241 was used for this study. Virus propagation was performed in C6/36 cells, and viral titers were determined by focus-forming assays with BHK-21 cells [32,33].

2.2. Preparation of recombinant proteins

The amino acid sequence of the dengue-4 envelope protein domain III (D4ED III) was described previously [33]. Based on the amino acid sequence of D4ED III, the DNA sequence was determined via *Escherichia coli* codon usage and was fully synthesized by a biotechnology company (Purigo Biotechnology Co., Taipei, Taiwan). The synthesized DNA was then amplified by PCR to generate pD4DE III and pLD4DE III plasmids. *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) and C43(DE3) (Lucigen, Middleton, WI) cells were transformed with pD4DE III and pLD4DE III cells to express D4ED III and LD4ED III, respectively.

After isopropylthiogalactoside (IPTG) induction, recombinant protein was purified by immobilized metal affinity chromatography (IMAC) columns (QIAgen, Hilden, Germany). The fractions from each critical step were analyzed by SDS-PAGE and immunoblotted with anti-His-tag antibodies. The lipid moiety in LD4ED III was further identified by a MALDI micro MX mass spectrometer (Waters, Manchester, UK). All the details of preparation of recombinant antigens were in Supplementary data.

2.3. Inhibition of dengue virus infection in BHK-21 cells by D4ED III and LD4ED III

To test whether D4ED III and LD4ED III blocked dengue virus infection of BHK-21 cells, the virus was pre-mixed with different amounts of D4ED III, heat-denatured D4ED III, LD4ED III, heat-denatured LD4ED III, or control bovine serum albumin (BSA) as indicated for 10 min at 4 °C. The viral titer prior to pre-mixing was approximately 20–40 FFUs per well. Viral adsorption was allowed to proceed for 3 h at 37 °C. The FFUs were determined by focus-forming assays.

2.4. Experimental mice and immunization

Female BALB/c mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The mice were maintained at the Laboratory Animal Center of the National Health Research Institutes (NHRI). All animal studies were approved and performed in compliance with the guidelines of the Animal Committee of the NHRI. Groups of mice (6–8 weeks of age) were immunized subcutaneously with recombinant D4ED III or LD4ED III. The lyophilized D4ED III and LD4ED III were reconstituted with PBS. Each mouse received a 10 μg/0.2-mL dose. Mice were given 2 immunizations at a 4-week interval with the same regimen. This immunization protocol was used throughout the present study. Blood was collected by tail bleeding for 0.1–0.2 mL from each mouse at different time points as indicated. Sera were prepared and stored at −20 °C until use.

2.5. Flow cytometry

To determine the number of D4ED III-specific B cells, bone marrow cells were collected at 3–4 weeks after the second immunization. Single-cell suspensions were prepared for flow cytometry. Nonspecific staining was blocked by incubation with a rat anti-mouse CD16/CD32 antibody (93, eBioscience) in PBS for 10 min at 4 °C. Cells were stained with phycoerythrin-cyanine 7-conjugated anti-B220 (RA3-6B2, eBioscience), allophycocyanin-conjugated anti-CD19 (1D3, BD Biosciences), and D4ED III. After washing, cells were incubated with biotin-conjugated anti-His-tag antibodies, and the D4ED III-bound cells were stained with phycoerythrin-conjugated avidin (Sigma-Aldrich). The results were acquired using the CellQuest Pro software on a BD FACSCalibur and were analyzed using FACS 3 software.

2.6. Enzyme-linked immunospot (ELISPOT) assays

To detect and quantify individual anti-D4ED III antibody-secreting B cells, bone marrow cells were analyzed by ELISPOT. All the details were in Supplementary data.

2.7. Measurement of antibody titers

The levels of anti-D4ED III IgG in serum samples were determined by titration. All the details were in Supplementary data.

2.8. Focus reduction neutralization tests (FRNT)

Sera were diluted using a 2-fold serial dilution (starting at 1:8), and the sera were heat-inactivated prior to testing. A monolayer of BHK-21 cells in 24-well plates was inoculated with dengue-4 virus that had been incubated at 4 °C overnight with pre-immunization or post-immunization sera in a final volume of 0.5 mL. The FFUs were determined by focus-forming assays. The neutralizing antibody titer $FRNT_{70}$ was calculated as the highest dilution that produced a 70% reduction in FFUs compared with control samples containing the virus alone. For calculation purposes, the neutralizing antibody titer was designated as 4 when the neutralizing antibody titer was less than 8.

2.9. Challenge

Sixteen weeks after the second immunization, mice were intraperitoneally injected with 5×10^7 dengue-4-infected K562 cells suspended in 0.5 mL of serum-free RPMI medium [34]. Blood samples were collected 8 h after K562 injection. The blood (0.2 mL) was mixed with 0.02 mL of 3.8% sodium citrate pre-chilled on ice. Plasma was isolated, and the viral titer was determined by focus-forming assays with BHK-21 cells. The detection limit of the assay was 2.3 $\log_{10}$ FFU/mL. Any infective titers below the limit of detection were assigned a value of 2.0.

2.10. Statistical analyses

Statistical analyses were performed with ANOVA and a Bonferroni post test using GraphPad Prism software version 5.02 (GraphPad Software, Inc.). Differences with a p-value of less than 0.05 were considered statistically significant.