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Analysis of recombinant, multivalent dengue virus containing envelope (E) proteins from serotypes-1, -3 and -4 and expressed in baculovirus

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ABSTRACT

Dengue virus has four serotypes that cause a public health problem in Indonesia. Currently, there is no preventative vaccine for this disease, but some model vaccines are in development. The envelop (E) protein genes from three isolates of dengue virus (DENV-1, -3 and -4) were isolated, cloned into *Escherichia coli* and then sub-cloned into a baculovirus vector before co-transfection into Sf9 cells. Recombinant E genes were inserted between the *SmaI* and *SacI* sites of the plasmid, adjacent to the baculoviral structural gene, polyhedrin. The sequence of recombinant E gene was relatively stable with 97–98% homology, although there were amino acid substitutions in some regions. The recombinant protein was more antigenic when exposed to polyclonal sera from infected humans than sera from immunized mice, but its binding to monoclonal antibodies IgG1a and IgG2b was stronger than other isotopes, including IgM, IgG and Ig1b. Recombinant E protein induced cellular immune responses in immunized mice, as demonstrated by lymphocyte secretion of IL-3. This study indicates that recombinant E protein expressed in a baculovirus system can induce humoral and cellular immune responses.

Introduction

Dengue virus is negative-sense, single-stranded RNA virus of the Flavivirus family; it has four serotypes that are endemic across Indonesia [12,15] and other tropical countries. The virus causes mostly pediatric morbidity and mortality [5]. The two types of dengue virus protein are structural and non-structural; their functions are genome replication (polymerase) and induction of antibodies. Structural proteins include the capsid (C), premembrane (prM), matrix (M) and envelop (E) proteins. E protein is important for attachment to cell membranes during infection, and it has many epitopes that react with neutralizing antibodies (Rey et al., 1995). Envelope protein is a glycoprotein, which has been studied for its antigenicity and immunogenicity [2,6] and especially explored an epitope type [10]. The severity of dengue virus disease was correlated with serotype. Serotypes 2 and 3 are more virulent than serotypes 1 and 4 [15], although antibody dependent enhancement has an effect [11]. Thus, vaccines would be effective in preventing dengue virus infections.

Potential dengue virus vaccines have been developed, but they may not protect against all serotypes. Recombinant protein vaccines with many epitopes have been produced for yellow fever virus and baculovirus (lobigs et al., 1987; [17], but it is not clear which antigen determines the induction of neutralizing antibodies. The baculovirus expression system has been used extensively for the expression of recombinant proteins [7] because it is a large, enveloped virus with double-stranded, circular DNA genome [4]. In this paper, we used a baculovirus system to express a multivalent, recombinant E protein for vaccine subunit development. The baculovirus insect cell expression system has been extensively developed. The most commonly used insect host cells include the Sf9 and sf21AE cell lines, originally derived from *Spodoptera frugiperda* pupal ovarian tissue [7]. In our studies, Sf9 cells were used.

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Materials and methods

Virus and clinical specimens

The DENV-1, -3 and -4 strains were isolated from the sera of 62 human patients. Serum samples were collected by Dr. Soegijanto of the Child Health Department, Dr. Soetomo Teaching Hospital/ School of Medicine, Airlangga University, from 2000 to 2008. Dengue virus isolates were selected by their biological properties, including cytopathic effect (CPE) and plaque-forming unit titers on infected Vero cells. Vero cells were kindly provided by Dr. Morita at Nagasaki University. DENV-1, -3 and -4 strains were isolated in 2008, 2004 and 2003, respectively, at the Dengue Hemorrhagic Fever laboratory, Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia, and were identified by multiplex RT-PCR as described by Lanciotti et al. [8].

RNA extraction, RT-PCR amplification, cloning and transfection

RNA was extracted from the supernatants of Vero cells infected with DEN-1, -3, or -4 and reverse transcribed to cDNA using random hexamer primers. cDNA was amplified using PCR conditions modified from Bielefeldt-Ohmann et al. [1] and Dos Santos et al. (2004). The following primers were used: forward Den1/Env/5′-G GGGGCTTCAACATCCCAAG-3′, reverse Den1/Env/5′-GTGCTCC ACG GGCAGTTGTC-3′, forward Den3/Env/5′-AGGGGCTACAAC AGAA AC AC-3′, reverse Den3/Env/5′-TTGCACCTCTGGCAGTGGCC-3′, forward Den4/Env/5′-AGGAGCAGACACATCAGAAG-3′, reverse Den4/Env/5′-TTGCACCTCTGTATGTGGAC-3′. PCR products were digested with the restriction endonuclease *EcoR1*, which cleaves at the joining site of the three E proteins, to minimize disruptions to protein secondary structure. The E protein genes were cloned individually into plasmid pGem and shuttle vectors (Promega) and subsequently ligated at the *EcoR1* sites. The sequences were subcloned into the baculovirus transfer vector pVL (Stratagene). For cloning and transfer into the baculovirus transfer vectors, *Smal* and *Sac1* were used at the 5′ and 3′ ends, respectively, according to the modified method of Deuble et al. [3] and Kost and Condreay [7]. The recombinant virus was transfected according to the method of Bielefeldt-Ohmann et al. [1]. To ensure the molecular properties of recombinant E protein, the truncated protein E PCR product was sequenced using a method modified from Ong et al. [12], and amino acid analysis was performed using ClustalW Mega 5 software.

Production of recombinant E protein

Confluent Sf9 cell monolayers in 75-cm^2 tissue culture flasks were inoculated with the recombinant baculoviruses at an MOI of 1 in 3 ml medium. After 1 h, 7 ml serum-free medium was added. The conditioned medium was collected after 72 h, and the proteins were precipitated with 9% final concentration sterile polyethylene glycol 6000 (PEG, BDH) and overnight incubation with stirring at 4 °C. The precipitates were pelleted by centrifugation at 14,000*g* for 5 h and resuspended in sterile TNE buffer. The protein was used directly for analysis using a method modified from Bielefeldt-Ohmann et al. [1].

SDS–PAGE and western blotting

The recombinant dengue virus envelope proteins were separated using non-reducing 12% SDS–PAGE. The proteins were transferred onto nitrocellulose membranes by electroblotting in a methanol, glycine and Tris-buffer, and membranes were blocked with 1% bovine serum albumin (BSA) for 1 h [14]. The membranes were incubated with dengue virus-specific primary monoclonal antibody (mAb). After washing with Tris-buffered saline containing 0.1% Tween 20 and 1% BSA, primary polyclonal antibody was added. Following the washes, alkaline phosphatase-conjugated rabbit anti-mouse antibody (Dako) was added. Finally, nitrocellulose membranes were stained with Fast Red.

Immunotyping – ELISA

Microplates (96-well, Nunc) were coated with purified recombinant protein overnight at 4 °C, washed with PBS containing Tween 20 (PBST) and blocked for 1 h at room temperature with 1% BSA. After three washes with PBST, the primary mAb goat anti-mouse IgM, IgG, Ig1a, Ig1b and Ig2b (Dako) were diluted appropriately in PBS and incubated on the plates for 1 h at 37 °C. After three washes with PBST, horseradish peroxidase-conjugated anti-mouse immunoglobulin (Dako) was diluted in PBS and added to the wells. Following incubation for 45 min at 37 °C, the plates were washed three times with PBST, and ortho-phenylenediamine (OPD) substrate (Sigma) was added. The plates were incubated 10 min in the dark. After the addition of 1 M HCl, the plate was read on an ELISA reader (Bio-Rad) with the 450-nm wavelength. This method was modified from Bielefeldt-Ohmann et al. [1] and Rantam [14].

Immunization of mice and spot-ELISA

Three groups of 15 BALB/c mice (male, Veterinary Farma, Surabaya, Indonesia) were immunized by the intraperitoneal route with 15 μg of purified recombinant E protein mixed with Montaned 70 adjuvant (Seppic, Institute Pasteur). All mice were sacrificed at 14 days post-immunization, and spleen cells were isolated as described by Bielefeldt-Ohmann et al. [1] to analyze the secreted cellular immune response by ELISpot as described by Mabtech. All animal experiments were conducted with institutional ethics approval in accordance with the Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia.

Results

Virus culture and identification

Dengue viruses caused cytopathogenic effect (CPE) in 60–80% of Vero cells after passage 3–4, indicating that the virus was growing in these cells. Viruses were harvested, and RNA was extracted for serotype identification. The virus titers were determined by indirect ELISA (data not shown). While all dengue virus serotypes induced CPE in cell culture, DENV-4 replicated less rapidly than DENV-1 and DENV-3, as shown in Fig. 1. RT-PCR was used to identify dengue virus isolate serotypes.

In Fig. 2b, cDNA was amplified from the selected serotypes. The E protein genes of DENV-1, -3 and -4 were cloned individually and subsequently fused in a single plasmid, as illustrated in Fig. 3. To construct the recombinant envelope protein gene, a DNA fragment of 1744 nucleotides was cloned. The dengue virus E protein gene in this construct was placed under the transcriptional control of the baculovirus polyhedrin promoter. Additionally, the dengue virus RNA transcripts contained an authentic initiation codon and produced recombinant E proteins (Fig. 3). The recombinant DNA and baculovirus genomic DNA were co-transfected into Sf9 cells, resulting in homologous recombination, as indicated by western blotting and plaques in Sf9 cells (Fig. 4a, b and Fig. 5a).