An Envelope Domain III–based Chimeric Antigen Produced in Pichia pastoris Elicits Neutralizing Antibodies Against All Four Dengue Virus Serotypes

Behzad Etemad, Gaurav Batra, Rajendra Raut, Satinder Dahiya, Saima Khanam, Sathyamangalam Swaminathan, and Navin Khanna*

Recombinant Gene Products Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India

Abstract. There is currently no vaccine to prevent dengue (DEN) virus infection, which is caused by any one of four closely related serotypes, DEN-1, DEN-2, DEN-3, or DEN-4. A DEN vaccine must be tetravalent, because immunity to a single serotype does not offer cross-protection against the other serotypes. We have developed a novel tetravalent chimeric protein by fusing the receptor-binding envelope domain III (EDIII) of the four DEN virus serotypes. This protein was expressed in the yeast, *P. pastoris,* and purified to near homogeneity in high yields. Antibodies induced in mice by the tetravalent protein, formulated in different adjuvants, neutralized the infectivity of all four serotypes. This, coupled with the high expression potential of the *P. pastoris* system and easy one-step purification, makes the EDIII-based recombinant protein a potentially promising candidate for the development of a safe, efficacious, and inexpensive, tetravalent DEN vaccine.

INTRODUCTION

There are four closely related, yet antigenically distinct, serotypes of dengue (DEN) viruses (DEN-1, -2, -3, and -4), that are members of the *Flaviviridae* family.¹ DEN virus infection can result in a spectrum of clinical symptoms ranging from inapparent or mild dengue fever (DF) to severe and fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Approximately 2.5 billion people, in over a hundred tropical and sub-tropical countries, representing ~40% of the world’s population, are at risk of DEN infections. The World Health Organization estimates that there may be ~100 million cases of DEN infections worldwide every year.²-four Infection with any one DEN serotype provides lifelong homologous immunity to that serotype, with only transient cross-protection against the remaining three.²⁵ Epidemiologic and laboratory data suggest that cross-reactive antibodies produced during a primary infection can predispose an individual to potentially fatal DHF and DSS during a subsequent infection through antibody-dependent enhancement (ADE).²⁶ A role for cross-reactive T cells in mediating immunopathology has also been proposed.²⁶ This has prompted the view that a DEN vaccine must be “tetravalent,” affording protection against all four DEN virus serotypes.²⁶ This, together with the lack of a suitable animal model, has made the development of a DEN vaccine a challenging task. Current efforts to develop tetravalent DEN vaccines, which are in advanced stages of development, are based on live attenuated DEN viruses,⁶–¹¹ or genetically manipulated chimeric flaviviruses.¹²,¹³ All these vaccine viruses are monovalent, in that each one is specific to a single serotype.⁶ Recent studies have shown that tetravalent formulations, obtained by mixing these monovalent vaccine viruses, corresponding to the four serotypes, are prone to elicit an unbalanced immune response, predominantly to one serotype, caused by “viral interference.” This has been observed both in non-human primates⁷,⁸ and human volunteers.⁹–¹¹ This poses a serious risk in the context of the ADE phenomenon. In addition, another reason for concern is that live flaviviruses have the potential to undergo genetic recombination.¹⁴

In parallel, several alternative approaches for the development of recombinant DNA- and protein-based subunit vaccines are being explored by many groups,¹⁵–⁴³ including ours.³⁴–³⁶ The genetic vaccines being explored use naked plasmid DNA,²⁷–²⁹ pox virus,²⁰ or, more recently, adenovirus,³²,³³,³⁵,³⁶ vectors encoding DEN virus antigens. The protein-based approaches are based on both prokaryotic¹⁷,²¹,²⁵,²⁶,³⁰,³¹ and eukaryotic,¹⁵,¹⁶,¹⁸,²²–²⁴ expression hosts. The majority of current efforts that seek to develop such recombinant subunit candidate vaccines focus on the major envelope (E) protein, a ~500 amino acid (aa) residues long, cysteine-rich, multifunctional protein. Its structure is stabilized by six disulfide (S-S) bridges and is organized into three discrete domains: a central domain (I), a dimerization domain (II), and an immunoglobulin (Ig)-like domain (III).³⁷,³⁸ The E protein binds to host cells through as yet unidentified receptor(s),³⁹ contains multiple serotype-specific, conformation-dependent neutralizing epitopes,⁴⁰–⁴² elicits long-lasting antibody response,⁴³ and, most importantly, confers protective immunity.¹⁵,²⁰,²³,²⁸

A growing body of evidence, accumulated in recent years by several groups,¹⁷,²¹,²³,³⁴–³⁶,⁴⁰–⁴⁸ has shown that many of the properties of the E protein, important from a vaccine perspective, are associated with domain III, referred to as EDIII, spanning aa residues 300–400. Its structural and antigenic integrity depends on a single S-S bond.⁴⁹ The host cell receptor–binding motif has been localized to EDIII.⁴⁰,⁴⁷ Structural studies of intact virions have shown that EDIII is exposed and accessible on the virion surface.⁵⁰ Consistent with this, it has been shown that recombinant EDIII proteins can block DEN-2 virus infectivity.⁴⁷,⁴⁸ Multiple type- and subtype–specific neutralizing epitopes have been mapped to this domain.⁴⁰ A recent study that analyzed a large panel of anti-E monoclonal antibodies (mAbs) showed that those that bound to EDIII were the most powerful blockers of virus infectivity.⁴⁴ Others,¹⁷,²¹,²³,²⁵,²⁶,³⁰,³¹ and our group,³⁴–³⁶ have shown that EDIII-based gene and protein vaccine candidates can elicit virus-neutralizing antibodies. Importantlly, EDIII has only a very low intrinsic potential for inducing cross-reactive antibodies¹⁷,²¹ implicated in the pathogenesis of DHF/DSS. Taken together, these attributes of the EDIII make it an
excellent vaccine candidate. Important from the perspective of recombinant protein expression, the Ig-like flavivirus EDIII has been shown to be an independent folding domain, as evidenced by its release as a discrete fragment on tryptic digestion of DEN virions, and exhibits a very high degree of stability.

In this study, we designed a chimeric tetravalent protein by joining the EDIIIs of the four DEN virus serotypes using flexible pentaglycyl peptide linkers. We expressed the gene encoding this protein in the methylotropic yeast, Pichia pastoris, which combines the advantages of both prokaryotic (high expression levels, easy scale-up, inexpensive growth media) and eukaryotic (capacity to carry out most of the post-translational modifications characteristic of higher eukaryotes) expression systems. In the last several years, the methylotropic yeast P. pastoris has emerged as a powerful and inexpensive heterologous system for the production of high levels of functionally active recombinant proteins of commercial and academic interest. The availability of the strongly regulated methanol-inducible alcohol oxidase 1 (AOX1) promoter for high-level expression is a distinct advantage for heterologous protein production. Importantly, P. pastoris is well suited for the expression of the disulfide-rich tetravalent EDIII-based antigen. Several S-S linked proteins such as recombinant hepatitis B surface antigen and insulin have been successfully produced in P. pastoris. The strong preference of P. pastoris (unlike Saccharomyces cerevisiae) for respiratory growth allows it to be cultured at extremely high cell densities of ~100 g/L dry cell weight or greater. This greatly enhances productivity to gram quantities per liter. Because manipulating the carbon source added to the culture medium controls the AOX1 promoter, growth and induction can be easily performed at all scales ranging from shake flasks to large fermenters. Finally, P. pastoris is a non-pathogenic organism; recombinant proteins expressed in it will be free of pyrogens (unlike Escherichia coli-expressed proteins), toxins, and viral inclusions (unlike tissue culture expressed proteins), making them safe for human use.

In this paper, we describe the recombinant EDIII-based tetravalent antigen (rEDIII-T), its expression, purification, and a preliminary evaluation of its immunogenic potential in eliciting immune responses specific to each of the four DEN virus serotypes.

MATERIALS AND METHODS

DEN viruses, mammalian, and insect cells. DEN-1 (Nauru Island), DEN-2 virus (NGC), DEN-3 (H87), and DEN-4 (Dominica) viruses were kind gifts of Dr. A. Falconar (University of Oxford, Oxford, UK). Baby Hamster Kidney (BHK 21) cell line, the monkey kidney cell line LLCMK2, and the mosquito cell line C6/36 were from American Type Culture Collection (Mannassas, VA). BHK21 and LLCMK2 cell lines were maintained in Dulbecco modified Eagle medium (DMEM), supplemented with 10% (vol/vol) fetal calf serum (FCS), in a 10% CO2 humidified incubator at 37°C. C6/36 cells were maintained in Leibovitz L-15 medium, supplemented with tryptose phosphate broth (0.3% wt/vol) and 10% (vol/vol) heat-inactivated (Δ) FCS, in a CO2-free incubator at 28°C.

Microbial host strains and plasmid vector. The P. pastoris host strain used in this study was the histidine-requiring auxotroph, GS115 (his4). It has a mutant histidinol dehydrogenase gene (his4) to allow for selection of expression vectors containing the wild-type allele (HIS4) on transformation. For expression in P. pastoris, the plasmid pPIC9K was used. This plasmid contains the P. pastoris AOX1 promoter fused to the S. cerevisiae pre-pro α factor secretory signal-encoding sequence, followed by a multicloning site for insertion of the foreign gene of interest and the P. pastoris transcription termination sequence. It also contains a wild-type copy of the HIS4 gene and the kanamycin resistance marker necessary for selection of P. pastoris transformants. In addition, the plasmid contains the ampicillin selection marker and ori sequences for selection and propagation in E. coli. Plasmid pPIC9K, the host strains P. pastoris GS115 (his4), for recombinant protein production, and E. coli DH5a, for recombinant plasmid construction, were purchased from Invitrogen, Carlsbad, CA.

Others. Anti-penta His monoclonal antibody (mAb), Ni-NTA superflow resin and Ni-NTA His-Sorb plates were from Qiagen, Germany. Anti-mouse IgG-fluorescein isothiocyanate (FITC), anti-mouse IgG-alkaline phosphatase (AP), anti-mouse IgG-horseradish peroxidase (HRPO) and the AP substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium were from Calbiochem. The HRPO substrate 3, 3′, 5′, 5′-Tetramethylbenzidine (TMB) was from Kirkegaard and Perry laboratories, USA. The murine cytokine ELISA kits were from eBioscience.

Creation of recombinant P. pastoris clone expressing the EDIII-based tetravalent gene. Fusing EDIII-encoding sequences corresponding to the E proteins of all four DEN virus serotypes generated a tetravalent gene, rEDIII-T. The gene was designed to carry a 6x-His Tag-encoding sequence at the 3′ end, followed by a stop codon. This gene, custom synthesized by Geneart AG (Regensburg, Germany), was codon-optimized for expression in P. pastoris and cloned into pPIC9K as an EcoRI/NorI fragment. The AOX1 promoter-driven rEDIII-T expression cassette of the resultant vector was integrated into the AOX1 locus of the P. pastoris genome, as described previously.

Expression and purification of recombinant EDIII-T protein. A 1-L culture of the P. pastoris transformant, harboring the rEDIII-T gene growing at logarithmic phase, was spun down, and induction was initiated by re-suspending the cell pellet in 100 mL of 1% (vol/vol) methanol-containing medium as described before. Because the recombinant protein failed to be secreted into the culture supernatant (based on ELISA and immunoblot analyses), we purified it from the induced cells. Cells were harvested 96 hours after induction. The pellet (~40 g wet weight) was washed twice with 500 mL cold distilled water and re-suspended in 200 mL lysis buffer (50 mmol/L phosphate buffer [pH 8.0]/500 mmol/L NaCl/10 mmol/L imidazole/6 mol/L guanidine-HCl/1 mmol/L phenyl methyl sulfonyl fluoride), followed by stirring for ~90 minutes at room temperature. This suspension was mixed with 300 g of 425- to 600-μm glass beads (catalog G-8772; Sigma, St. Louis, MO), and the cells were disrupted using a bead mill (Dyno Mill; Multi Laboratory, WAB, Basel, Switzerland) in four 10-minute cycles at 4°C. The lysate was collected and kept on ice. The beads were washed twice with ~150 mL cold lysis buffer and pooled with the lysate. The pooled lysate (volume ~500 mL) was clarified by centrifugation in a Sorvall GSA rotor at 10,000 rpm for 1 hour at 4°C. The resultant superna-
tant was further clarified by passing it through a 0.45-μm membrane filter, adjusted to pH 8 (using 1 N NaOH), and allowed to bind to 20 mL of Ni-NTA superfowl resin (50% [wt/vol] slurry) overnight at room temperature. Lysate/Ni-NTA mixture was loaded into a column, and the flow-through was collected. The column was washed with 15 bed volumes of wash buffer A (50 mmol/L phosphate [pH 6.3]/8 mol/L urea/150 mmol/L NaCl/10 mol/L imidazole) followed by 15 bed volumes of wash buffer B (50 mmol/L phosphate [pH 5.9]/8 mol/L urea). Finally, the bound proteins were eluted using buffer C (50 mmol/L phosphate [pH 4.5]/8 mol/L urea). Fractions of 5 mL were collected and analyzed by sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE). Peak fractions were pooled together and dialyzed successively against 1× phosphate-buffered saline (PBS) containing progressively decreasing urea concentrations (4, 2, 1, 0.5, and 0 mol/L).

**His-Sorb ELISA for the determination of rEDIII-T protein.** Recombinant 6×-His-tagged EDIII-T protein levels in soluble cell extracts and Ni-NTA column eluates were analyzed using Ni-NTA His-Sorb plates, essentially as described by the manufacturer (Qiagen, Inc., Valencia, CA) (QIAexpress detection and assay handbook), with minor modifications. Briefly, protein in sample aliquots (100 μL) was allowed to bind to the wells for 4 hours at 37°C. After washing, bound protein in each well was detected using 100 μL of 1/4,000 diluted anti-EDIII-T polyclonal murine antiserum (raised against *E. coli*–expressed rEDIII-T protein) in conjunction with horseradish peroxidase (HRPO)-conjugated secondary antibody and 3,3′,5,5′-tetramethyl benzidine (TMB) substrate, as previously described.

**Protein blot assays.** The purified, *P. pastoris*–expressed rEDIII-T protein was detected in Western blots. The primary antibodies used for detection were anti penta-His mAb and anti-EDIII-T polyclonal serum (raised in mice using *E. coli*–expressed EDIII-T protein) at dilutions of 1:2,000 and 1:5,000, respectively. The rest of the procedural details were essentially the same as described before. To detect glycosylation, blots were probed with Concanavalin A (Con A)–fluorescein isothiocyanate (FITC) (catalog 61761; Fluka, Seelz, Germany) and visualized using Ettan DIGE imager (excitation/emission: 495 nm/518 nm) from GE Healthcare (Uppsala, Sweden).

**Immunization of mice.** Groups of four Balb/c mice (4–6 weeks old) were immunized intraperitoneally on Days 0, 21, 42, and 84 with 20 μg purified *P. pastoris*–expressed rEDIII-T protein. The recombinant protein antigen was formulated using alum, Freund complete adjuvant, and montanide ISA 720. In the case of the Freund group alone, the booster doses were formulated using Freund incomplete adjuvant. Sera were collected from the animals 1 week after the final immunization by retro-orbital puncture. Animal experiments were reviewed and approved by the International Center for Genetic Engineering and Biotechnology Institutional Animal Ethics Committee and adhered to the guidelines of the Government of India.

**Detection of anti-DEN virus antibodies in immune sera.** Antibodies specific for each of the four DEN viruses in the sera of immunized animals were detected in ELISAs using tissue culture–derived DEN viruses as coating antigens. Starting from 100-fold dilution, serial 2-fold dilutions of each serum sample were assayed in duplicate against each of the four DEN virus serotypes. The ELISA protocol adopted was essentially as described previously.

**Immunofluorescence assay.** BHK 21 cells seeded on coverslips were infected separately with each of the four DEN virus serotypes. At 24 hours after infection, viruses in the infected cells were detected by an indirect immunofluorescence assay, using murine pre-immune and immune sera (from rEDIII–T antigen–immunized mice), as described earlier.

**Plaque reduction neutralization test.** DEN-1, DEN-2, DEN-3, and DEN-4 viruses (~120 PFU each), prepared from infected tissue culture supernatants by polyethylene glycol precipitation, were separately pre-incubated with serial 2-fold dilutions of heat-inactivated (56°C/10 minutes) pooled serum (200 μL final volume) collected from rEDIII–T–immunized mice for 1 hour at 37°C and plaqueed on LLCMK2 cells seeded in 24-well plates (50 μL virus-antisera mix/well), as described before. Negative (mock-infected) and positive (virus infection in the absence of antiserum) controls were set up in parallel. Plaques shown after staining were counted, and the antiserum dilution resulting in 50% reduction in plaque count (with reference to the number of plaques generated by the virus in the absence of antiserum) was expressed as the plaque reduction neutralization test (PRNT)50 titer. The amount of viruses used in the PRNT assay is within the range normally used in the reported literature.

**Proliferation and cytokine release assays.** Spleens were harvested from mice that had been primed 10 days earlier. Splenocytes were seeded in 96-well plates in DME + 10% FCS (2.5 × 105 cells in 0.1 mL/well) and either mock-stimulated (no antigen) or stimulated in vitro, separately, with each of the four DEN viruses and assayed for proliferation and cytokine secretion as described before.

## RESULTS

**Design of a novel tetravalent DEN antigen encoding the P. pastoris construct.** The tetravalent antigen was designed to contain the EDIIIs of the four DEN serotypes, arranged like beads on a chain. Adjacent EDIIIs were joined using flexible pentaglycine peptide linkers. Each EDIII is composed of ~120 aa residues spanning aa 296–599 of the corresponding E protein. Each of these domains possesses the single S-S bond (Cys 202–Cys 333) that is critical for antigenicity. A 6× His tag was engineered at the carboxy terminus to aid in the detection and purification of this recombinant protein. The tetravalent antigen is predicted to be ~55 kDa in size. A gene encoding this antigen, codon-optimized for expression in *P. pastoris*, was chemically synthesized and cloned into the *P. pastoris* integrative vector pPIC9K as a ~1.5 kb *EcoRI/NotI* restriction fragment. In this construct, the tetravalent antigen-encoding gene is fused in-frame with the *S. cerevisiae* α factor secretory signal, under the transcriptional control of the strong methanol-inducible *AOXI* promoter. This resultant plasmid, pPIC-EDIII-T, is shown in Figure 1A. A schematic representation of the rEDIII–T protein (precursor and its processed forms) is shown in Figure 1B. The pPIC-EDIII-T plasmid was digested with *BgIII* to release the tetravalent antigen expression cassette together with the *HIS4* and *Kan* antibiotic selection markers and transformed into the *P. pastoris* host strain GS115, which carries an intact *AOXI* locus (Mut′). Because both ends of this *BgIII* fragment are homologous to the *AOXI* region of the GS115 genome, it can integrate into
under denaturing conditions (6 mol/L guanidine HCl). After washing away the non-specifically bound contaminants using a progressively decreasing pH step gradient (pH 8, 6.3, and 5.9), bound proteins were eluted at pH 4.5. A single major homogeneous peak emerged in the pH 4.5 eluate as shown in Figure 2A. This was dialyzed against 1× PBS (pH ~ 8.5) to eliminate urea and neutralize the acidic pH. A summary of the purification is presented in Table 1. Starting from ~40 g of induced cell mass, we could obtain ~54 mg of purified tetravalent DEN antigen, representing a ~60-fold purification with a ~40% yield.

An SDS-PAGE analysis of the peak fractions is presented in Figure 2B. It is evident that the homogenous peak contains a major band with an apparent molecular weight of ~80 kDa and a minor band of ~55 kDa. Although it could be argued that the ~55-kDa protein represents the intended rEDIII-T protein, the identity of the ~80-kDa protein was not clear. To investigate this issue further, we generated another version of the rEDIII-T protein, lacking the S. cerevisiae α factor secretory signal peptide, in an E. coli expression system, and used this protein to raise anti-rEDIII-T polyclonal antibodies in mice. We next proceeded to analyze the P. pastoris–expressed purified peak material (shown in Figures 2A and 2B) in immunoblot assays using the polyclonal anti-rEDIII-T antibodies and a commercially available penta-His mAb (Figure 2C). Both the ~80- and ~55-kDa forms of the P. pastoris–expressed proteins possess the C-terminal 6× His Tag, as evidenced by their reactivity to penta-His mAb (Lane 6), consistent with their tight binding to the Ni-NTA affinity matrix (Figure 2B).

Furthermore, the ~55-kDa yeast protein co-migrated with the E. coli–expressed tetravalent antigen (Lane 4). These results were mirrored in the immunoblot analysis performed using the polyclonal antiserum raised against the E. coli–expressed tetravalent antigen (Lanes 1 and 3). Although these data suggest that the ~80-kDa protein is presumably the unprocessed precursor of the ~55-kDa protein, it is difficult to reconcile the apparent size difference between these two given that the size of the α factor secretory signal peptide is ~10 kDa. An obvious possibility is that the P. pastoris–expressed protein may be heavily glycosylated. This indeed turned out to be the case as shown in the experiment in Figure 2D. In this experiment, both the E. coli– and P. pastoris–expressed rEDIII-T protein preparations were electrophoresed, blotted onto nitrocellulose, and probed with Con A-FITC. This experiment showed that the ~80-kDa protein is indeed heavily glycosylated (compare Lanes 1 and 3). In contrast, the ~55-kDa protein does not bind Con A-FITC (see Lanes 2 and 3). Because there are no glycosylation sites in EDIII,37 our data suggest that the unprocessed signal peptide of the ~80-kDa protein is presumably the substrate for glycosylation in P. pastoris. This suggestion is consistent with the presence of two N-glycosylation sites in the S. cerevisiae α factor secretory signal peptide. Taken together, these data indicate that the two forms present in the protein preparation purified from P. pastoris represent the rEDIII-T antigen and that signal cleavage is relatively inefficient. The purity of the Ni-NTA–purified yeast recombinant tetravalent antigen was judged to be ≥ 95%.

Tetravalent DEN antigen elicits antibodies that can recognize and neutralize all four serotypes of DEN viruses. The major objective of this work was to examine whether it is possible to create a single DEN antigen capable of eliciting
antibodies specific to each one of the four DEN serotypes simultaneously. To this end, mice were immunized with the yeast-expressed and affinity-purified recombinant tetravalent antigen. The immunization was carried out with three different adjuvants, and the sera were analyzed for the presence of anti-DEN antibodies by a variety of criteria. To begin, an ELISA approach was used wherein the purified rEDIII-T protein was used as the coating antigen. This showed that the tetravalent antigen is highly immunogenic (data not shown). Next, to detect the presence of virus-specific antibodies in murine immune sera, the ELISA was repeated using DEN viruses of each serotype, separately, as controls. The data from this experiment shown in Figure 3 clearly show that the immune sera did indeed contain antibodies specific to each of the DEN virus serotypes. Among the three adjuvants tested, the use of Freund adjuvant elicited antibodies that manifested maximal ELISA reactivity toward each serotype, with antibody levels against DEN-1 and DEN-2 being slightly higher than those against DEN-3 and DEN-4. However, the difference was < 2-fold. For example, at a 100-fold serum dilution, anti-DEN-3 and anti-DEN-4 ELISA reactivities were, respectively, ~30% and ~40% lower. The use of montanide and alum as adjuvants produced comparable, but slightly lower, levels of antibodies against all the four serotypes. Again, differences were minimal. In fact, ELISA profiles obtained using DEN-2, DEN-3, and DEN-4 as capture antigens, were practically indistinguishable from each other. ELISA reactivities of the immune sera against DEN-1 were slightly higher, but within 2-fold, than those against the remaining three serotypes.

The ELISA data were essentially corroborated by the immunofluorescence experiment shown in Figure 4. In this experiment, BHK cells, infected separately with each of the four DEN virus serotypes, were probed using either murine pre-immune or immune sera, and virus-bound antibodies, if any, were visualized with a secondary anti-murine IgG-FITC conjugate. Pre-immune serum, as expected, did not produce immunofluorescence with any of the four DEN viruses. However, consistent with the ELISA data, immune sera picked up

<table>
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<tr>
<th>Table 1</th>
<th>Purification of P. pastoris–expressed rEDIII-T protein</th>
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<tr>
<td>Step</td>
<td>Total protein (mg)*</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>8,030</td>
</tr>
<tr>
<td>Ni-NTA chromatography</td>
<td>54</td>
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</table>

* Total protein was estimated with Bradford reagent using BSA as reference.
† ELISA ODs in appropriately diluted samples were measured at 450 nm using Ni-NTA His Sorb kit.
‡ Represents ELISA OD units per milligram protein.
§ Obtained by dividing specific activity at a given step by the specific activity of the crude lysate.
¶ Yields are based on ELISA ODs, taking the total ELISA ODs in the cell lysate as 100%.
all four DEN viruses as evidenced by immunofluorescence. Although the immune serum used in the experiment shown in Figure 4 was from the Freund group, essentially similar results were obtained using immune sera from the alum and montanide groups (data not shown). Positive control experiments were run in parallel, as reported earlier, to ensure that each of the four DEN viruses had successfully infected the BHK cells.36

Both the preceding experiments show that the tetravalent antigen has the potential to induce antibodies that can recognize and bind to each of the four DEN serotypes. The question that arises at this point is will such binding neutralize the infectivity of these viruses? To address this, we pre-incubated each of the four DEN viruses separately with the anti- rEDIII-T immune serum and infected BHK cells. Unbound viruses were washed away, and bound virus, if any, was visualized by indirect immunofluorescence assay as described previously.34 This experiment showed that anti-rEDIII-T antiserum, from all three adjuvant groups, could effectively block the binding of all four DEN viruses to BHK cells (data not shown). This leads to the conclusion that immunization with the P. pastoris–expressed rEDIII-T antigen can induce virus-neutralizing antibodies specific to all four DEN virus serotypes simultaneously. To quantitate the levels of neutralizing antibodies, we performed a PRNT assay wherein each of the DEN viruses was separately pre-incubated with the immune serum and plaqued on LLCMK2 cells to determine the residual virus titers. Figure 5 depicts the percent inhibition of infectivity of each DEN virus serotype as a function of dilution of the immune serum. In this experiment, serial dilutions of pooled immune sera from each of the three adjuvant groups were tested against each one of the four DEN virus

**FIGURE 3.** Comparative analysis of serum antibody titers elicited in mice by P. pastoris–expressed recombinant rEDIII-T protein formulated with different adjuvants. Anti-DEN antibodies in murine pre-immune sera (triangles) and immune sera from mice vaccinated with rEDIII-T antigen formulated in Freund (diamonds), alum (squares), or montanide (circles) adjuvants were determined by ELISA using (A) DEN-1, (B) DEN-2, (C) DEN-3, and (D) DEN-4 as the coating antigen. Sera at each dilution were assayed in duplicates, and the data shown represent the average.

**FIGURE 4.** Indirect immunofluorescence analysis of antibodies in sera of mice immunized with P. pastoris–expressed rEDIII-T protein. BHK cells were infected with each of the four DEN viruses as indicated. One day after infection, the virus-infected cells were fixed and probed with either sera drawn before immunization (Pre-imm) or after immunization with rEDIII-T formulated in Freund adjuvant (Imm).
lated splenocytes were pulsed with \[^3H\]-thymidine, followed by measurement of radioactivity, as shown in Figure 6A. Although DEN-1, DEN-2, and DEN-4 stimulated splenocytes from mice immunized in the presence of Freund adjuvant manifested a significant proliferative response (≥ 10-fold over unstimulated control cells), DEN-3 stimulation resulted in a very modest response (~4-fold increase). In contrast, when alum was the adjuvant, splenocyte proliferation was significantly stimulated by DEN-2 (~21-fold) and DEN-4 (~11-fold), in comparison to DEN-1 and DEN-3 (~4-fold in both cases). When splenocytes from mice immunized with montanide adjuvanted tetravalent antigen were tested, DEN-1, DEN-2, and DEN-3 stimulated more or less comparable increases in secreted IFN-\(\gamma\) levels, but DEN-2 and DEN-4 resulted in ~2.8- and ~1.8-fold, respectively. Similarly, when the adjuvant used during immunization was alum, fold enhancements in IFN-\(\gamma\) levels ranged from ~1.3- to 3.3-fold. Likewise, DEN-1, DEN-2, and DEN-3 produced very modest increase in the levels of IFN-\(\gamma\)-secretion, which ranged from ~2- to 4-fold. The noteworthy exception was DEN-4-stimulated splenocytes from the montanide group. These splenocytes manifested an ~9-fold increase in IFN-\(\gamma\)-secretion. In contrast, IL-4 secretion was enhanced significantly in response to in vitro stimulation by each one of the DEN virus serotypes (Figure 6C). This was true for splenocytes obtained from mice of all three adjuvant groups. In most instances, there was a ≥10-fold increase in IL-4 secretion compared with unstimulated controls. DEN-3-stimulated splenocytes obtained from mice that had been immunized with montanide adjuvanted antigen manifested a slightly lesser increase in IL-4 secretion levels (~7-fold increase).

### Table 2

Neutralizing antibody responses in rEDIII-T-immunized mice

<table>
<thead>
<tr>
<th>Adjuvant group</th>
<th>DEN-1 (PRNT(_{50}) titer(^\ast))</th>
<th>DEN-2</th>
<th>DEN-3</th>
<th>DEN-4</th>
</tr>
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<tbody>
<tr>
<td>Alum</td>
<td>197</td>
<td>363</td>
<td>47</td>
<td>546</td>
</tr>
<tr>
<td>Montanide</td>
<td>153</td>
<td>293</td>
<td>271</td>
<td>588</td>
</tr>
<tr>
<td>Freund</td>
<td>160</td>
<td>118</td>
<td>234</td>
<td>479</td>
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\(^\ast\) Geometric mean titers (\(N = 3\)).
DISCUSSION

A dengue vaccine has been an elusive goal thus far. Efforts to develop live attenuated virus vaccines, although promising, are beset with problems of “viral interference” and unbalanced immune response. Efforts in primates foreshadow a similar outcome for live chimeric vaccines. Although an overwhelmingly large proportion of recombinant subunit vaccines have thus far targeted single serotypes, a few tetravalent vaccine candidates are also being developed. Recently, two groups have developed tetravalent DNA vaccines, both based on full-length E protein genes. Although one is based on mixing four monovalent plasmid vaccines, the other is a plasmid encoding a single shuffled E gene chimera, carrying epitopes representative of all four serotypes. Yet another group has used a mixture of four EDIII-encoding plasmids as an experimental vaccine. Using the Drosophila cell expression system, Hawaii Biotech, is reported to have developed a tetravalent vaccine, prepared by mixing the E proteins of all four DEN serotypes plus the NS1 protein of DEN-2. This has been reported to elicit PRNT<sub>50</sub> titers > 1:100 in animals. Another tetravalent vaccine, based on a recombinant protein mixture, has been reported by Simmons and others. We showed previously that it is possible to express high levels of DEN-2 EDIII in E. coli without a fusion partner. In a subsequent study, we showed that an E. coli–expressed, renatured bivalent chimera, consisting of the EDIIIs of DEN-2 and DEN-4 viruses linked together, retained the antigenic integrity of its precursors and was capable of eliciting neutralizing antibodies toward DEN-2 and DEN-4 but not to DEN-1 and DEN-3. We built on this and created the tetravalent EDIII-based chimeric protein, rEDIII-T, by including the EDIIIs of the remaining two serotypes. Because this tetravalent protein would be disulfide rich with each of the four EDIII components contributing one S-S bridge apiece, we decided to express the rEDIII-T protein using a eukaryotic rather than a prokaryotic host. We chose to express the rEDIII-T protein in P. pastoris, a yeast host, which combines the high expression potential of the prokaryotic systems and the capacity of eukaryotic systems to facilitate post-translational modifications. In addition, from a production scale-up perspective, this yeast offers several distinct advantages, as mentioned earlier.

To design the tetravalent antigen, we spliced the EDIIIs of DEN-1, DEN-2, DEN-3, and DEN-4 viruses using flexible pentaglycyl linkers. Using the Drosophila cell expression system, Hawaii Biotech, is reported to have developed a tetravalent vaccine, prepared by mixing the E proteins of all four DEN serotypes plus the NS1 protein of DEN-2. This has been reported to elicit PRNT<sub>50</sub> titers > 1:100 in animals. Another tetravalent vaccine, based on a recombinant protein mixture, has been reported by Simmons and others. In this instance, the vaccine contained four recombinant EDIII proteins, each one expressed in E. coli as a maltose binding protein (MBP) fusion.

More recently, DEN-1, DEN-2, and DEN-3 EDIII proteins have been expressed using the Neisseria meningitides P64K protein as a carrier. These proteins have been shown to be partially purified (~35–70%), with purity ranging from 5% to 70%. Virus-neutralizing antibodies induced by these proteins, in mice, were found to vary over a wide range. For example, PRNT<sub>50</sub> titers ranged from as low as 1:15 for DEN-3 to 1:640 for DEN-2. Recent work from this group has indicated that not all EDIII sequences in the context of the P64K carrier protein may be adequately antigenic. We showed previously that it is possible to express high levels of DEN-2 EDIII in E. coli without a fusion partner. In a subsequent study, we showed that an E. coli–expressed, renatured bivalent chimera, consisting of the EDIIIs of DEN-2 and DEN-4 viruses linked together, retained the antigenic integrity of its precursors and was capable of eliciting neutralizing antibodies toward DEN-2 and DEN-4 but not to DEN-1 and DEN-3. We built on this and created the tetravalent EDIII-based chimeric protein, rEDIII-T, by including the EDIIIs of the remaining two serotypes. Because this tetravalent protein would be disulfide rich with each of the four EDIII components contributing one S-S bridge apiece, we decided to express the rEDIII-T protein using a eukaryotic rather than a prokaryotic host. We chose to express the rEDIII-T protein in P. pastoris, a yeast host, which combines the high expression potential of the prokaryotic systems and the capacity of eukaryotic systems to facilitate post-translational modifications. In addition, from a production scale-up perspective, this yeast offers several distinct advantages, as mentioned earlier.

To design the tetravalent antigen, we spliced the EDIIIs of DEN-1, DEN-2, DEN-3, and DEN-4 viruses using flexible pentaglycyl linkers. We previously used a similar linker successfully to create a chimeric protein encoding multiple DEN epitopes for diagnostic use. To facilitate purification of the rEDIII-T protein, we engineered a 6× His tag at its carboxy terminus. With a view to simplifying downstream processing, we attempted to secrete the rEDIII-T protein by inserting the S. cerevisiae α-factor secretion signal at its amino terminus. However, we found that signal peptide cleavage was incomplete, and secretion was consequently inefficient. This obser-
viation is consistent with others who have also reported difficulty in secreting P. pastoris–expressed DEN-1 and DEN-4 E proteins into the culture supernatant.

The intracellularly expressed rEDIII-T protein was purified under denaturing conditions because its efficient extraction from the induced biomass could be achieved only in the presence of 6 mol/L guanidine hydrochloride. However, the purified protein remained soluble in the absence of denaturant. Based on Coomassie-stained SDS gel analysis, we estimate the level of purity of rEDIII-T to be ≥95%. The two forms seen on the gel presumably represent the precursor and mature forms of the rEDIII-T protein based on the protein blot data using penta-His mAb and anti-rEDIII-T antibodies and Con A–FITC. The larger than expected size of the precursor seems to be caused by heavy glycosylation of its signal peptide. The yield of rEDIII-T protein from a liter culture of P. pastoris was ~54 mg. This represents a significantly high yield reported for a DEN antigen.

To evaluate its immunogenicity, the purified rEDIII-T protein was injected intraperitoneally into BALB/c mice with various adjuvants such as Freund adjuvant, alum, and montanide. We used Freund adjuvant as a control to evaluate alum, an adjuvant approved for human use, and montanide ISA 720, an experimental adjuvant intended for human use.26 The questions we addressed were would the antibodies elicited by rEDIII-T bind to all serotypes of DEN viruses? If so, would such binding block virus adsorption to host cells and neutralize virus infectivity? Using an immunofluorescence approach to visualize cell surface–bound virus, we observed that the anti-rEDIII-T antisera could detect all the four DEN virus serotypes in infected BHK cells. It is likely that because EDIII is involved in host receptor recognition, antibodies elicited by the rEDIII-T protein may specifically bind to EDIII on the DEN virion surface and thereby preclude its binding to host cell surface. This was borne out by PRNT data, which showed that the infectivity of all four DEN viruses could be effectively neutralized by the anti-rEDIII-T antisera obtained from both the Freund and montanide adjuvant groups. For reasons that are not clear, alum, which served as a good adjuvant in eliciting high PRNT50 titers against DEN-1, DEN-2, and DEN-4, was not as good with regard to PRNT50 titers against DEN-3. The neutralizing titers reported recently, using the EDIII–p56 plasmid mixture referred to earlier, were ~1:10 for each of the four DEN serotypes.27 In contrast, a tetravalent mixture of EDIII-MBP proteins was reported to elicit PRNT50 titers of 1:240, 1:1,024, 1:510, and 1:80 against DEN-1, DEN-2, DEN-3, and DEN-4, respectively.21 The observed differences are very likely a reflection of the nature of antigen, route of immunization, and, most importantly, differences in the experimental parameters of the PRNT assay. In the absence of a good animal model for dengue, neutralizing antibody titers are widely accepted as surrogate markers of protective immunity. The PRNT50 titers of 1:10 are considered indicative of protective immunity.15,16 Our data clearly show the potential of the rEDIII-T protein to elicit neutralizing, and therefore, presumably protective antibodies against all four DEN virus serotypes.

T-cell responses were studied by monitoring the magnitude of cell proliferation and production of the cytokines IFN-γ and IL-4 in splenocytes obtained from immunized mice in response to virus stimulation in vitro. Splenocytes from all the three groups of mice displayed pronounced proliferative response on incubation with any of the four DEN viruses. Of the two cytokines studied, IL-4 secretion by splenocytes from rEDIII-T–immunized animals (from all three adjuvant groups) was enhanced significantly in response to in vitro stimulation by each one of the DEN virus serotypes. IL-4, which is a B-cell stimulatory cytokine, may contribute to the observed DEN virus-neutralizing antibody response. In striking contrast, in vitro–stimulated splenocytes manifested barely discernible increases in secreted IFN-γ levels. Overall, the data indicate that the T-cell response elicited is predominantly Th2 type. This is consistent with the known tendency of protein vaccines to be presented to T lymphocytes in association with major histocompatibility complex (MHC) class II molecules. Recently, investigators have begun testing protein vaccines in association with DNA vaccines as a way to elicit Th1 responses as well, which are important in clearing virus-infected cells.63

In conclusion, we developed a non-replicating subunit vaccine prototype based on EDIII, a critical domain of the major DEN virus E protein that mediates viral entry and contains multiple neutralizing epitopes and induces robust protective immunity. Importantly, the EDIII precursor has intrinsically low potential for eliciting enhancing antibodies. We endeavored to develop a single tetravalent vaccine antigen based on EDIII as a simpler and less expensive alternative to expressing and purifying four antigens. We have successfully expressed this protein in the yeast P. pastoris and obtained purified protein using a one-step affinity protocol in high yields. However, the failure to secrete the rEDIII-T protein into the medium and the necessity to carry out its purification under denaturing conditions are issues that need to be resolved. Work is underway to address these concerns.

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Authors’ addresses: Behzad Etemad, Gaurav Batra, Rajendra Raut, Satinder Dahiya, Saima Khanam, Sathyamangalam Swaminathan, and Navin Khanna, RGP Group, PO Box 10504, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India, Tel: 91-11-26742357, Fax: 91-11-26742316.

Reprint requests: Navin Khanna, RGP Group, PO Box 10504, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India, Tel: 91-11-26742357, ext. 272, Fax: 91-11-26742316, E-mail: navin@icgeb.res.in.

REFERENCES


