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Carboxy-terminally truncated Dengue 4 virus envelope glycoprotein expressed in *Pichia pastoris* induced neutralizing antibodies and resistance to Dengue 4 virus challenge in mice

Brief Report

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Summary. We have expressed a recombinant Dengue 4 virus envelope glycoprotein (E4rec), truncated at its C-terminus by 53 amino acids, in *Pichia pastoris*. The presence of E4rec was confirmed by Western-blot using anti-DEN 4 hyper immune mouse ascitic fluid. E4rec migrated during SDS-PAGE as a 64 kDa protein. Treatment with endoglycosidases showed that the E protein was modified by the addition of short mannose chains and the absence of hyperglycosylation. When administered to BALB-C mice, E4rec elicited a DEN 4 neutralizing antibody response haemagglutination inhibition antibodies and specific memory T cell response. Mice immunized were also significantly protected against lethal DEN 4 virus challenge (86.6%, $p < 0.001$).

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The dengue (DEN) virus serocomplex contains four serotypes: DEN 1, 2, 3 and 4. These viruses are human pathogens and a significant threat to world health. *Dengue virus* is estimated to cause several hundred thousand cases of dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) annually. Dengue viruses are members of the family *Flaviviridae* and are transmitted by *Aedes aegypti* mosquitoes [4, 11]. The flavivirus genome is a single stranded, positive-sense RNA molecule of 11 kb containing a single open reading

frame arranged as follows: C-preM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. The flavivirus envelope (E) protein, the major surface protein of the virion, elicits the production of highly neutralizing antibodies and protective immunity. It is involved in virion attachment to host cells and can be detected by its ability to haemagglutinate goose erythrocytes. These biological functions are dependent upon six disulfide bridges and on the topographical arrangement of discontinuous domains [13]. Recombinant flavivirus E proteins produced in different expression systems such as *E. coli*, vaccinia and baculoviruses have elicited variable degrees of protective immunity when tested experimentally in animal models [6, 7, 9, 18]. Best results in terms of neutralizing antibodies and protection against challenge have been obtained when the E protein is truncated at the C terminus [5, 6].

In spite of the advantages of yeast as a protein expression system, few studies have addressed the question of flavivirus recombinant E protein expressed in this system. Recently, Bisth et al have expressed the E protein of DEN 2 virus (NGC strain) as a chimera with hepatitis B surface antigen (HBsAg). The protein was efficiently expressed in *Pichia pastoris* [1]. Yeast represents an alternative eukaryotic expression system for the generation of flaviviral glycoproteins. Previous studies have demonstrated that Japanese encephalitis virus (JEV) E protein expressed in *Saccharomyces cerevisiae* can elicit neutralizing antibodies in experimental animals [10]. More recently, Sugrue et al., 1997, have demonstrated expression of DEN 1 E protein in *Pichia pastoris* [24].

We have constructed a recombinant DEN 4 E protein from which the C-terminal 53 amino acids (the C-terminal anchor) have been deleted. The protein was expressed in *Pichia pastoris*. RNA was extracted from AP61 cells infected with DEN 4 814669 strain. A full-length cDNA fragment comprising the E gene was made by reverse transcriptase-polymerase chain reaction (RT-PCR) [8]. The glycoprotein E gene was amplified by PCR using specific primers (sense, 5′ GG GAATTCT ATGCGATGCTTAGGAGTAGGA 3′; antisense 5′ GG GAATTCTTA AAACATCCT GCC AAT GGA ACT 3′).

The resulting PCR fragment of 1202 bp was digested with EcoRI and ligated into the dephosphorylated EcoRI site of a plasmid vector, pfAO. This vector is basically identical to vector ppS-7 that was previously reported (EP European Patent Application EP 438200), but it contain MF_{α} propeptide leader sequence from *Saccharomyces cerevisiae* as the signal sequence, generating the clone $pDfE_4$-47.

The presence of the DEN E translation initiation and stop codons, the correct orientation of the insert and consequently the correct orientation of the truncated DEN 4 E coding region were verified by DNA sequencing. The yeast recombinant $pDfE_4$-47 vector was digested with the restriction endonuclease *Pst*I and used to transform *P. pastoris* strain MP-36. Electroporation was performed essentially according to Martinez et al. [17]. The E gene was integrated into the genome of *P. pastoris* under the control of the methanol – inducible alcohol oxidase (AOX1) promoter. After induction the Den 4 E recombinant protein (E4rec) was expressed and partially purified using the procedure described by Sariol et al. [21].

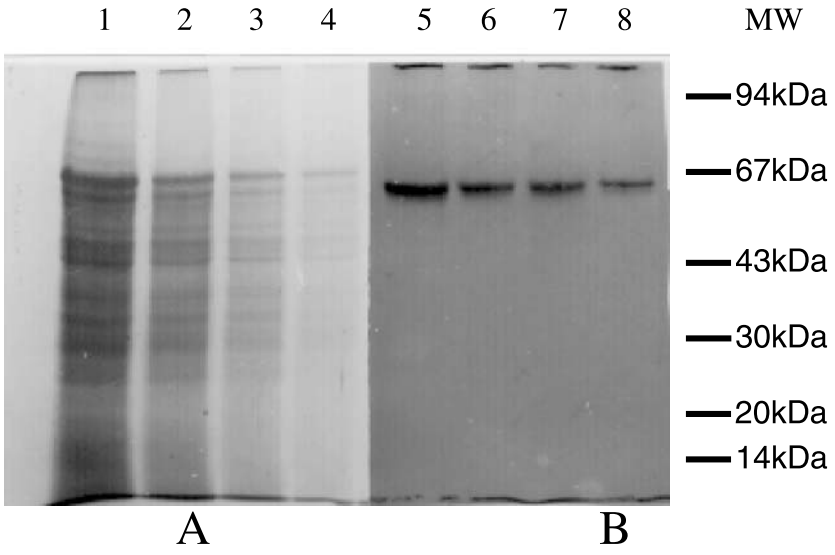

Fig. 1. Polyacrylamide gel electrophoresis and Western blot analysis of E4rec. **A** Polyacrylamide gel stained with Coomassie blue, *1–4*: 1/2, 1/4, 1/8, 1/16 dilutions of E4rec. **B** Separated protein bands were transferred to a nitrocellulose membrane and reacted with DEN-4 polyclonal hyperimmune mouse ascitic fluid (*5–8*)

E4rec was detected on Western blots (Fig. 1) using anti-DEN 4 hyper immune ascitic fluid (HMAF) [2, 15]. The immunoblot revealed the presence of a specific 64 kDa antigenic band. E4rec was associated with the insoluble fraction after cellular disruption and it was not detected in the culture medium despite repeated screening. Sugrue et al., reported that DEN 1 E recombinant protein expressed in *Pichia pastoris* was not detected in the culture medium presumably due to the complex nature of the yeast cell wall [24]. Men et al. observed that DEN 4 E protein with a 95 amino acid C-terminal truncation was not secreted and accumulated in small amounts on the cell surface [19]. DEN 1 and DEN 4 E proteins produced in baculoviruses were not secreted suggesting that the absence of secretion was due to the inappropriate length or nature of the signal sequence such that it could not be cleaved properly [16]. The N-terminal amino acid sequence analysis of the E4rec indicated the presence of the MF_α propeptide leader sequence (from *Saccharomyces cerevisiae*), suggesting that the MF_α was incompletely processed. This could be one of the reasons the lack of secretion of the recombinant protein.

The glycosylation state of E4rec was analyzed using endoglycosidase H (Endo H). One hundred μg of E4rec in 50 mM phosphate- citrate buffer at pH 6.5 was digested for 18 hours at 37 °C with 0.01 units of Endo H. E4rec protein was sensitive to treatment with this glycosidase. The difference in size of the glycosylated and deglycosylated proteins was approximately 6 kDa which is consistent with the modification of both potential glycosylation sites in the E protein and demonstrating the absence of hyperglycosylation.

Groups of 15 BALB-C mice were immunized by the intramuscular, intraperitoneal and subcutaneous routes on days 1, 7 and 21 with 100 μg of the E4rec

protein emulsified in complete (first injection on day 1) or incomplete (second and third injections on days 7 and 21) Freund adjuvant. A group of control mice were immunized with control yeast.

Blood was drawn from the retro-orbital sinus on day 28 after the first inoculation and sera were assayed for DEN 4 antibodies by enzyme linked immunosorbent assay (ELISA). Briefly, plates were coated with human anti-dengue immunoglobulin. After 18 h (overnight) incubation at 4 °C, plates were washed with PBS-Tween 0.05% and incubated for 1 h at 37 °C with DEN 4 antigen prepared from infected suckling mouse brain. After washing the plates, serial dilutions of sera from immunized and control mice were added and incubated for 1 h at 37 °C.

Signal was detected using peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham-Pharmacia, UK) and H_2O_2 ortho-phenylenediamine. The reaction was stopped by addition of 12% H_2SO_4 and the color intensity was read at 490 nm in an ELISA reader. Wells containing serial dilutions that showed half or less than half the OD obtained for the negative control serum were considered to be positive.

The memory T cell immune response in mice immunized with E4rec and DEN 4 virus respectively was evaluated. To estimate the lymphoproliferative response, mononuclear cells were collected and adjusted to 2×10^6 cells. Cells were cultured in 100 μl of supplemented RPMI 1640 for 4 days at 37 °C, 5% CO_2 in the presence of 20 μg/ml of dengue 1, 2, 3 and 4 virus antigens, control antigen or yeast control respectively. Phytohaemagglutinin at 5 μg/ml was used as a positive control for proliferation. To measure the proliferative response, cells were pulsed with 1 μCi of tritiated thymidine ([^{3}H] TdR, Amersham) for 6 h before harvesting. [^{3}H] TdR incorporation was measured using a liquid scintillation counter (LKB 1214 Wallac rack β).

We found a E4rec specific memory T cell response in immunized mice. Splenocytes from E4rec immunized mice recognized DEN 4 virus without serotype cross reactivity. A lymphoproliferative response to E4rec was also found in mice immunized with DEN 4, suggesting to us that immunodominant epitopes were present in the E4rec protein sequence. These results show the ability of our protein to elicit a T cell response, which is an essential attribute for a good immunogen.

Sera were also tested for DEN antibodies by haemagglutination inhibition (HI) and plaque reduction neutralization test (PRNT) [3, 20]. Protection against experimental dengue encephalitis was evaluated by intracerebral inoculation of 100 LD_{50} of mouse adapted DEN 4 814669 strain. Data were analyzed for statistical significance using the chi- square or Fisher's exact tests as appropriate.

Sera from individual mice developed strong IgG responses to the homologous DEN 4 antigen as detected by ELISA. The geometric mean antibody titer (GMT) was 1/141323 (Table 1). Western-blots showed that the reactivity was specifically directed towards the native DEN 4 E protein (data not shown). Some individual sera (50%) exhibited HI antibodies at dilutions of 1/5 to 1/20. Seven sera (70%) showed neutralizing antibody titers higher than 1/10 (Table 1). Control mice,

Table 1. Serum antibody responses of mice immunized with E4rec protein by ELISA, HI and PRNT

Serum	ELISA[a] Reciprocal end-point titers	HI	PRNT[b]
1	32000	1:5	>10
2	256000	1:10	>10
3	128000	<1/5	>10
4	64000	<1/5	>10
5	64000	<1/5	<10
6	64000	<1/5	>10
7	256000	<1/5	>10
8	256000	1:20	<10
9	256000	1:20	>10
10	256000	1:20	<10

[a]Sera were analyzed at 1/100 dilution

[b]Results are expressed as the reciprocal of 50% plaque reduction neutralization titer against DEN 4 serotype

Table 2. Survival of mice immunized with E4rec after challenge with live DEN 4 virus

Immunizing inoculum	No. mice surviving/no. mice tested
E4rec	13/15[a]
Negative control	0/15

[a] $p < 0.001$ when compared to yeast negative control

immunized with control yeast showed antibody titers lower than 1/100 by ELISA and lower than 1/5 and 1/10 by HI and PRNT respectively.

Immunized mice were challenged with DEN 4 virus, Thirteen out of fifteen immunized animals resisted the challenge (survived), indicating that E4rec had induced good protection against lethal encephalitis (86.6%, $p < 0.001$) (Table 2). None of the non-immunized mice survived the challenge.

The level of the antibodies against dengue virus and the presence of neutralizing and HI antibodies we obtained is in agreement with the results from other authors on the usefulness of recombinant E protein as an immunogen with a similar number of immunizations. For example, Feighny et al. obtained 90% and 80% viral protection using truncated E protein with 20% of the C terminal region removed [9].

In addition a recombinant Dengue envelope B domain against DEN 2 virus infection showed good neutralizing antibody response and partial protection against DEN 2 challenge infection in mice [23]. Another report that used recombinant proteins containing the B domains of all four dengue serotypes fused to the maltose

binding protein (MBP) of *E. coli* showed that sera from mice immunized with the tetravalent DEN subunit vaccine neutralized all 4 DEN viruses. Moreover, sera from mice immunized with monovalent DEN-MBP recombinant protein vaccines developed a strong IgG antibody responses to the homologous purified whole virus as detected by ELISA [22].

Recently, our E4rec purified by either immune-affinity chromatography or immobilized metal ion adsorption chromatography (IMAC) produced comparably functional antibodies in mice, as assayed by HI and PRNT [14]. In addition, monkeys immunized with E4rec were partially protected against viremia [12].

In this report we demonstrated that Den 4 truncated E recombinant protein expressed in *Pichia pastoris* was able to elicit protection levels in mice similar to other well characterized recombinant proteins and more specifically to produce neutralizing antibodies, protection to challenge, and specific lymphoproliferative T cell responses to both the E4rec protein and dengue 4 virus. This study is the first to evaluate protection against challenge and T cell responses in small animals (mice) immunized with recombinant dengue protein expressed in *Pichia pastoris*. Our results suggest that this system should be considered a viable candidate for expression of dengue recombinant proteins.

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