

Antigenic cell associated dengue 2 virus proteins detected in *vitro* using dengue fever patients sera

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Abstract

At least three major antigenic dengue 2 virus proteins were recognized by pooled dengue fever patients' sera in infected *Aedes albopictus* (C6/36) mosquito cells. Dengue virus envelope (E), pre-membrane (PrM) and non-structural protein 1 (NS1) dimer were detected beginning on day 3 post-infection in both the cell membrane and cytosolic fractions. Using the patients' sera, the presence of antigenic intermediate core protein (C)-PrM and NS1-non-structural protein 2a (NS2a) in the cytoplasmic fraction of dengue 2 virus infected cells was revealed. The presence of a ~92 and ~84 kDa NS1 dimer in the membrane (NS1_m) and cytosolic (NS1_c) fractions of C6136 cells, respectively, was also recognized. Using individual patient's serum, it was further confirmed that all patients' sera contained antibodies that specifically recognized E, NS1 and PrM present in the dengue 2 virus-infected cell membrane fractions, suggesting that these glycosylated virus proteins were the main antigenic proteins recognized *in vivo*. Detection of dengue 2 virus C antibody in some patients further suggested that C could be antigenic if presented *in vivo*.

Key words: antigenic, dengue fever, dengue virus, mosquito, patients

INTRODUCTION

Dengue virus infection is endemic in most South East Asian countries. In recent years, this mosquito borne disease has spread to many other countries in the tropics and subtropical regions.¹ The disease is caused by any one of the four serotypes of dengue virus; dengue 1 virus, dengue 2 virus, dengue 3 virus and dengue 4 virus. This positive single stranded RNA virus replicates efficiently in the mosquito vector, *Aedes aegypti* and the human host. In the laboratory, dengue viruses have been shown to infect various cell lines with marked differences in efficiency depending on the cell type. *Aedes albopictus* mosquito cells (C6/36), in particular allow efficient virus replication *in vitro*.^{2,3} In contrast, cells from human origin, including monocytes, fibroblasts and endothelial cells did not support dengue virus replication well.^{4,6} This is in spite that human is the intermediate host for dengue virus and it is apparent that the virus replicates and spreads efficiently throughout the body to result in clinical manifestations of the infection and high presence of virus in the blood during the viremic phase.⁷

Attempts to increase infectivity of human cells *in vitro* using antibody treated virus resulted in only marginal increase in productive dengue

virus infection.^{6,8} These observations have led to the suggestion that specific cellular factors are probably involved in determining the efficiency of dengue virus replication *in vivo* and *in vitro*.⁹ These factors could exert directly or indirectly specific effects on the mechanisms that affect the efficiency of viral polymerase activities and processing of viral proteins. It is also possible that some of these cellular factors could act differently *in vitro* versus *in vivo*. Using various non human cell culture systems, it was established that dengue virus initiates infection by binding to receptors present on the cell surface.¹⁰⁻¹² Following attachment, the virions enter the host cell and the genome is released into the cytoplasm.¹³⁻¹⁵ The genomic RNA is translated into polyprotein that are then cleaved by viral and cellular proteases to generate three major glycosylated structural proteins and at least seven nonstructural proteins.^{16,17} The core protein (C) made-up the capsid of the virus, whereas, the pre-membrane protein (PrM) is cleaved during maturation of the virions to release the matrix protein (M).¹⁸⁻²⁰ The envelope (E) protein facilitates binding of the virus to host cells.^{21,22}

Among the nonstructural proteins, only non-structural protein 1 (NS1) was found to be glycosylated and it has been shown to form

dimers, tetramers and hexamers with the dimers being the most commonly detected.²³ In general, expression of dengue viral proteins has been studied using *in vitro* cell culture systems. Whether there are differences in dengue virus protein expression and processing in human cells *in vivo* is not presently known. Furthermore, due to limited productive dengue virus replication in human cells *in vitro*, it is not possible at this time to deduce how dengue virus proteins are expressed *in vivo*. In the present investigation, dengue 2 virus infected *Aedes albopictus* cells were used to examine for the presence of dengue virus specific antibodies in sera of clinically and serologically confirmed dengue fever patients. It is suggested that detection using the patients' sera would also enable examination of the kinetics of expression and processing of antigenic dengue virus proteins.

MATERIALS AND METHODS

Cells and virus cultures

Aedes albopictus cells (C6/36) used in the study were purchased from the American Type Culture Collection (ATCC, USA). Cells were cultured at 28°C in RPMI 1640 (Gibco BRL, USA) supplemented with 10% fetal bovine serum (Flowlab, Australia). The C6/36 cells were also used for propagation of dengue 2 virus (New Guinea C strain, NGC). The initial virus inoculum was purchased from ATCC (USA) and the subsequent inocula prepared from the stock were used throughout the study. Virus inoculum was prepared as previously detailed²⁴ and the supernatant was harvested when the virus hemagglutination titer (HA) reached 1:256. Virus titer was determined by performing virus plaque assay as previously described.²⁵

Cell membrane preparation

Cells were infected with the virus inoculum to give an estimated multiplicity of infection (MOI) of 3-5 plaque forming unit (PFU)/ cell. At selected intervals post-infection (PI) or on day 0 (1-2 hr PI), cells were rinsed with serum free medium, harvested using sterile plastic cell scrapers (Costar, USA) and the cell suspension was then centrifuged at 800X g. The pellet was resuspended in HOMO buffer (10 mM Tris-HCl, 150 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂) and cells were sonicated using the Branson Sonifier 250 (USA) at output level 6 with constant duty cycle for 9 minutes in ice-cold water. The lysate was spun at 800X g (Biofuge 17RS, Heraeus, USA) to remove cell debris and

the unbroken cells. The supernatant was then pelleted at 40,000X g (F-20 MICRO, Sorvall RC 26 Plus, Dupont, USA). The supernatant was reserved and used as the cytosolic fraction. The crude cell membrane pellet was resuspended in solubilization buffer (10 mM Tris-HCl, 150 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 6 mM 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propane-sulfonate (CHAPSO), 15 mM n-Octylglucoside, 2.1 μM leupeptin, 2.9 μM pepstatin A, 1 μg/ml aprotinin²⁶ and incubated overnight with constant agitation at 4°C. Subsequently, the cell suspension was spun at 40,000X g and the resulting supernatant consisting of the solubilized cell membrane proteins was reserved. Protein concentration was assayed using the Micro BCA Protein Assay Kit (Pierce, USA) and samples were stored at -70°C in 20% glycerol.

Immunoblot Assays

Equal amount of protein prepared in native form without heat and reducing agent was electrophoresed in 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).²⁷ The separated polypeptides were electrotransferred^{28,29} onto nitrocellulose membrane (MSI, Westborough, MA, USA) and following thereafter, the membrane was blocked with 5% skim milk in Tris buffered saline (TBS; 100 mM Tris base, pH7.5, 150 mM NaCl) and washed rigorously in TBS-Tween 20 (0.05% Tween 20 in TBS).³⁰ The membrane was cut into strips and incubated overnight at 4°C with preadsorbed sera (1:500) of confirmed (based on IgM capture enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition assay and virus isolation) dengue fever patients. The strips were then washed, treated with biotinylated goat anti-human IgG (Kirkegard & Perry Laboratories, USA; 1:500) pre-incubated with alkaline phosphatase-conjugated streptavidin (SA-AP; 1:1000) and developed using NBT-BCIP (Kirkegard & Perry Laboratories, USA). At each washing step, the membranes were washed rigorously to ensure low background. Results were digitized using Epson Expression 1600Pro (Epson, Japan) scanner and analysed using BioNumerics ver. 2.0 (Applied Maths, Kortrijk, Belgium). Identification of the specific protein bands was made using monoclonal antibodies specific against dengue 2 virus E, NS1 and C as previously described.²⁴

RESULTS AND DISCUSSION

The kinetics of antigenic dengue 2 virus protein expression on the cell membrane of C6/36 cells was investigated using pooled dengue fever patients' sera as a probe. Using this method, substantial presence of specific antigenic dengue virus proteins of about 92, 58, 48 and 23 kDa, corresponding to dengue virus NS1 dimer, E, NS1 monomer and PrM, respectively, were detected in the cell membrane fraction beginning on day 3 PI (Fig. 1(a), lane 4). These proteins were present in the cell membrane fraction throughout the duration of the investigation. On the other hand, faint presence of other antigenic proteins of about 34, 28 and 27 kDa, were also detected on day 3 PI (Fig. 1a, lane 4 and Fig. 1b). The presence of these proteins was substantially diminished by day 7 PI (Fig. 1a, lane 6), suggesting that these proteins could be the intermediate precursor proteins of one of the major but smaller antigenic protein. Faint presence of antigenic proteins of ~ 32-31 kDa

were also detected using the patients' sera beginning on day 5 PI (Fig. 1b). Considering that dengue 2 virus PrM is highly antigenic and has an estimated molecular mass of only about 23 kDa, it was likely that these faint proteins were the less abundant intermediate C-PrM (~ 34 kDa) and perhaps the ~ 32-31 kDa proteins could represent variations in the glycosylation of this intermediate precursor protein. Variation in sizes of the C-PrM could also explain the presence of antigenic protein bands of about 28 to 27 kDa which represent perhaps the different species of the PrM with the major one detected as a 23 kDa protein. Thus, the lower molecular mass protein (6-14 kDa) bands (Fig. 1a, lanes 5 and 6) were most likely the resulting C protein from the cleavage of C-PrM. The presence of C-PrM intermediate precursor protein in dengue virus infected C6/36 mosquito cells has been reported previously¹⁹ which they attributed it to perhaps inefficient processing of the protein at lower incubation temperature normally used for culturing mosquito cells. Furthermore, variation

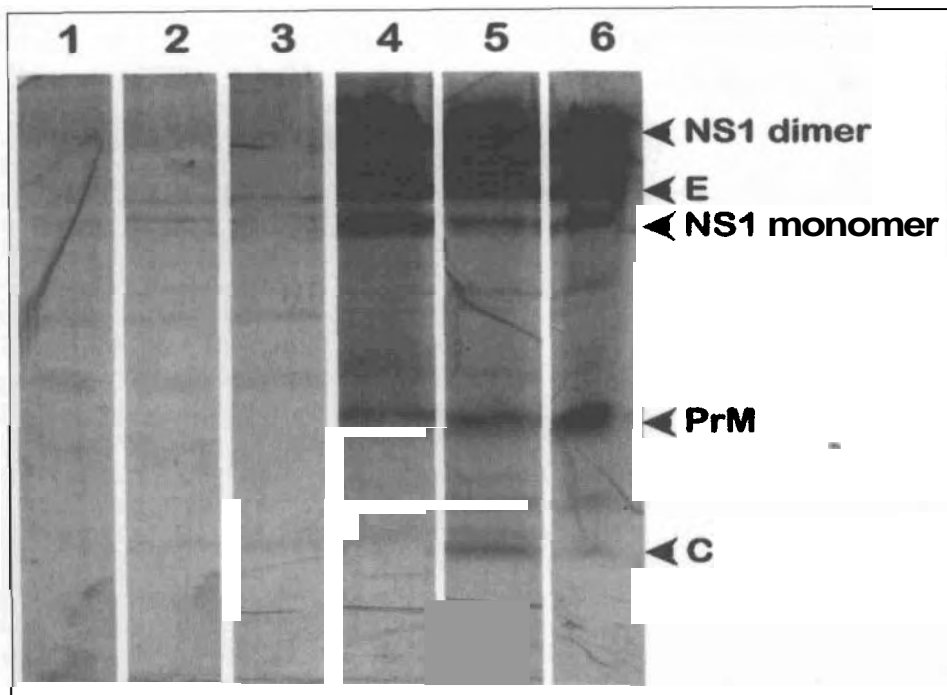


FIG. 1a: Antigenic dengue 2 virus proteins detected in the membrane fraction of C6/36 mosquito cells using pooled dengue fever patients' sera. Membrane fractions of dengue 2 virus infected cells were prepared on day 0 (lane 2), day 1 (lane 3), day 3 (lane 4), day 5 (lane 5) and day 7 (lane 6) post-infection. Whereas, membrane fraction of the mock-treated control was prepared on day 7 PI (lane 1). Patients' IgG against the antigenic dengue 2 viral proteins were detected using biotinylated anti human IgG monoclonal antibody, alkaline phosphatase-conjugated streptavidin, and NBT/BCIP substrate. Designation of the protein bands was made following identification using monoclonal antibodies specific against E, NS1, and C (data not shown).

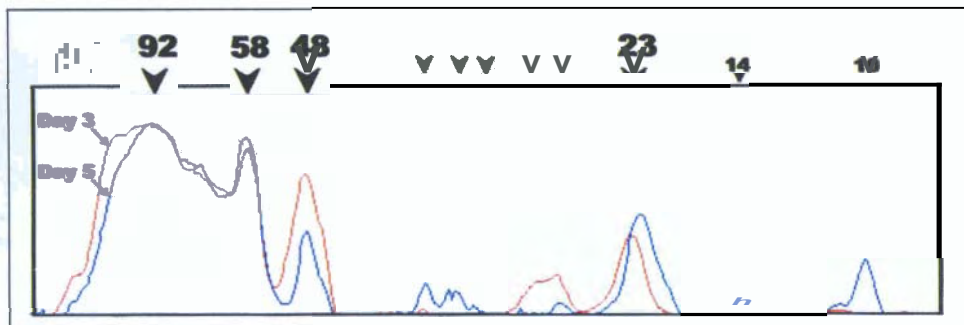


FIG. 1b: Schematic of a pairwise comparison between dengue 2 virus proteins detected in the membrane fraction on day 3 and 5 PI. Estimation of the molecular mass of the protein bands relative to the internal markers was performed using BioNumerics ver. 2.0 (Applied Maths, Kortrijk, Belgium).

in glycosylation of dengue 2 virus glycoproteins has been demonstrated previously with the recovery of glycopeptides of at least three different sizes from Pronase digested dengue 2 virus glycoproteins.³¹ Detection of the intermediate C-PrM proteins in the C6136 cell membrane fraction also suggested that processing of these antigenic structural proteins took place on the cell membrane and this supported earlier observations made using *in vitro* protein processing system in which cleavage of the C-PrM occurred only in the presence of pancreatic microsomal membranes.¹⁸

Examination of the infected C6/36 cells cytosolic fraction using the similarly pooled dengue fever patients' sera, on the other hand, revealed the presence of at least 15 antigenic protein bands. Apart from those major proteins detected in the membrane fraction, additional antigenic protein bands of about 71, 62, 40, 37 and 25 kDa were noted in the C6/36 cell cytosolic fraction beginning on day 3 PI (Fig. 2a, lane 4). These proteins were present in the cytosolic fraction at different intensities until at least day 7 PI except for the 71 kDa protein which became completely undetectable after day 3 PI (Fig. 2b). The presence of the 84, 58 and 23 kDa proteins corresponding to dengue 2 virus NS1 dimer, E and PrM proteins, respectively, increased gradually from day 3 PI (Fig. 2a, lane 4) until day 7 PI (Fig. 2a, lane 6). In contrast, the presence of the 40, 37, 28, 27 and 25 kDa proteins became diminished by day 7 PI (Fig. 2b). The C (10 kDa) protein, on the other hand was detected in the cytosolic fraction on day 5 PI (Fig. 2a, lane 5) but diminished substantially by day 7 PI (Fig. 2a, lane 6). These results obtained using the cytosolic fractions of C6136 cells supported the earlier finding using the membrane fraction that the main antigenic

proteins recognized by dengue fever patients *in vivo* were NS1, E and PrM. However, since C which appeared only transiently on day 5 in both the membrane and cytosolic fractions was also recognized by the pooled sera, it suggested that C was also antigenic *in vivo* though the antibody response was perhaps not as high as the other antigenic dengue 2 virus proteins. This could be due to in general C is not present on the infected cell membrane but is only transiently associated with the membrane during processing of the intermediate precursor C-PrM which takes place in the membrane. Previous study has shown that C is indeed localized mainly in the nucleus,³² suggesting that perhaps following cleavage of C-PrM, C is immediately transported into the nucleus³³ which account for the lack of detection of C in either the membrane or the cytosolic fraction before and after 5 days PI. Nonetheless, results obtained indicating the presence of antibody against dengue 2 virus C in patients' sera suggest that C could be antigenic if exposed to the host immune system *in vivo*. Detection of the 62 kDa antigenic protein only in the cytoplasmic fraction, on the other hand, suggested the presence of the NS1-non-structural protein 2a (NS2a) intermediate protein. The presence of this intermediate protein in the cytoplasm of dengue virus infected cells has been previously reported.³⁴ Its presence only in the cytoplasm suggested that cleavage of the protein took place in the cytoplasm resulting in the relatively more NS1 monomer in the cytoplasm then in the membrane fractions.

In the present study, it was also noted that under the experimental conditions used in which the samples were not treated with heat or any reducing agent, both the monomeric and dimeric forms of NS1 were detected in the cell membrane fraction using the patients' sera (Fig. 1a). The

dimeric form, however, was relatively more abundant than the monomeric form suggesting that in general dengue virus NS1 dimer is preferentially extruded into the cell membrane. Both forms, however, were detected by the patients' sera suggesting that the antigenic sites recognized by the host immune response lies within the exposed monomeric NS1. It was further noted that the NS1 dimer detected in the infected cell cytosolic fraction (NS1_c) using the dengue fever patients' sera had a much lower (84 kDa) molecular mass (Fig. 2a) than those detected in the membrane fraction (92 kDa; NS1_m) (Fig. 1a). This finding, however, was not surprising since NS1, similar to other dengue virus glycoproteins is posttranslationally modified. Thus, it is possible that NS1_m is the mature glycosylated form of the NS1_c. Dimerization and glycosylation of the NS1 involving at least two different glycans have been demonstrated before³¹ and it was suggested that the dimerization occurred even prior to glycosylation of the proteins.^{35,36} Detection of NS1 monomer of similar sizes in both the cytoplasmic and membrane fractions added further support to the suggestion. The presence

of two different forms of the NS1 dimer differing in molecular mass attributable to glycosylation has also been shown for Japanese encephalitis virus, a closely related *Flavivirus*.³⁷ It was suggested that during translocation of the dimer from the cytoplasm to the membrane, glycosylation occurs at two positions on the monomer resulting in the additional mass.²³

In contrast to NS1, no differences in the molecular mass of cytoplasmic and membrane-associated dengue 2 virus E and PrM were noted suggesting that the mature form of the proteins were found in both fractions. It is also suggested that processing of the PrM, nonetheless, do involved the formation of several C-PrM intermediates detected in the cytoplasmic fraction as 40 – 30 kDa immunogenic bands and the resulting PrM following cleavages at 28 – 23 kDa with the 23 kDa being the mature predominant form. Also similar to results obtained using the membrane fraction, the presence of C (~ 10 kDa) was detected only transiently on day 5 PI (Fig. 1a, lane 5). Whereas, the 71 kDa antigenic protein detected transiently on day 3 PI has been reported by others as the NS3 viral protein.³⁸ This protein, similar to non-

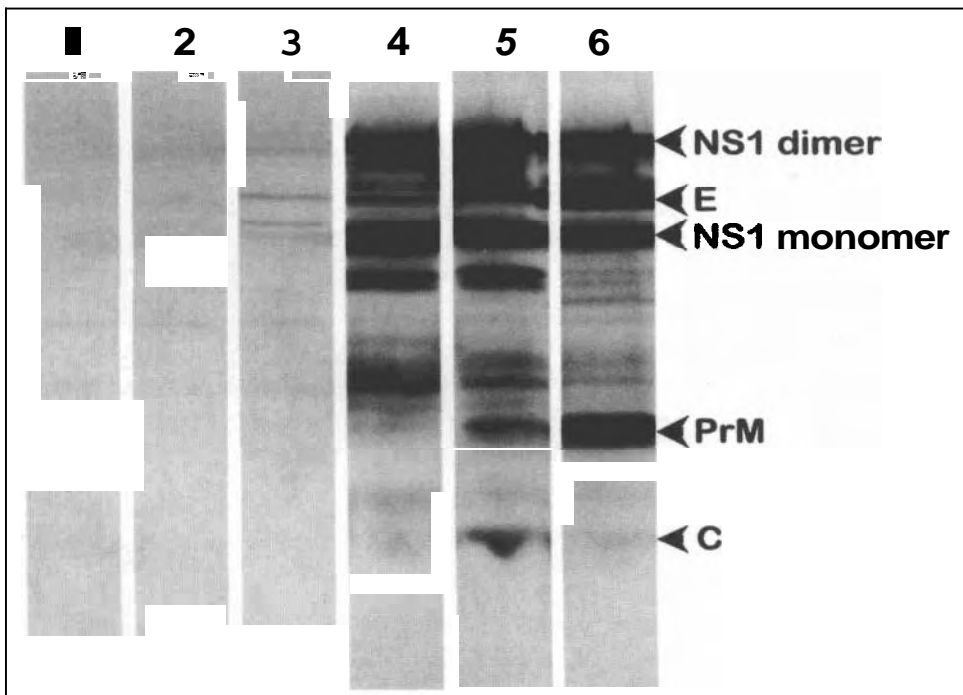


FIG. 2a: Detection of antigenic dengue 2 virus proteins in the cytosolic fraction of C6/36 mosquito cells using pooled dengue fever patients' sera. Cytosolic fractions of dengue 2 virus infected cells were prepared on day 0 (lane 2), day 1 (lane 3), day 3 (lane 4), day 5 (lane 5) and day 7 (lane 6) post-infection. Samples from the mock-treated cells were prepared on day 7 PI (lane 1). Patients' IgG against the antigenic dengue 2 viral proteins were detected as described in Figure 1a.

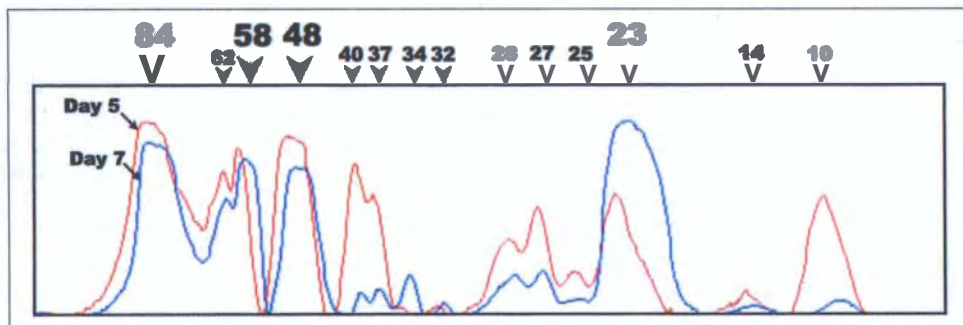


FIG. 2b: Schematic of a pairwise comparison between dengue 2 virus proteins detected in the cytosolic fraction on day 5 and 7 PI. Estimation of the molecular mass of the protein bands relative to the internal markers was performed using BioNumerics ver. 2.0 (Applied Maths, Kortrijk, Belgium).

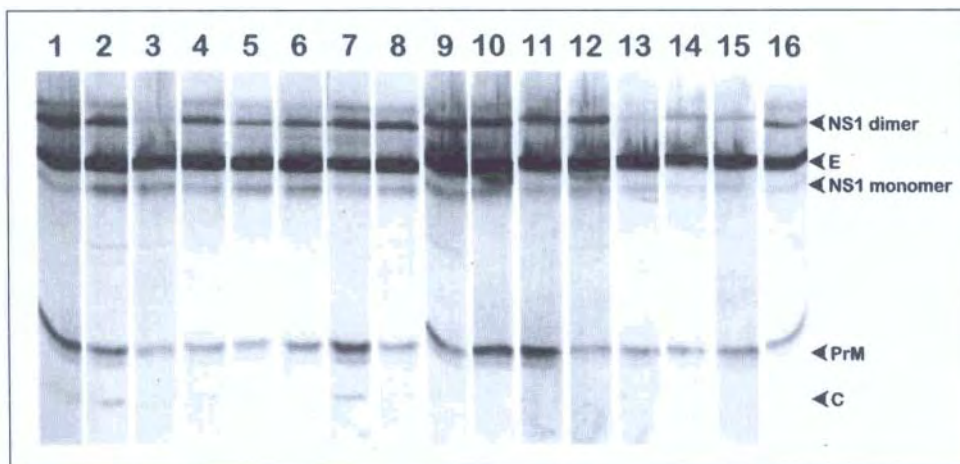


FIG. 3: Detection of antigenic membrane-associated dengue 2 virus proteins by dengue fever patients. Membrane fractions of dengue 2 virus infected cells were prepared on day 5 post-infection. Individual dengue fever patient's serum (lanes 1-16) was used in the immunoblot assays. Patients' IgG against the antigenic dengue 2 viral proteins were detected using biotinylated anti human IgG monoclonal antibody as above.

structural protein 5 (NS5) has been shown to posses catalytic activities and could be antigenic if used as antigen.^{39,40} Since these proteins were not detected in the membrane fraction, it was likely that the proteins are cytosolic proteins and how it could become antigenic is not known. Peptides corresponding to dengue virus non-structural protein 3 (NS3) sequences, however, have been shown to be associated with the major-histocompatibility complex (MHC) molecule for recognition by specific thymus (T) cells,⁴¹ suggesting that the NS3 could be important for activation of the cell-mediated immune responses. Findings presented here nonetheless supported previous observation that some dengue fever patients sera contained antibodies specific against dengue virus NS3.^{42,43}

To investigate if similar proteins present on the infected cell membrane were generally recognized by all dengue fever patients, individual patient's serum was used to probe the infected C6/36 cell membrane fractions harvested after 5 days of infection. From the study it was found that all 16 patients' sera had detectable antibodies against E, PrM, and NS1 (dimer and monomer). Only three of the patients' sera showed the presence of antibodies specific against C (Fig. 3 lanes 1, 2 and 7). These results supported the importance of the three glycosylated dengue virus proteins in stimulating the host immune responses. However, since C was also recognized by at least three patients' sera, the importance of immune responses against C needs to be assessed further.

In summary, results from the present study confirmed and extend previously reported findings⁴²⁻⁴⁴ that the major antigenic dengue virus proteins recognized by dengue fever patients *in vivo* are the E, NS1 (dimer and monomer), and PrM.²⁴ Detection of these glycosylated proteins in the membrane fraction of infected cells suggested that the host immune responses involving specific antibodies, antibody dependent cell cytotoxicity and the complement pathways could play a major role in protection and perhaps also pathogenesis of dengue. Since results presented in the present study along with those previously reported by others suggested the presence of antibodies in dengue fever patients sera against C and NS3, it is conceivable that the host immune response to these proteins could also be important in providing protection against dengue. Hence, in our effort to develop effective candidate dengue virus vaccine, it is imperative that we take into consideration not only those virus proteins that are highly antigenic but also the minor antigenic protein such as C that could potentially be just as important.

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REFERENCES

1. Monath TP. Dengue: the risk to developed and developing countries. *Proc Natl Acad Sci* 1994;91:2395-400.
2. Igarashi A. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. *J Gen Virol* 1978;40:531-44.
3. Igarashi A. Characteristics of *Aedes albopictus* cells persistently infected with dengue viruses. *Nature* 1979;280:690-1.
4. Andrews BS, Theofilopoulos AN, Peters CJ, Loskutoff DJ, Brandt WE, Dixon FJ. Replication of dengue and junin viruses in cultured rabbit and human endothelial cells. *Infect Immun* 1978;20:776-81.
5. O'Sullivan MA, Killen HM. The differentiation state of monocytic cells affects their susceptibility to infection and the effects of infection by dengue virus. *J Virol* 1994;75:2387-92.
6. Diamond MS, Edgil D, Roberts TG, Lu B, Harris E. Infection of human cells by dengue virus is modulated by different cell types and viral strains. *J Virol* 2000;74:7814-23.
7. Henchal EA, Putnak JR. The dengue viruses. *Clin Microbiol Rev* 1990;3:376-96.
8. Wang S, He R, Patarapotikul J, Innis BL, Anderson R. Antibody-enhanced binding of dengue-2 virus to human platelets. *Virus* 1995;213:254-7.
9. Ong CC, Lam SK, AbuBakar S. Cellular proteins bind to the 3' and 5' untranslated regions of dengue 2 virus genome. *Malays J Pathol* 1998;20:11-7.
10. Putnak JR, Kanesa-Thasan N, Innis BL. A putative cellular receptor for dengue viruses. *Nat Med* 1997;3:828-9.
11. Salas-Benito JS, del Angel RM. Identification of two surface proteins from C6/36 cells that bind dengue type 4 virus. *J Virol* 1997;71:7246-52.
12. Munoz ML, Cisneros A, Cruz J, Das P, Tovar R, Ortega A. Putative dengue virus receptors from mosquito cells. *FEMS Microbiol Lett* 1998;168:251-8.
13. Hase T, Summers PL, Eckels KH. Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. *Arch Virol* 1989;104:129-43.
14. Heinz FX, Auer G, Stiasny K, *et al.* The interactions of the flavivirus envelope proteins: implications for virus entry and release. *Arch Virol Suppl* 1994;9:339-48.
15. Hung SL, Lee PL, Chen HW, Chen LK, Kao CL, King CC. Analysis of the steps involved in dengue virus entry into host cells. *Virus* 1999;257:156-67.
16. Biedrzycka A, Cauchi MR, Bartholomeusz A, Gorman JJ, Wright PJ. Characterization of protease cleavage sites involved in the formation of the envelope glycoprotein and three non-structural proteins of dengue virus type 2, New Guinea C strain. *J Gen Virol* 1987;68:1317-26.
17. Lai CJ, Pethel M, Jan LR, Kawano H, Cahour A, Falgout B. Processing of dengue type 4 and other flavivirus nonstructural proteins. *Arch Virol Suppl* 1994;9:359-68.
18. Markoff L. *In vitro* processing of dengue virus structural proteins: cleavage of the pre-membrane protein. *J Virol* 1989;63:3345-52.
19. Murray JM, Aaskov JG, Wright PJ. Processing of the dengue virus type 2 proteins prM and C-prM. *J Gen Virol* 1993;74:175-82.
20. Stocks CE, Lobigs M. Posttranslational signal peptidase cleavage at the flavivirus C-prM junction *in vitro*. *J Virol* 1995;69:8123-26.
21. Anderson R, King AD, Innis BL. Correlation of E protein binding with cell susceptibility to dengue 4 virus infection. *J Gen Virol* 1992;73:2155-59.
22. Chen Y, Maguire T, and Marks RM. Demonstration of binding of dengue virus envelope protein to target cells. *J Virol* 1996;70:8765-72.
23. Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. *J Virol* 1999;73:6104-10.
24. AbuBakar S, Azmi A, Mohamed-Saad N, Shafee N, Chee HY. Antibody responses of dengue fever patients to dengue 2 (New Guinea C strain) viral proteins. *Malays J Pathol* 1997;19:41-51.
25. Clark DH, Cassals J. Techniques for hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg* 1958;7:561-73.
26. Rizvi N, Chaturvedi UC, Mathur A. Inhibition of the presentation of dengue virus antigen by serine-protease inhibitors. *Int J Exp Path* 1991;72:23-9.
27. Laemli UK. Cleavage of structural proteins during

- the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
28. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci* 1979;76:4350-4.
 29. Talbot PJ, Knobler RL, Buchmeier MJ. Western and dot immunoblotting analysis of viral antigens and antibodies: Application to murine hepatitis virus. *J Immuno Meth* 1984;73:177-88.
 30. Birk HW, Koepsell H. Reaction of monoclonal antibodies with plasma membrane proteins on nitrocellulose: renaturation of antigenic sites and reduction of nonspecific antibody binding. *Ana Biochem* 1987;164:12-22.
 31. Smith GW, Wright PJ. Synthesis of proteins and glycoproteins in dengue type 2 virus-infected vero and *Aedes albopictus* cells. *J Gen Virol* 1985;66:559-71.
 32. Bulich R, Aaskov JG. Nuclear localization of dengue 2 virus core protein detected with monoclonal antibodies. *J Gen Virol* 1992;73:2999-3003.
 33. Tadano M, Makino Y, Fukunaga T, Okuno Y, Fukai K. Detection of dengue 4 virus core protein in the nucleus. I. A monoclonal antibody to dengue 4 virus reacts with the antigen in the nucleus and cytoplasm. *J Gen Virol* 1989;70:1409-15.
 34. Lindenbach BD, Rice CM. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. *J Virol* 1999;73:4611-21.
 35. Winkler G, Randolph VB, Cleaves GR, Ryan TE, Stollar V. Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. *Virology* 1988;162:187-96.
 36. Winkler G, Maxwell SE, Rueemmler C, Stollar V. Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. *Virology* 1989;171:302-5.
 37. Mason PW. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* 1989;169:354-64.
 38. Teo KF, Wright PJ. Internal proteolysis of the NS3 protein specified by dengue virus 2. *J Gen Virol* 1997;78:337-41.
 39. Tan CHC, Yap EH, Singh M, Deubel V, Chan YC. Passive protection studies in mice with monoclonal antibodies directed against the non-structural protein NS3 of dengue 1 virus. *J Gen Virol* 1990;71:745-8.
 40. Bartholomeusz AI, Wright PJ. Synthesis of dengue virus RNA in vitro: initiation and the involvement of proteins NS3 and NS5. *Arch Virol* 1993;128:111-21.
 41. Okamoto Y, Kurane I, Leporati AM, Ennis FA. Definition of the region on NS3 which contains multiple epitopes recognized by dengue virus serotype-cross-reactive and flavivirus-cross-reactive, HLA-DPw2-restricted CD4⁺ T cell clones. *J Gen Virol* 1998;79:697-704.
 42. Churdboonchart V, Bhamarapravati N, Peampramprecha S, Sirinavin S. Antibodies against dengue viral proteins in primary and secondary dengue hemorrhagic fever. *Am J Trop Med Hyg* 1991;44:481-93.
 43. Valdes K, Alvarez M, Pupo M, Vazquez S, Rodriguez R, Guzmán MG. Human dengue antibodies against structural and nonstructural proteins. *Clin Diagn Lab Immunol* 2000;7:856-7.
 44. Se-Thoe SY, Ng MML, Ling AE. Retrospective study of western blot profiles in immune sera of natural dengue virus infections. *J Med Virol* 1999;57:322-30.