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Full Length Research Paper

The contribution of host cells to dengue virus infectivity

Woraphol Rattanachuen, Lukkana Suksanpaisan, Nitwara Wikan and Duncan R. Smith*

Molecular Pathology Laboratory, Institute of Molecular Biosciences, Mahidol University, Thailand.

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Dengue virus infections remain a significant worldwide public health problem, causing millions of new infections each year. Transmitted by mosquitoes of the *Aedes* genus, the virus is capable of replicating in a number of different cells of both mosquito and human origin, but the molecular mechanism of infection remains largely unknown. This study sought to determine if the host cell in which a virus was produced had an effect on its subsequent ability to infect other cells. The study used dengue serotype 1 and 2, propagated in both insect (C6/36) and mammalian (Hep3B liver cells) to infect both C6/36 and Hep3B cells. Results showed clearly that both serotype and host cell modulated the ability of the virus to infect cells.

Key word: Aedes, dengue, flavivirus, infection, liver.

INTRODUCTION

The mosquito borne dengue virus (DENV) is endemic or hyperendemic in many tropical and sub-tropical countries of the world, and infections with dengue represent a potential health risk to as much as one third of the world's population (Gubler, 1997; Guzman and Kouri, 2002). Infection with any one of the four DENVs may be asymptomatic, or may result in a wide range of illness ranging from a relatively mild, undifferentiated fever to the severe, life threatening dengue hemorrhagic fever or dengue shock syndrome (Gubler, 1998; Halstead, 2007; Henchal and Putnak, 1990). While infection with one DENV results in lifelong immunity to that DENV, only transient protection is offered against heterotypic DENVs and second, third or even fourth infections with heterotypic DENVs are possible (Guzman et al., 2000; Sangkawibha et al., 1984). Considerable evidence suggests that subsequent infections are associated with a more severe disease presentation that may be mediated through either the process termed antibody dependent enhancement (ADE) of infection in which non-neutralizing antibodies from a prior infection potentiate the disease by enabling virus entry into Fc bearing cells (Halstead et al., 2010; Halstead and O'Rourke, 1977; Halstead et al., 1980) or by the process termed "original antigenic sin" in which a previous infection results in a mis-priming of the immune system to deal with a subsequent heterotypic infection (Midgley et al., 2010; Mongkolsapaya et al., 2003). In addition, some evidence suggests that the particular genotype of a virus may play a role in determining disease severity. In support of this, there are reports of severe (hemorrhagic) dengue disease in response to primary DENV infection (Murgue et al., 2000; Vaughn et al., 2000; Wang et al., 2006). None of the three models however are mutually exclusive, and pathogenesis is almost certainly determined by a number of factors, including genetic makeup of the individual (Rico-Hesse, 2007).

The initial interaction between a DENV and a susceptible cell (whether mosquito or mammalian) occurs at the point of virus binding to the host cell, and evidence suggests that this interaction is mediated by both non-specific and specific interactions of the virus will cell surface expressed proteins (Cabrera-Hernandez and Smith, 2005). Non specific interactions may include the interaction of the virus with glycosaminoglycans such as heparan sulfate which may serve to concentrate the virus at the cell surface (Chen et al., 1997; Hilgard and Stockert, 2000; Thepparit et al., 2004), while specific interactions include those associated with the binding of

*Corresponding author.E -mail: duncan_r_smith@hotmail.com. Tel: +662 800 3624, Ext. 1266.

the virus to a specific receptor protein. To date, several proteins have been implicated as DENV receptor proteins, and the evidence would suggest that receptor usage is cell type specific, as well as serotype specific (Jindadamrongwech et al., 2004; Reyes-del Valle et al., 2005; Tassaneetrithep et al., 2003; Thepparit and Smith, 2004).

Several studies have shown the requirement for N-linked glycosylation of the DENV E protein as being important for DENV entry (Mondotte et al., 2007; Pryor et al., 1998; Pryor and Wright, 1994). The DENV E protein contains two glycosylation sites at Asn-67 and Asn-153, and while the glycosylation site at amino acid Asn-153 is conserved in flaviviruses, the site at Asn-67 is unique for DENV (Heinz and Allison, 2003). Studies have shown that the glycosylation of Asn-153 is important in virus entry while Asn-67 seems to have a greater effect in virus assembly (Mondotte et al., 2007). In particular Asn-153 has been implicated as critical in mediating the binding of DENV to DC-SIGN an attachment molecule implicated in mediating DENV entry to dendritic cells (Lozach et al., 2005). Both insect cells and mammalian cells have been shown to glycosylate Asn-153, although it has been proposed the glycosylation of Asn-153 may not be advantageous for viral replication in insect cells, while in contrast in mammalian cells glycosylation at this site offers a significant advantage in infectivity in addition to a smaller advantage in replication (Mondotte et al., 2007). Besides glycosylation, how the host cell affects the fitness of the mature virion to infect subsequent cells remains largely unexplored.

MATERIALS AND METHODS

Cells and virus

The *Aedes albopictus* cell line C6/36 was cultured in MEM (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco, Grand Island, NY) and maintained at 28°C. The human liver cell line Hep3B was maintained in DMEM (Gibco, Grand Island, NY) medium supplemented with 10% FBS and incubated at 37°C, 5% CO_2. The monkey kidney cell line LLC-MK_2 was cultured in DMEM medium supplemented with 5% FBS and incubated at 37°C, 5% CO_2. Fifth passage DEN-1 (strain 16007) and DEN-2 (strain 16681) were used as stock viruses and additionally passaged once through Hep3B cells.

Detection of virus infectivity and production

C6/36 or Hep3B cells were infected with either DEN-1 or DEN-2 at a multiplicity of infection (m.o.i.) of 10, for 2 h. Cells were then washed three times with PBS and incubated for up to 3 days. Infected cells and culture medium were collected daily. Infected cells were quantitated by flow cytometry and viral production was determined by standard plaque assay. For flow cytometry, total cells were harvested and blocked with 10% normal goat serum for 30 min, on ice. Cells were washed once with 1% BSA follow by fixation in 4% paraformaldehyde at room temperature for 20 min. Subsequently cells were permeabilized with 0.2% saponin in 1% BSA for 10 min at room temperature. Cells were then incubated with a pan-specific mouse anti-dengue E monoclonal antibody, HB114 (Henchal et al., 1982) at a dilution of 1:10 at 4°C for overnight. After three washes with 1% BSA, cells were incubated with a FITC conjugated goat anti-mouse IgG polyclonal antibody (KPL, Gaitherburg, MD) at dilution of 1:10 at room temperature for 1 h. Cells were washed three times with 1% BSA and resuspended 500 µl of PBS and analyzed by flow cytometry (FACS Calibur™, BD Biosciences, San Jose, CA) using the CELLQuest™ software (BD Biosciences). All experiment undertaken independently in triplicate. Culture supernatant containing infectious viruses was quantified by standard plaque assay using LLC-MK2 cells as previous described (Sithisarn et al., 2003) each experiment as undertaken independently in triplicate, with duplicate assay of titer.

RESULTS

To investigate the effect of the host cell on subsequent DENV infectivity, laboratory adapted, passage 5 DENV-1 and DENV-2 grown in C6/36 were passaged one additional time through Hep3B cells. Hep3B was selected as a representative liver cell line as our previous studies have shown that liver cells represent a valid mammalian DENV target cell type (Suksanpaisan et al., 2007) and that Hep3B releases significantly more infectious virus than the comparable HepG2 cell line (Thongtan et al., 2004). Quantitation of virus titers at all stages was undertaken on LLC-MK2 cells as a common reference point. Hep3B or C6/36 cells were then infected at m.o.i. 10 using either DENV-1 or DENV-2 produced from either C6/36 or Hep3B cells. On days 0 (input virus in the medium) 1, 2 and 3 post-infection the levels of infectious virus in the culture medium was assayed by standard plaque assay on LLC-MK2 cells, and the percentage of cells infected was determined by flow cytometry, using a pan-specific anti DENV E protein monoclonal antibody.

Results of the infection experiment are shown in Figure 1. DENV-2 produced in both C6/36 and Hep3B cells showed high levels of infectivity towards Hep3B cells (80% or greater as assessed by flow cytometry) from as early as 24 h post-infection, while in contrast DENV-1 produced in these two cells showed a strongly dichotomous result with DENV-1 produced in Hep3B cells showing significantly higher degree of infectivity towards Hep3B cells than DENV-1 produced in C6/36 cells. DENV-2 produced from both C6/36 and Hep3B cells showed a markedly lower infectivity towards C6/36 cells than was observed for Hep3B cells (Figure 1). At day 1 post infection, less than 10% of C6/36 cells were infected by DENV-2 produced from Hep3B cells (compared with 80% of Hep3B cells). Similarly, DENV-2 produced from C6/36 only infected some 40% of C6/36 cells at 24 h post-infection (compared to 80% of Hep3B cells). By day 3 post infection however, infection rates were approximately 80% (Figure 1) for C6/36 cells infected with DENV-2 produced from Hep3B and C6/36. Similarly, for DENV-1, virus produced from both C6/36 and Hep3B cells showed extremely low initial infectivity towards C6/36 cells, but by day 3 post-infection around 50 to 70% of C6/36 cells were infected.

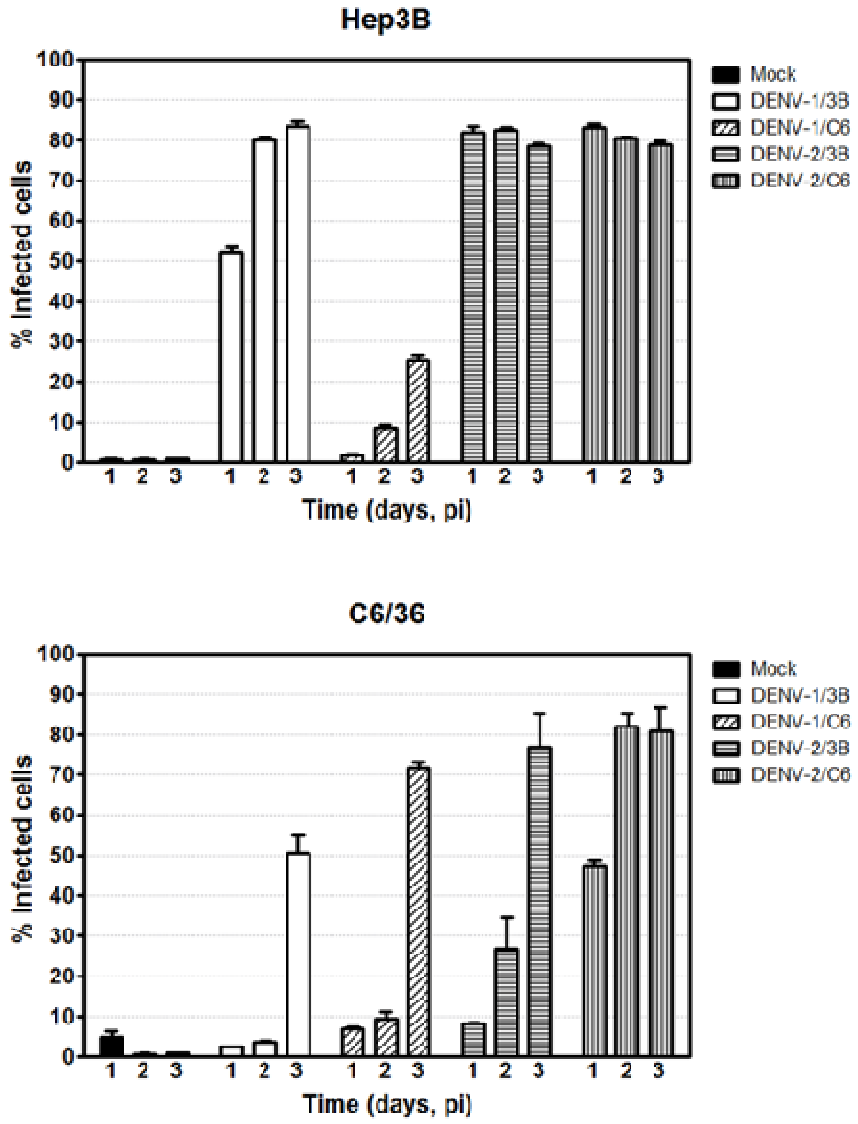

Figure 1. Flow cytometry analysis of DENV infected Hep3B (top) and C6/36 (bottom) cells. Cells were infected at m.o.i. 10 with DENV-1 propagated in Hep3B cells (DENV-1/3B), DENV-1 propagated in C6/36 cells (DENV-1/C6), DENV-2 propagated in Hep3B cells (DENV-2/3B) or DENV-2 propagated in C6/36 cells (DENV-2/C6) as indicated and analyzed for infectivity on days 1 to 3 post-infection after staining with a pan-specific mouse anti-dengue E monoclonal antibody. Results shown are the means ± SEM of triplicate independent experiments.

No difference in virus production was seen in Hep3B cells infected with DENV-2 from either Hep3B or C6/36 cells (Figure 2), and in both cases output virus was some 3log higher than input virus by 24 h post-infection. In contrast, virus production from Hep3B cells infected with DENV-1 was lower than for DENV-2, with only a 2log (Hep3B infected with DENV-1 produced in Hep3B cells) or a 1 log (Hep3B infected with DENV-1 produced in C6/36 cells) increase in virus output as compared to virus input at 24 h port infection. At 3 days post infection only 25% of Hep3B cells are infected with DENV-1 produced in C6/36 cells as compared to over 80% of Hep3B cells infected with DENV-1 produced in Hep3B cells (Figure 1), and this is reflected in an approximately 1.5 log difference in virus output (Figure 2).

For both DENV-1 and DENV-2 virus production was markedly slower in C6/36 cells as compared to Hep3B cells. For DENV-2, virus production at 24 h post-infection was either 1 log (C6/36 cells infected with DENV-2 produced from Hep3B cells) or 2 log (C6/36 cells infected with DENV-2 produced from C6/36 cells) above input virus, but virus titer by day 3 post-infection in both cases

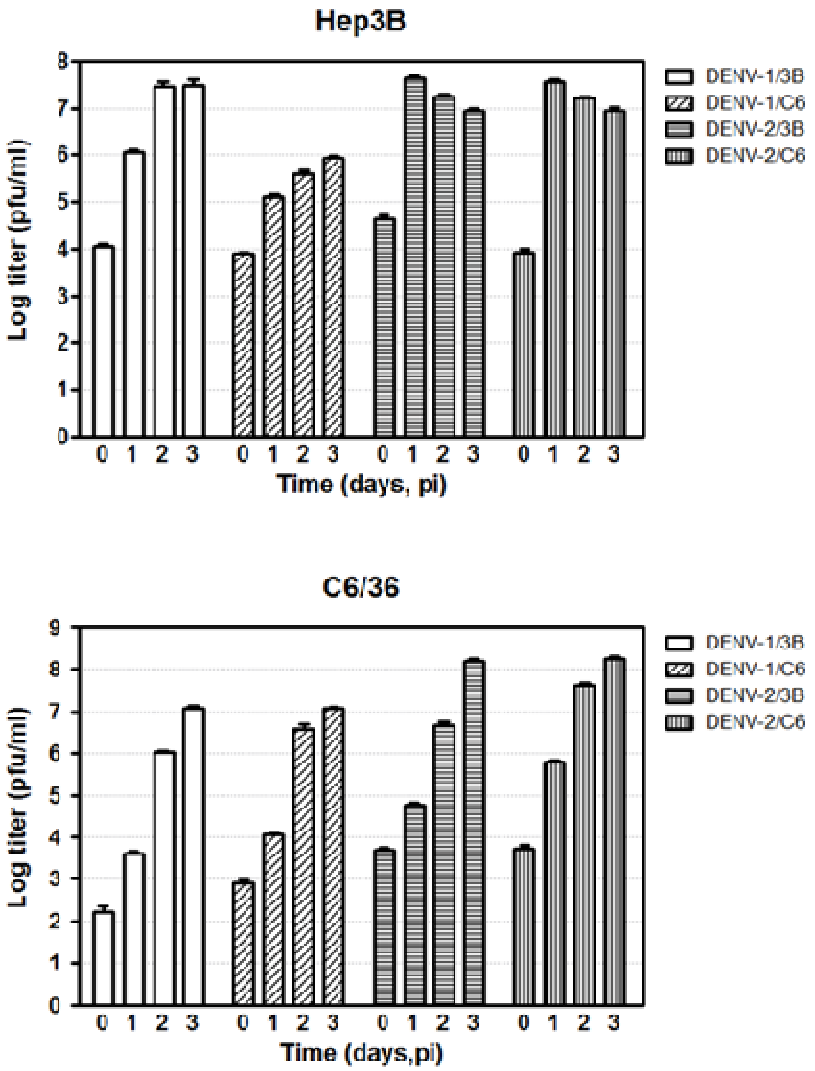

Figure 2. Plaque assay of virus production from DENV infected Hep3B (top) and C6/36 (bottom). Cells were infected at m.o.i. 10 with DENV-1 propagated in Hep3B cells (DENV-1/3B), DENV-1 propagated in C6/36 cells (DENV-1/C6), DENV-2 propagated in Hep3B cells (DENV-2/3B) or DENV-2 propagated in C6/36 cells (DENV-2/C6) as indicated and analyzed for infectious plaque titer by plaque assay on LLC-MK2 cells immediately after infection (day 0) and on days 1, 2 and 3 post-infection. Results shown are the means ± SEM of triplicate independent experiments.

was approximately 1 log above the levels seen in Hep3B infected cells. C6/36 cells infected with DENV-1 produced from either Hep3B or C6/36 cells showed 1 to 2 log lower virus production at 24 h post infection compared to the corresponding infection of Hep3B cells (Figure 2) but by day 3 post-infection levels of virus production from C6/36 cells were comparable to Hep3B when the original infecting virus was produced in Hep3B cells, and was 1 log higher when the original infecting virus was produced from C6/36 cells. Overall however, DENV-1 virus production was 1 log lower than DENV-2 production in C6/36 cells on day 3 post-infection, irrespective of the cellular origin of the initial infecting virus.

DISCUSSION

While significant advances have been made in understanding the process and mechanism by which DENV infects host cells, there remain a number of areas where less progress has been made. Productive infection of a host cell can be broadly divided into three stages: virus entry and uncoating, virus replication and packaging and virus exit from the cell (Clyde et al., 2006;

Rodenhuis-Zybert et al., 2010; Urcuqui-Inchima et al., 2010). Virus entry into a host cell is generally believed to occur after binding of the virus to a specific high affinity receptor or receptor complex, and a number of receptor proteins have been implicated in this process (Cabrera-Hernandez and Smith, 2005). Overall, the evidence suggests that receptor usage is cell type specific with the virus using different receptor proteins in different cell types and, at least in some cell types, receptor usage shows evidence of serotype specificity (Cabrera-Hernandez and Smith, 2005).

The factor or factors determining the specific receptor usage in specific cell types remains poorly understood. Obviously some contribution to the cell type specificity lies in the distribution of specific receptor molecules on specific cell types, but this alone is insufficient to explain all the observed results. For example Reyes-del Valle and colleagues proposed that Hsp70 and Hsp90 form a complex mediating the entry of DENV to monocytic cells (Reyes-del Valle et al., 2005), but these molecules were excluded as playing a role in the entry of DENV to liver cells (Cabrera-Hernandez et al., 2007). While Hsp70 and Hsp90 are primarily classified as cellular chaperone molecules, there is a significant amount of evidence showing their widespread cell surface expression (Jang and Hanash, 2003; Shin et al., 2003), and thus the usage of these chaperones as entry molecules is mediated by another factor or factors.

In this work we show that both serotype and the host cell in which the virus was propagated played a significant role in determining infectivity of the virus. DENV-2 was shown to infect mammalian liver cells extremely well, irrespective of source, while DENV-1 prepared from Hep3B showed high infectivity to Hep3B cells, while DENV-1 prepared from insect cells showed low infectivity towards these cells. Similarly, DENV-2 prepared from Hep3B was significantly less infective towards insect cells (C6/36) than was DENV-2 prepared from insect cells.

These results have several important implications. Firstly studies that use multiple serotypes in determining the pathobiology of DENV infection need to ensure that they are in fact comparing similar situations. For example the study by Umareddy and colleagues show the apparent serotype specific induction of ER stress in the A549 (human alveolar adenocarcinoma) cell line cells using a common multiplicity of infection (Umareddy et al., 2007). As shown here however, the same apparent multiplicity of infection can result in markedly different initial infection rates. For example in Hep3B cells a common multiplicity of 10 resulted in either an infection rate of less than 10% (DENV-1, propagated in C6/36) or more than 80% (DENV-2 propagated in C6/36 cells) at 24 h post infection (Figure 1). Secondly studies evaluating receptor usage maybe markedly altered by the source of the virus used in the experiments. This is clearly demonstrated by the low infection rate of C6/36 cells seen with DENV-2 prepared from Hep3B, as compared to the high infection rate seen with the same virus prepared in C6/36 cells, which may indicate that the viruses prepared in either C6/36 cells or Hep3B cells are using different receptor proteins.

Clearly, in determining infectivity of the viruses used, the data obtained during the first 24 h is the most robust. Subsequent days will reflect a combination of infection from the original virus used to infect the cells, as well as the virus produced from those cells infecting other cells. For this reason in practically determining infectivity of viruses, data that is obtained from less than 2 replication cycles is more robust than data obtained from multiple rounds of infection and production, and it is also clear that determination of virus titer is not a particularly useful indicator of the degree of infection.

In this experiment the viruses used were from either passage 5 (DENV-1 or DENV-2 grown in C6/36 cells) or with one additional passage (DENV-1 or DENV-2 grown in Hep3B cells) and as such the differences seen are unlikely to result from adaptation of the virus to the cell which normally takes several passages (Lee et al., 1997, 2006). Previous work has established that the nature of the carbohydrate moieties on the DENV E glycoprotein is a critical factor in the use of DC-SIGN (Navarro-Sanchez et al., 2003), and while both insect and mammalian cells glycosylate DENV (Mondotte et al., 2007), there are differences in the nature of the glycosylation. In this regard, the exact nature and extent of glycosylation may play a role in mediating the interaction of DENV with its cognate receptors in a number of different cell types, but that this may be further modulated by other factors, including DENV serotype.

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