Immunoglobulin M Enzyme-Linked Immunosorbent Assay Using Recombinant Polypeptides for Diagnosis of Dengue

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We demonstrate that a mixture of four recombinant dengue virus E polypeptides corresponding to the N-terminal region of the envelope protein from all serotypes substitutes for standard antigens in two immunoglobulin M enzyme-linked immunosorbent assay formats with 100% concordance, making these polypeptides a useful and accessible reagent for serological diagnosis of dengue in endemic countries.

Dengue is a major global public health problem with increasing numbers of cases and expanded geographic distribution (5). The four serotypes of the dengue flavivirus (DENV1 through DENV4) have a positive-sense RNA genome that is translated as a single polyprotein and posttranslationally cleaved into three structural proteins and seven nonstructural proteins (8). The envelope protein (E) is considered to be the immunodominant protein (10).

Large-scale dengue diagnosis relies on serological testing. While analysis of the change in immunoglobulin M (IgM) or IgG antibody titer in paired acute- and convalescent-phase sera is considered the "gold standard," in practice, the most commonly used diagnostic test is the IgM capture enzyme-linked immunosorbent assay (MAC-ELISA) (13) with a single specimen. Viral antigen, commonly prepared in cell culture or in suckling mouse brain, is often the limiting reagent in developing countries in which in-house ELISAs are used instead of expensive commercial kits. The simplified production of recombinant DENV antigen in bacteria avoids problems associated with the quality and standardization of conventional DENV antigen preparations (2, 4, 7, 9, 11, 14). Previously, we identified and produced a candidate diagnostic polypeptide corresponding to the N-terminal portion (166 amino acids) of the immunogenic E protein of DENV2 (3). Here, we produced homologous recombinant E antigens from DENV1, DENV3, and DENV4, which, in combination with the DENV2 polypeptide, have great potential as a low-cost and accessible tetravalent dengue diagnostic reagent.

A ~500-bp fragment from the N terminus (nucleotides 1093 through 1585) of the DENV E gene was amplified by reverse transcriptase PCR (3) using specific primers for each serotype (D1E-F [GTCGGATCCCCTGCCGTCCTGCGCAAACTG TG] and D1E-R [GGGCTGCAGTCATAAGTCTAGAAAC CATTGTTTGTG], D3E-F [GCCGGATCCCTGGCGACCC TAAGGAAGCTATG] and D3E-R [GGGCTGCAGTCAGT CAAAGAACCATTGTCTATG], and D4E-F [GCCGGATC CGTGGCTCTGTTAAGAACCTATTG] and D4E-R [GGGC TGCAGTCAATTCAAAAACCATTGCTTATG]), inserted into a TA cloning vector (Invitrogen, Carlsbad, CA) (3), and sequenced. The identities of the products were confirmed by comparison to known sequences (GenBank). The cDNAs were then cloned in-frame into the pQE-30 expression vector (3) immediately downstream of the hexahistidine tag.

Expression of the recombinant polypeptide clones in transformed *Escherichia coli* M15 was assessed by induction with 1 mM isopropyl-D-thiogalactoside (IPTG) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of aliquots at various time points. Large-scale expression of the expected ~20-kDa recombinant proteins (200 ml Super-Broth) yielded enough soluble protein for nickel affinity purification. Bacterial lysis and binding to affinity resin were conducted under denaturing conditions, whereas elutions were performed under native conditions, according to the protocol previously established for DENV2 (Fig. 1A) (3). DENV2 clone pD2-3 (3) was used to prepare the DENV2 E polypeptide.

The specificity of the polypeptides was tested by Western blot analysis as described before (3) with minor modifications, using sera from Nicaraguan dengue patients and polyclonal anti-E antibodies. Two acute-phase sera (with high and low titers of anti-DENV IgM), one convalescent-phase serum sample (high anti-DENV IgG titer), rabbit anti-E antibodies, and a serum sample negative for anti-DENV IgM were tested. The positive sera (Fig. 1B) and the polyclonal antibodies recognized all the recombinant polypeptides, while the negative control did not (data not shown). The sensitivities of the individual polypeptides were compared to that of a mixture of the four by dot blot using serial dilutions of each antigen. Serum known to contain anti-DENV IgM recognized the mixture of the four recombinant antigens at a dilution of 1:15 (66 ng/ml), whereas the thresholds of detection of single polypeptides ranged from 100 to 200 ng/ml (Fig. 1C).

The performance of the novel tetravalent polypeptides was compared to that of antigen prepared from infected cell lysates (cellular antigen) in a modified in-house MAC-ELISA (1). Briefly, plates coated with anti-human IgM (Sigma Chemical Co., St. Louis, MO) were incubated with 1:20 dilutions of

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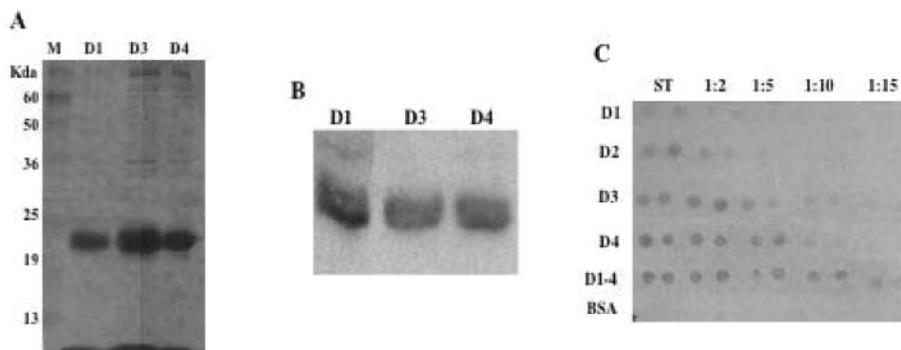
FIG. 1. Purification and evaluation of DENV E polypeptides. (A) SDS-PAGE analysis of fractions eluted from nickel affinity chromatography containing the DENV1, DENV3, and DENV4 ~20-kDa E polypeptides induced with 1 mM IPTG in *E. coli* M15 transformed with the pQE-30 expression plasmids. (B) Western blot. Two micrograms of DENV1, DENV3, and DENV4 E peptides were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with serum from an acute-phase dengue patient with DENV-specific IgM antibodies. (C) Dot blot. Serial dilutions (starting at 1 μg/ml) of individual or a mixture of the four recombinant E peptides were applied to a nitrocellulose membrane and exposed to acute-phase dengue serum containing DENV-specific IgM antibodies. Bovine serum albumin (BSA) was used as a negative control.

serum samples, followed by the tetravalent recombinant polypeptides (30 μg/ml) and then the secondary antibody (anti-DENV IgG-peroxidase conjugate prepared from hyperimmune sera) diluted 1:5,000. The reaction with substrate tetramethylbenzidine (Sigma) was stopped with 2.5% sulfuric acid after 30 min. Negative controls consisted of sera from healthy donors confirmed as being negative for DENV-specific antibodies by ELISAs using cellular antigen (6), while positive controls were sera from patients serologically confirmed as being positive for anti-DENV IgM by MAC-ELISA (6). These sera were collected during a period when DENV2, DENV3, and DENV4 were circulating in Nicaragua. Fifteen acute-phase sera, 15 convalescent-phase sera, 11 sera from yellow fever (YF) vaccinees, and 10 sera positive for anti-rubella IgM were tested. An excellent correlation between the two assays was found ($R^2 = 0.98$), with 14 of the 15 acute-phase sera defined as positive and 1 as negative by both assays. Likewise,

11 convalescent-phase serum samples were positive and 4 were negative by ELISA with either antigen. None of the YF-vaccinated or rubella-positive sera were positive by either assay (Fig. 2A).

An indirect IgM ELISA (6) with antigen bound to the polystyrene plate was evaluated by using four sources of antigen (the four recombinant E polypeptides, DENV2 recombinant E protein, cellular antigen [DENV1 through DENV4] [1], and mouse brain antigen [DENV1 through DENV4] [Instituto de Medicina Tropical Pedro Kouri, Havana, Cuba]). Recombinant E protein was expressed from baculovirus $AcD2E\Delta H_6$ in Sf9 cells and purified as described previously (12). Human rheumatoid factors (anti-IgG IgM antibodies) were removed from all serum specimens (previously diluted 1:20) by pretreatment with a 1:5 dilution of anti-human IgG (Sigma). For the
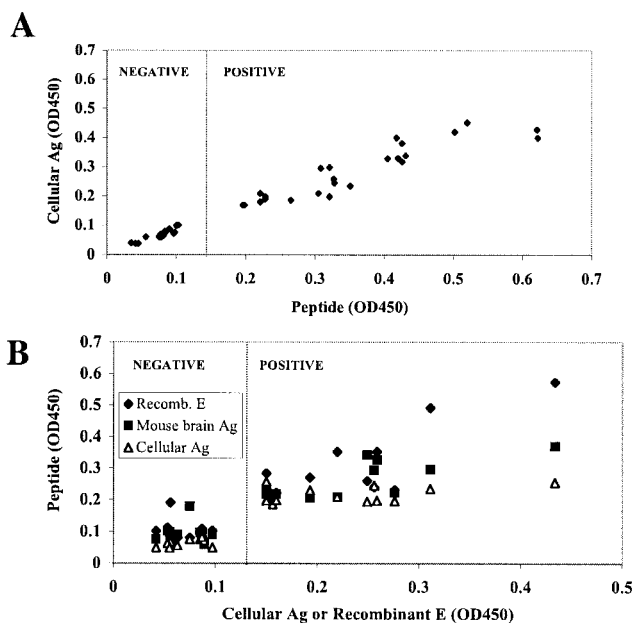

FIG. 2. Comparison of tetravalent E polypeptides with conventional antigens in ELISA assays. (A) IgM capture ELISA. The results obtained using cellular antigen and a mixture of the four DENV E polypeptides as an antigen were plotted against each other. Acute-phase sera (taken within the first 7 days since the onset of symptoms) and convalescent-phase sera (taken 8 or more days since symptom onset) from suspected dengue cases and sera from YF vaccinees and from rubella-positive cases were tested. The sera from YF vaccinees were a gift of X. Aguilar, Universidad Mayor de San Andrés, La Paz, Bolivia; the rest were samples sent to the Virology Laboratory at the Centro Nacional de Diagnóstico y Referencia, Nicaraguan Ministry of Health, for routine testing for either dengue or rubella. Cutoff values were 0.160 for E polypeptides and 0.106 for cellular antigen. (B) Indirect IgM ELISA. Tetravalent recombinant E polypeptides were compared with three different antigens: DENV2 recombinant E protein (Recomb. E), antigen from DENV1 through DENV4 produced in suckling mouse brain (mouse brain Ag), and antigen produced by lysis of cultured C6/36 cells infected with DENV1 through DENV4 (cellular Ag). Cut-off values were 0.145 for E polypeptides, 0.138 for recombinant E protein, 0.160 for mouse brain antigen, and 0.164 for cellular antigen. For both assays, the cutoff was twice the average optical density (OD) (450/630 nm) of the negative controls (included in triplicate per plate). R^2 analysis of correlation was performed with Excel. Chi-square analysis conducted using Epi-Info (Centers for Disease Control and Prevention) yielded a P value of <0.001 for comparison of results obtained with the E polypeptides versus each of the other DENV antigens.

ELISA, plates were coated with 2.5 μg/ml of each antigen and blocked with 3% nonfat dry milk. Serum samples were incubated for 1 h at 37°C, followed by incubation with a goat anti-human IgM-horseradish peroxidase conjugate (Sigma) diluted 1:5,000 in blocking buffer, and detected with tetramethylbenzidine as described above. Three washes with phosphate-buffered saline–Tween followed each step. Twelve acute-phase sera previously confirmed as positive, 5 convalescent-phase sera previously confirmed as negative for DEN-specific IgM antibodies, 6 sera from YF vaccinees, and 10 rubella-positive sera were tested in all assays in triplicate. The acute-phase sera were positive for IgM antibodies with all three antigens, whereas the convalescent-phase, YF-vaccinated, and rubella-positive control sera were negative in all ELISAs (Fig. 2B and data not shown). Correlations (R^2) of 0.91, 0.90, and 0.84 were obtained by comparing the four E polypeptides with recombinant E protein, mouse brain antigen, and cellular antigen, respectively. The polypeptides and standard cellular antigen discriminated between positive and negative samples with 100% concordance (Fig. 2B). Both the DENV polypeptides and cellular antigen were more specific than recombinant E or mouse brain antigen, each of which detected one low false positive from YF-vaccinated serum samples (Fig. 2B).

Routine diagnosis of dengue is performed predominantly by IgM capture ELISA, which requires large amounts of DENV antigen produced in mouse brain or cell culture, often unavailable in laboratories across the developing world. We have produced an alternate source of DENV antigen by expressing in *E. coli* a recombinant fragment of the E protein from all four DENV serotypes that, according to our calculations, costs approximately one-half as much to prepare as does cellular antigen. Our data suggest that a tetravalent preparation of the recombinant DENV E polypeptides can be used as a substitute for the traditional DENV antigens in a MAC-ELISA and indirect ELISA format.

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