Final Report

Identification and Characterization of the Human Hepatocyte Receptor for Dengue Virus Infection

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Visiting Professor Duncan R. Smith PhD.

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Institute of Molecular Biology and Genetics

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The Thailand Research Fund

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Masters student from 2000 to 2002 (Completed)
Miss Suksanpaisan was awarded the Mahidol University "Outstanding Masters Thesis in Science and Technology, 2003" prize for her work in this project.

Miss Patchima Sithisarn
Masters student from 2001 to 2003 (Completed)

Miss Waranyoo Phoolcharoen
Masters student from 2001 to 2003 (Completed)

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ABSTRACT

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Project title:
Identification and Characterisation of the Human Hepatocyte Receptor for Dengue Virus Infection.

Investigator:
Visiting Professor Duncan R. Smith
Institute of Molecular Biology and Genetics
Mahidol University

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Over 2 billion people live in areas at risk of infections with the dengue virus, making the dengue virus a significant world public health issue. In Southeast Asia dengue virus infection is the leading cause of hospitalization amongst children, and it is predicted that the rate of infections will increase in the future. The dengue virus is transmitted by mosquitoes and replicates in the cells of the human host. The mechanism by which the virus enters into cells is of significant interest as it determines the tissue tropism of the virus, and ultimately the pathogenicity of the disease.

Our studies have focused on dissecting out the mechanism by which the virus enters into mammalian cells, and has primarily centered on the use of liver cells given that the liver is known to be a true target tissue for dengue virus replication. In addition we have undertaken studies that utilize strains of all four serotypes of the dengue virus. The times for the virus to internalize, replicate and be produced from the liver cells has been determined for each serotype, as well as the contribution of extracellular moieties such as glycosaminoglycans and proteins to the internalization process.

Using the technique of virus overlay protein binding assays, we have demonstrated that the proteins used to internalize the virus (the receptor proteins) are used in a manner that has a significant serotype specific element, and more over we have isolated and characterized a protein that acts as a receptor element for dengue virus serotype 2. This protein, GRP78 (BiP) normally functions as a molecular chaperonin, but its expression on the surface of a wide variety of cells has been well established. Blocking this protein on the surface of a cell with specific monoclonal antibodies reduces, but does not abolish entirely, the entry of dengue virus serotype 2 into liver cells. This protein does not seem to be involved with the entry of other serotypes into liver cells, suggesting that a wide variety of receptors can be use by the virus to enter into cells. Future work will involve isolating receptor proteins used by other serotypes to enter into liver cells.

Key words: BiP, dengue, hepatocyte, flavivirus, receptor.

SUMMARY

The mechanism by which the dengue virus enters into human or insect cells is still unknown. Evidence from electron microscopy studies suggests that the virus enters by a process of direct cell fusion, while other studies suggest that the process is mediated by receptor molecules expressed on the surface of the cell, leading to entry of the virus into clathrin coated pits followed by un-coating and internalization of the virus. Despite many studies, the specific nature of the cell surface expressed receptor remains unknown. The situation is compounded by studies that suggest the mechanism of virus internalization may be mediated both by cell type specific factors as well as by the specific serotype of the virus being investigated.

It is known, however, that in cases of secondary dengue infection, where the virus becomes complexed with cross reacting but non-neutralizing antibodies from a primary infection with a different serotype the Fc receptor is used by the virus to gain entry to monocytes [12]. However the identification of proteins used in primary infections of the dengue virus has been less successful, although several groups have identified potential virus binding proteins using the virus overlay protein binding assay (VOPBA) technique [23, 24], and recently glycosaminoglycans [8], an LPS/CD14-associated binding protein [9] and DC-SIGN [27] have all been implicated as elements mediating dengue virus entry into cells.

The work undertaken in this grant period to investigate the specific nature of the dengue virus receptor relied upon two important principle that distinguish it from other laboratories investigations:

1. The identification of a cell line representative of a true target tissue

and

2. The use of all four serotypes of the dengue virus in the investigation.

Given the importance of the liver in the pathogenesis of dengue, liver cell lines were chosen as the representative cell line.

We initially sought to define the growth of all four serotypes of the dengue virus in HepG2 cells. HepG2 cells were able to support the growth of all serotypes, although yield of mature virus varied considerable (**Thepparit et al., In press**). These studies led us to further examine the nature of the dengue virus itself, and have demonstrated that the dengue virus has an inherent half life (**Sithisarn et al., 2003**).

We then focused on the macromolecular nature of the interaction by examining the binding of the virus to the cells (**Suksanpaisan and Smith, 2003**). We prepared radiolabeled dengue virus serotypes 1 and 2 through viral propagation in Vero cells. Increasing amounts of virus were then incubated with HepG2 cells to determine the ability of the virus to saturate binding on HepG2 cells. Results indicated that it was not possible to saturate binding under experimentally achievable conditions. These results were consistent with proposals that dengue virus binding to cells is mediated initially through a low affinity interaction with an abundant molecule on the surface of the cell, and that a second interaction with a less commonly expressed molecule is required for viral internalization.

We then further defined the nature of the extracellular binding elements by pre-treating cells with either heparinase III or trypsin to remove either glycosaminoglycans or all proteins. Results (**Thepparit et al., In press**) indicated that both of these elements contributed towards virus internalization, although the specific contribution of each class of molecule was serotype specific.

To determine the specific nature of the proteins involved in the binding and internalization, we employed the technique of Virus overlay protein binding assay (VOPBA), and adaptation of the Far-Western technique. Results indicated a significant serotype specific element in the binding of the virus to liver cells. The first serotype to be characterized more fully, dengue virus serotype 2, demonstrated two major virus binding bands, as well as several minor virus binding bands. One of these bands was characterized by mass spectroscopy, and determined to be GRP78 (BiP). Virus inhibition studies, as well as immunoflourescent binding studies confirmed

GRP78 (BiP) as a protein involved in the internalization of dengue virus serotype 2 into Hep G2 cells (**Jindadamrongwech et al., 2004**).

As a tool to aid further studies on the dengue virus/liver cell interaction, we have also developed a novel adaptation of the plaque assay to enable HepG2 cells to be assayed by direct plaque counting instead of by virus production **(Chingsuwanrote et al., 2004).**

Full details of methods and results, as well as a comprehensive review of the literature can be found in the accompanying papers, all of which have been either published or are in press:

Suksanpaisan, L. and Smith, D.R. Analysis of saturation binding and saturation infection for Dengue serotypes 1 and 2 in liver cells. Intervirology 46: 50 – 55, 2003.

Sithisarn, P., Suksanpaisan, L., Thepparit, C. and Smith, D.R. Behavior of the dengue virus in solution. Journal of Medical Virology 71: 532-539, 2003.

Jindadamrongwech, S., Thepparit, C. and Smith, D.R. Identification of GRP 78 (BiP) as a liver cell expressed receptor for dengue virus serotype 2. Archives of Virology doi: 10.1007/s00705-003-0263-x.

Chingsuwanrote, P., Suksanpaisan, L. and Smith, D.R. Adaptation of the plaque assay methodology to dengue virus infected Hep G2 cells. Journal of Virological Methods doi:10.1016/j.viromet.2003.11.002.

Thepparit, C., Phoolcharoen, W., Suksanpaisan, L. and Smith, D.R. Binding, internalization and propagation of all four dengue serotypes in human hepatoma (HepG2) cells. In press: Intervirology.

Original Paper

Intervirology

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Analysis of Saturation Binding and Saturation Infection for Dengue Serotypes 1 and 2 in Liver Cells

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Key Words
Dengue virus · Receptor · Binding · Saturation

Abstract

Objective: The liver has been increasingly recognized as a significant target organ in the pathogenesis of dengue virus infection. However, only two contradictory studies have examined the binding of the dengue virus to liver cells. This study therefore sought to investigate the binding of the dengue virus to HepG2 cells. ***Methods:*** Radiolabeled dengue virus serotypes 1 and 2 were prepared through viral propagation in Vero cells. Increasing amounts of virus were then incubated with HepG2 cells to determine the ability of the virus to achieve saturation of binding on HepG2 cells. ***Results:*** Results indicated that it was not possible to reach saturation of binding under experimentally achievable conditions. We then sought to determine whether it was possible to reach a state of saturation of infection, by using increasingly high titers of virus on a constant number of cells. Dengue serotype 1 showed no evidence of saturation of infection, even at titers of 5,000 viruses per cell. In contrast, dengue serotype 2 became saturated at levels of approximately 3,000 viruses per cell. ***Conclusions:*** These results are consistent with proposals that dengue virus binding to cells is mediated initially through a low-affinity interaction with an abundant molecule on the surface of the cell and secondly through interaction with a less commonly expressed molecule, which is required for viral internalization.

Introduction

Over half the world's population live in areas where there is a risk of infection by dengue viruses, and these are also popular tourist destinations [1, 2]. Typically, dengue fever is an acute febrile illness characterized by muscular aches and pains, nausea, vomiting, frontal headache and rash [3]. The more severe form of the disease, dengue hemorrhagic fever (DHF), is defined as an acute febrile illness with minor or major bleeding, thrombocytopenia and evidence of plasma leakage, pleural or other effusions, or hypoalbuminemia or hypoproteinemia [4]. When DHF is associated with signs of circulatory failure, hypotension or frank shock, the patient is classified as having dengue shock syndrome (DSS) [4]. While the majority of infections, especially in children, are either asymptomatic or minimally symptomatic, DHF and DSS are now a leading cause of hospital admissions and death among children in Asia [5, 6]. In DHF and DSS, the liver may be palpable and tender and liver enzymes are usually

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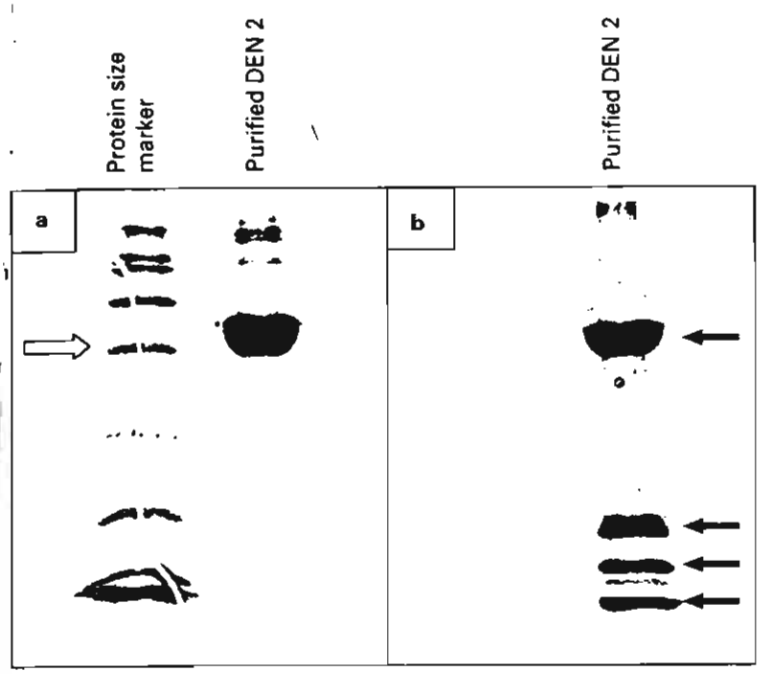


Fig. 1. Analysis of purified labeled dengue serotype 2 (DEN 2) by 15% SDS-PAGE gel electrophoresis (**a**) and autoradiography (**b**). Open arrow: 45-kD marker band. Closed arrows (top to bottom): dengue virus E, prM, C and M proteins.

of $CaCl_2 \cdot 2H_2O$, 7.5 g of glycine/l, pH adjusted to 3 with HCl) for 1 min at room temperature to inactivate noninternalized viruses. Cells were washed again with PBS and then incubated at 37° in DMEM supplemented with 10% FBS and antibiotics for 22 h. Duplicate 250-µl aliquots of media were assayed by plaque titration to determine levels of infectious viruses.

Statistical Analysis

Analysis of data from saturation binding experiments was undertaken using the GraphPad Prism program (GraphPad Software Inc., San Diego, Calif., USA).

Results

Preparation of Radiolabeled Virions

Time course titers of dengue virus production in Vero cells were undertaken to establish the optimum viral yield. Subconfluent cultures of Vero cells were infected with either dengue serotype 1 or 2, and the viral yield was established by titering the level of the virus in the media over a period of 10 days. The optimal time of viral production was established to be 4 days for dengue serotype 1 and 5 days for dengue serotype 2. For preparation of radiolabeled viruses, subconfluent cultures of Vero cells

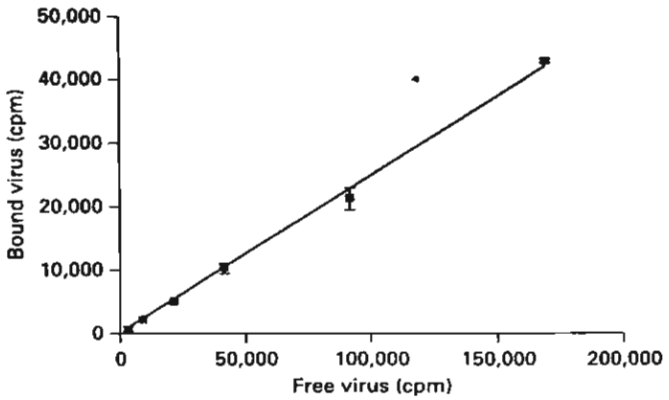


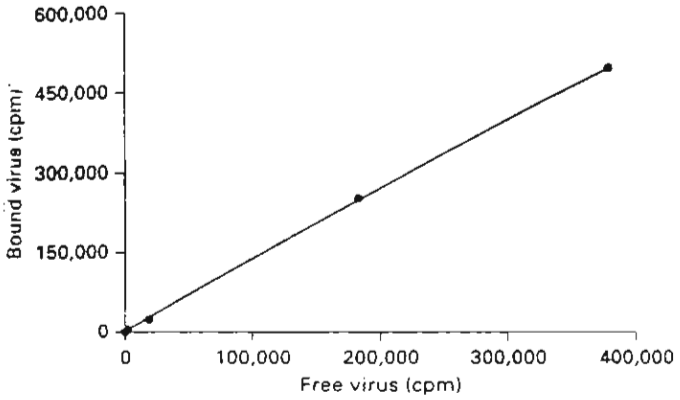
Fig. 2. Binding of dengue virus serotype 1 to HepG2 cells at increasing concentrations of radiolabeled virus. Each point represents the mean of three independent experiments.

were infected with the respective dengue serotype in supplemented DMEM. After 2 (dengue serotype 1) or 3 (dengue serotype 2) days, the medium was changed to DMEM minus methionine/cysteine and supplemented with ^{35}S-labeled methionine/cysteine. Cultures were incubated for a further 24 h. Purified viruses were obtained by a combination of polyethylene glycol precipitation and sucrose density centrifugation. Sample purity was confirmed by SDS-PAGE electrophoresis and autoradiography (fig. 1).

Saturation Analysis

To investigate the nature of dengue virus binding to liver cells, a series of virus binding experiments were undertaken. Initially, a constant number of cells were incubated with increasing amounts of ^{35}S-radiolabeled dengue virus. The binding with each amount of virus was studied in triplicate. After incubation for 1 h, free and bound virus were separated by centrifugation and fractions were counted. Initial experiments were undertaken with 10^5 cells per tube. Analysis of binding showed no saturation. To maximize virus cell ratios, further experiments were undertaken using 10^3 cells per tube, but again neither dengue 1 nor dengue 2 was able to achieve saturation of binding (fig. 2).

Saturation binding experiments do not normally require an accurate knowledge of the titer of the radiolabeled virus. However, to gain a greater understanding of the kinetics of binding, a further preparation of dengue serotype 2 was undertaken and the purified radiolabeled virus was titered prior to use. In this experiment, the virus



g. 3. Binding of dengue serotype 2 to HepG2 cells. Each point presents the mean of three independent experiments. The final int represents a virus to cell ratio of 200,000:1.

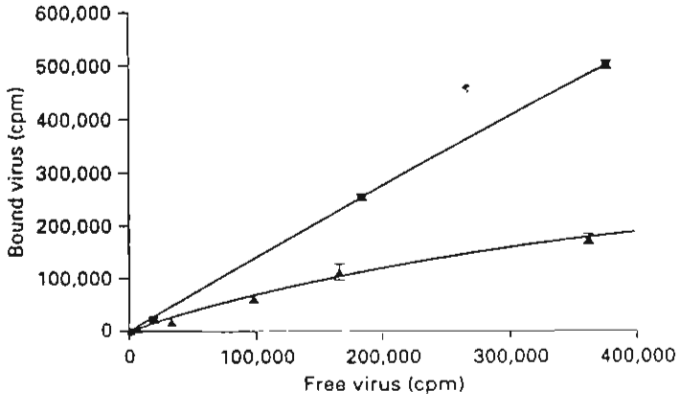


Fig. 4. Comparison of binding of dengue serotype 2 to HepG2 (■) and Vero (▲) cells. Each point represents the mean of three independent experiments.

ter at each point was known. In the experiment shown in gure 3, the final assay point represents a virus to cell atio of 200,000:1. Again, as can be seen, the virus was not ble to achieve saturation of binding on HepG2 cells, in ontrast to the result obtained when Vero cells were used fig. 4).

In a further experiment, the effect of the cell number ised in the reaction was analyzed, by undertaking an dentical binding experiment, but with different numbers of cells in the reaction. Cell numbers of 10^3, 10^4 and 10^5 were analyzed, with the exception that, due to material constraints, less radiolabel input levels were undertaken for the reactions containing 10^4 cells. Results are shown in figure 5. Perhaps most surprisingly, it can clearly be seen that the proportion bound increased with decreasing cell number.

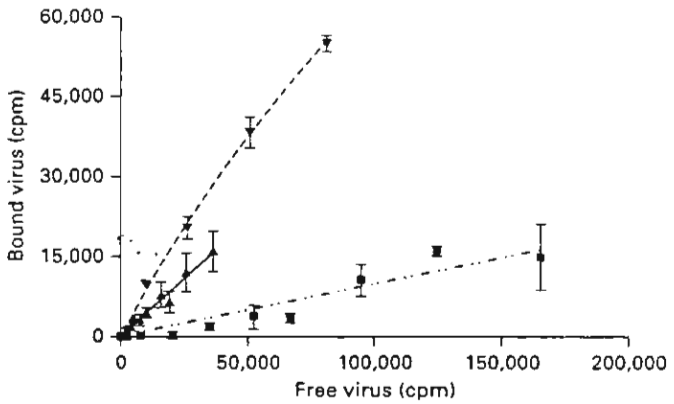


Fig. 5. Effect of cell number on binding of dengue serotype 2 to HepG2 cells. Each point represents the mean of three independent experiments. Lines represent 10^3 cells (dashed line), 10^4 cells (solid line) and 10^5 cells (dashed and dotted line).

Saturation Infection Analysis

In the absence of saturation of binding of radiolabeled virus of either serotype 1 or 2, we sought to determine the profile of infection of liver cells in the context of increasing virus to cell ratios. Cells were incubated for 90 min under standard infection conditions with different virus to cell ratios ranging from 1:1 to 5,000:1. After incubation, cells were treated with acid glycine to inactivate any noninternalized virus, and cells were incubated under optimal conditions. To avoid complications arising from reinfection of the cells by de novo produced viral particles, aliquots were taken and assayed for virus titer at 22 h, sufficient time to have allowed viral production from the first infection, but insufficient to allow reinfection and production [unpubl. observation]. Experiments were undertaken in triplicate with duplicate counting of each aliquot. Viral titer profiles are shown in figures 6 and 7. It is clear from the results that the response of liver cells to dengue serotype 1 was linear over the range investigated, while dengue serotype 2 became saturated with respect to infection at approximately 3,000 viruses per cell.

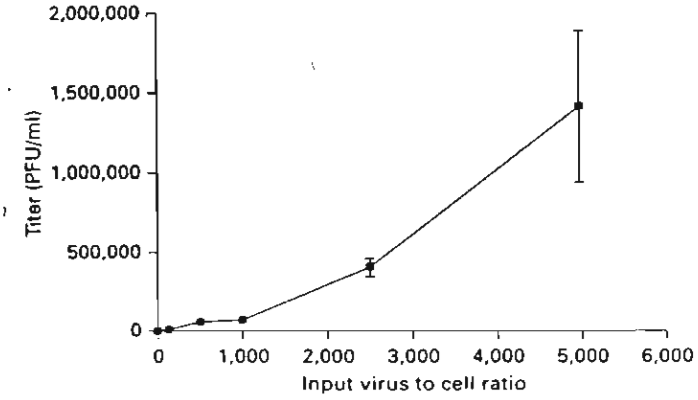


Fig. 6. Saturation of infection for dengue serotype 1. Viral progeny were assayed 22 h after infecting HepG2 cells with increasing ratios of virus to cells.

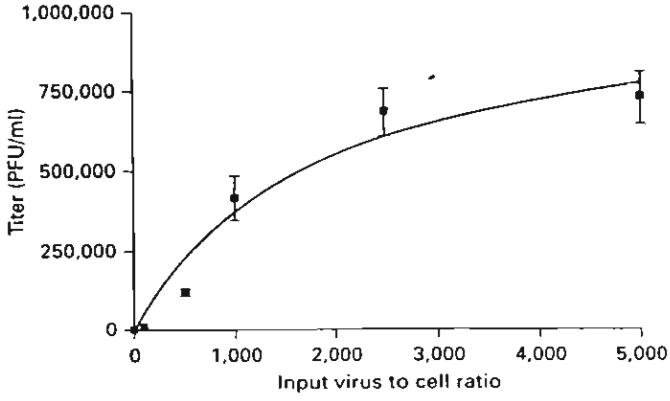


Fig. 7. Saturation infection of dengue serotype 2. Viral progeny were assayed 22 h after infecting HepG2 cells with increasing ratios of virus to cells.

Discussion

The binding of a virus to its cognate receptor is one of the most critical steps in determining the tissue tropism of a virus, and despite the importance of the liver as a target organ in the pathogenesis of the severe forms of dengue fever, namely DHF and DSS [9, 10], the nature of the interaction between dengue viruses and liver cells remains largely unexplored. Two studies which have investigated this relationship are contradictory [20, 21].

Our study with dengue serotypes 1 and 2 has shown that HepG2 cells are not saturable under experimentally obtainable conditions. Even at levels of 200,000 viral particles per liver cell, saturation was not obtained. In this we agree with the results of Hilgard and Stockert [21], who showed that Huh7 cells, also a hepatoma-derived cell line, were not saturable. In contrast, Marianneau et al. [20] showed obtainable saturation in HepG2 cells. Interestingly, however, our experimental methodology is consistent with that of Marianneau et al. [20] rather than with that of Hilgard and Stockert [21] in that our binding assay was undertaken in suspension after detaching the cells from the culture plate with trypsin. We note, however, that in our final experiment, a ratio of 200,000 viruses per cell was used, a figure far in excess of the 10^4 binding sites per cell calculated by Marianneau et al. [20].

Particularly interesting from our results is the evidence that there may be a degree of cooperation in dengue virus binding to liver cells. With the same viral input, a greater proportion of binding is noted with fewer cells. This trend was consistent over three different input cell values. This result suggests that the binding of one virus to a cell serves to facilitate the binding of further virus particles, and that this is cooperative in that the binding of each virus particle makes the binding of successive particles easier, perhaps through conformational changes induced in cell surface binding molecules. A degree of cooperation of binding would serve to concentrate viruses at the cell surface, and this may represent an important mechanism given that in vivo virus levels may be significantly below the levels used experimentally.

In light of the inability to demonstrate saturability of binding of the virus to the cell surface, we sought to establish whether it was possible to saturate the infectability of the cells by using increasingly high ratios of virus to cells. Interestingly, we note that increasing virus levels above 3,000 per cell for dengue serotype 2 does not increase the number of viral progeny. In contrast, dengue serotype 1 does not show a similar saturation of infection. The result with dengue serotype 2 suggests that the availability of receptors able to internalize the virus became exhausted. The results showing that serotype 1 does not become saturated may indicate that these two dengue serotypes utilize different receptors, i.e. ones that are present at different levels on the cell. It is, however, possible that other factors within the cell may serve to limit the production of virus-related proteins or the generation of intermediate forms of the virus, and as such, further experiments are warranted.

Overall, our results are consistent with proposed models of a two-step internalization of the dengue virus [21]. Glycosaminoglycans have been proposed to act as low-

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Behavior of the Dengue Virus in Solution

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The dengue virus consists of four antigenically related but distinct viruses, termed Dengue virus 1–4 (DEN 1–4). We have established that the dengue virus loses infectivity over time in solution in an exponentially declining manner. The four strains examined (one from each serotype) have half-lives that range from 2.5 to 7.5 hr in defined medium. The half-life is temperature and pH-dependent and is affected by the nature of the host cell in which it is produced, but is not dependent upon the presence of either Mg^{2+} ions or chelating agents. Electron microscopy (EM) of solutions of the dengue virus show almost complete virus aggregation after 24 hr at room temperature, while RT-PCR shows an intact RNA genome. These results show that the solution environment of the dengue virus is an important determinant of dengue virus infectivity. ***J. Med. Virol. 71:532–539, 2003.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: aggregation; electron microscopy; Flavivirus; infection; serotype

INTRODUCTION

Worldwide it is estimated that 2.5 billion people live at risk of infection with the dengue virus (family *Flaviviridae*, genus *Flavivirus*, species *Dengue virus*) with up to 100 million cases of infection each year [Halstead, 1988; Guzman and Kourf, 2002]. Infection of the dengue virus may be asymptomatic, but results more commonly in a relatively self-limiting febrile disease [Burke et al., 1988], termed dengue fever (DF). In up to 250,000 cases per year, however, the disease progresses to a more severe form characterized by significant bleeding normally known as Dengue Hemorrhagic Fever (DHF), or if coupled with hypovolemic shock, as Dengue Shock Syndrome (DSS) in which significant mortality may result [Halstead, 1989]. Dengue fever and its syndrome account for a significant number of hospitalizations each year, and in Southeast Asia represent the largest cause for hospital admission among children [Pinheiro and Corber, 1987].

The dengue virus consists of four antigenically related but distinct viruses, termed Dengue virus 1–4 (DEN 1–4). They are enveloped viruses, with a single-stranded positive polarity RNA genome and belong to the family *Flaviviridae*, which includes other viruses such as Japanese encephalitis and West Nile fever virus [Henchal and Putnak, 1990].

Why some infections result in a relatively mild (or even asymptomatic) disease, while others progress to DHF/DSS is unclear. Considerable data have suggested that the more severe forms of the disease are more likely to be associated with secondary dengue infections [Halstead et al., 1967, 1970; Russell et al., 1968; Halstead and O'Rourke, 1977; Sangkawibha et al., 1984]. It is hypothesized that non-neutralizing antibodies generated from a first round of infection serve to enhance the severity of the infection through a process termed antibody-dependent enhancement [Kliks et al., 1989; Morens, 1994], in which the complex of the dengue virus and the non-neutralizing antibodies enter monocytes through the use of the Fc receptor [Littaua et al., 1990]. However, while it is true that an association between Dengue hemorrhagic fever/Dengue shock syndrome (DHF/DSS) and secondary infection exists, it is believed to account for only some 15–80% of the overall risk of developing these syndromes. In particular, evidence that shows that DHF/DSS can occur in response to primary infections [Scott et al., 1976; Morens et al., 1987] serves to throw some question over this as a general mechanism to account for progression to the severe forms of infection.

During studies on the interaction between the dengue virus and liver cells [Suksanpaisan and Smith, 2003], we became interested in the ability of the dengue virus to retain its infectivity in solution. While it is known that the dengue virus becomes inactivated by exposure to a

Patchima Sithisarn and Lukkana Suksanpaisan contributed equally to this work.

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pH of 3 or lower [Hung et al., 1999], the mechanism of this inactivation is unclear, as is the long-term behavior of the dengue virus in a solution environment. This study therefore initially sought to determine the ability of the dengue virus to retain its infectivity over time in solution. As our studies investigate the dengue virus/liver cell interaction, we initially tested the retention of infectivity of the virus in the medium we used for growing the liver cell line and we undertook to investigate the retention of infectivity of strains of all four dengue serotypes.

MATERIALS AND METHODS

Dengue Viruses and Cells

Dengue serotypes 1 (strain 16007), 2 (strain 16681), 3 (strain 16562), and 4 (strain 1036) were a kind gift of Siritorn Butrapet (Center for Vaccine Development, Mahidol University, Thailand). Dengue viruses were propagated in Vero (African Green Monkey Kidney) cells except where stated. Vero cells were cultured at 37°C, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, UT) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD) and 100 U/ml of penicillin and 100 μg/ml streptomycin.

Virus Quantitation by Plaque Assay

Viral quantitation was undertaken by plaque assay using Vero cells. Cells were seeded in 6-well plates (4×10^5 cells/well) in DMEM supplemented with 5% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a humidified incubator at 37°C in 5% CO_2 for 2 days before the medium was removed and then infected with 200 μl of serially diluted virus solution. The plates were incubated for 1.5 hr at 37°C with agitation every 10 min. Subsequently, 1% SeaKem LE agarose (BMA, Walkersville, MD) mixed with nutrient overlay (Earle's Balanced salts supplemented with 0.5% (w/v) yeast extract, 2.5% lactalbumin hydrolysate, 3% FBS) was added to each well. The plates were incubated for a further 7 days at 37°C following which the agarose plugs were removed and cells fixed with 3.7% formaldehyde for 1 hr at room temperature. Plaques were visualized by staining with 1% crystal violet in 10% ethanol. All titration experiments were undertaken independently in triplicate, with duplicate assay of each point.

Dot Blot Analysis

Each dengue serotype was independently incubated in 2 ml Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat-inactivated FBS (Gibco-BRL) and 100 U of penicillin/ml, and 100 μg/μl streptomycin at 37°C with 10% CO_2 to a final viral concentration of 1×10^7 pfu/ml. Aliquots were withdrawn immediately and after every 12 hr for 48 hr and dotted onto nitrocellulose membranes (Protran, Schleicher & Schull, Keene, HN). Membranes were blocked with 5% skim milk in TBS (50 mM Tris-Cl, pH 7.6, 150 mM NaCl) at room temperature for 1 hr with constant agitation. Immunogenic dengue virus proteins were detected with human anti-dengue serum at a dilution of 1:100 in 5% skim milk in TBS. Incubation was undertaken at room temperature for 2 hr followed by washing three times with TBS. Subsequently, the membranes were incubated with HRP-conjugated anti-human IgG at a dilution of 1:3,000 in 5% skim milk in TBS for 1 hr at room temperature. After washing three times with TBS, detection was undertaken with the ECL+ plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, England) for 5 min at room temperature followed by exposure to autoradiographic film. The experiments were undertaken independently in triplicate.

Transmission Electron Microscopy (EM)

Virus solutions (purified dengue virus serotype 2 at a titer of 8.5×10^9 and 5×10^7 pfu/ml in DMEM with 20% FBS after 0 and 48 hr of incubation at 37°C, 10% CO_2) were diluted 1:1 with distilled water and applied to carbon-coated EM grids. Seven drops of 2% uranyl acetate solution were slowly applied to the grid, and excess liquid removed with Whatman paper (Whatman International Ltd., Maidstone, England). Grids were washed with 20 drops of distilled water and allowed to air dry. Grids were examined using a JOEL model JEM-1220 transmission electron microscope.

RT-PCR Analysis

Solutions of 2.5×10^7 pfu/ml of dengue virus serotype 2 in DMEM with 10% FBS were incubated for 0, 24, or 48 hr at 37°C, 10% CO_2 following which 250 μl was mixed with 750 μl Tri-Reagent LS (MRC, Cincinnati, OH) and RNA extracted according to the manufacturer's protocol. First strand cDNA synthesis was undertaken using the Impromp-II Reverse transcriptase system (Promega, Madison, WI) using 20 pmol of NS3 reverse primer according to the manufacturer's protocol. First strand cDNA product (5 μl) was used directly in the PCR amplification reaction by the addition of 20 pmol of NS3 forward and reverse primers, 0.2 mM dNTPs, and 1 U of cloned *pfu* polymerase (Promega) and *pfu* buffer. Samples were initially denatured at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 68°C for 1.4 min followed by a final extension at 68°C for 7 min. Amplification was carried out in an automated thermal cycler, GeneAmp PCR system model 2400 (Perkin Elmer, Foster City, CA). Positive PCR control was provided by a cloned-dengue serotype 2 NS2B-NS3 construct [Khumthong et al., 2002]. Forward and reverse primers used are expected to generate a 602 bp fragment of the protease domain of NS3 and were: NS3 (F) 5′-GAACAAACACTGTGGTACCTGTGGGAAGTGAAGAAAC-3′ and NS3 (R) 5′-CTTCTCTTTCAGGATCCCTAATCTTCGATCTCTGGGTTG-3′.

Data Analysis

All data were analyzed using the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA).

RESULTS

Loss of Dengue Virus Infectivity in Solution

A known titer of infectious dengue viruses (as determined by plaque titer assay) of each serotype were added independently to DMEM supplemented with 10% FBS, a medium normally employed for the propagation of HepG2 cells, to give a final viral titer of 1×10^7 pfu/ml. The virus/medium mixture was incubated at 37°C and 10% CO_2. At various time-points aliquots were taken and the level of infectious dengue viruses per milliliter assayed by standard plaque titration assay on Vero cells (Fig. 1A–D). All four serotypes demonstrated a rapid loss of viral viability. In each case, the loss of viral viability fitted an exponential decay equation, and gave significantly different ($P < 0.0001$) calculated half-life times of 2.5 hr (Dengue serotype 3), 4.2 hr (Dengue serotype 1), 6.8 hr (Dengue serotype 2), and 7.5 hr (Dengue serotype 4). Semi-log plots of loss of viral infectivity resulted in a straight line (Fig. 2). The loss of infectivity was shown to be concentration independent over a 100-fold range with starting titers of 1.2×10^7 and 1.2×10^5 pfu/ml for dengue serotype 2 (Fig. 3).

Exclusion of Enzymatic Factors

Having determined the basic profiles of loss of infectivity of the dengue virus in solution, we then sought to determine which factors were influencing the rate of loss of infectivity. As the dengue virus consists of a positive single-stranded RNA genome surrounded by a protein shell, we sought to determine if either the presence of RNases or proteases were causing the loss of viral infectivity. However, the time course profiles of dengue

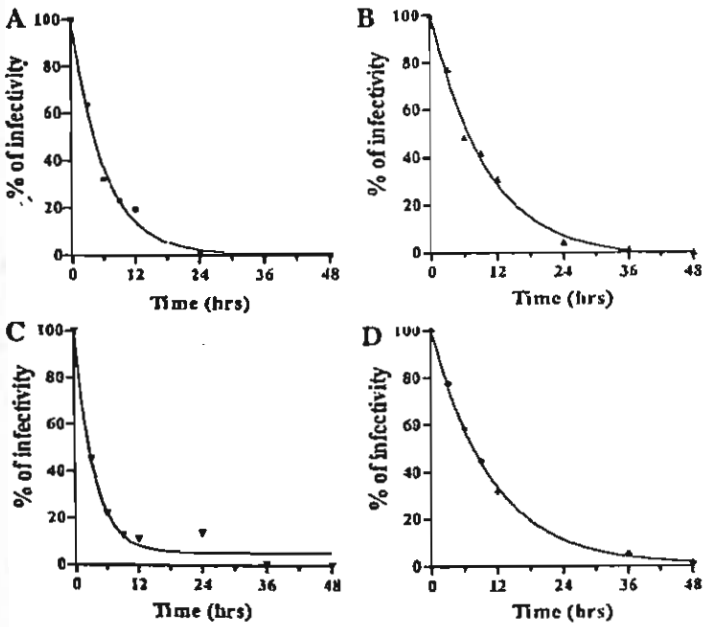

Fig. 1. Time course profiles of loss of dengue virus infectivity for dengue serotypes 1 (Panel A), 2 (Panel B), 3 (Panel C), and 4 (Panel D) in cell culture medium. Points represent the mean of three independent experiments.

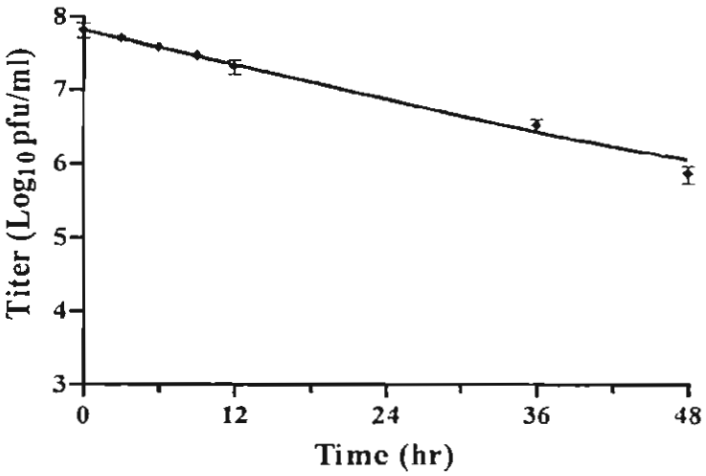

Fig. 2. Semi-log plot of time course profile for loss of infectivity for dengue serotype 4. Error bars indicate SEM.

serotype 2 in the presence or absence of RNase inhibitors or protease inhibitors were not significantly different (Fig. 4), suggesting that the loss of viral infectivity over time is not due to enzymatic factors but is rather an inherent characteristic of the dengue virus. The short half-life seen with and without protease inhibitor was later determined to be an effect of the solution pH (see later).

Effects of Solution Composition

The standard medium used in these titers is DMEM supplemented with 10% FBS. The exclusion of the bovine serum did not affect the rate of loss of dengue serotype 2 viral infectivity (Fig. 5) neither did the inclusion of EDTA in the medium (Fig. 5). The presence of Mg^{2+} ions at high concentration had some effect on the rate of loss of dengue serotype 2 viral infectivity (Fig. 5). The pH of the solution (Fig. 6) was, however, shown to have a marked effect on the loss of viral infectivity, with the slowest rate of loss of infectivity (longest half-life) being determined when the pH of the solution was pH 9.0. Solutions both above (data not shown) and below this pH showed a more rapid loss of virus infectivity (Fig. 6). Similarly, the temperature of the incubation was shown to have a dramatic effect on the rate of loss of infectivity. Incubation of dengue serotype 2 virus at 42°C produced an extremely rapid loss of viral infectivity, while incubation at 4°C showed a relatively slow loss of infectivity (Fig. 7). Interestingly, at 4°C the loss of infectivity seems to reach a plateau, at which point the virus remains relatively stable over time (Fig. 7).

Effect of Host Cell on Half Life

The viruses used in the experiments above were purified from infected Vero (African Green Monkey kidney) cells, a standard dengue virus propagation cell line. To investigate the nature of the host cell in which the virus was propagated, we also propagated and purified the dengue virus from a human cell line, namely

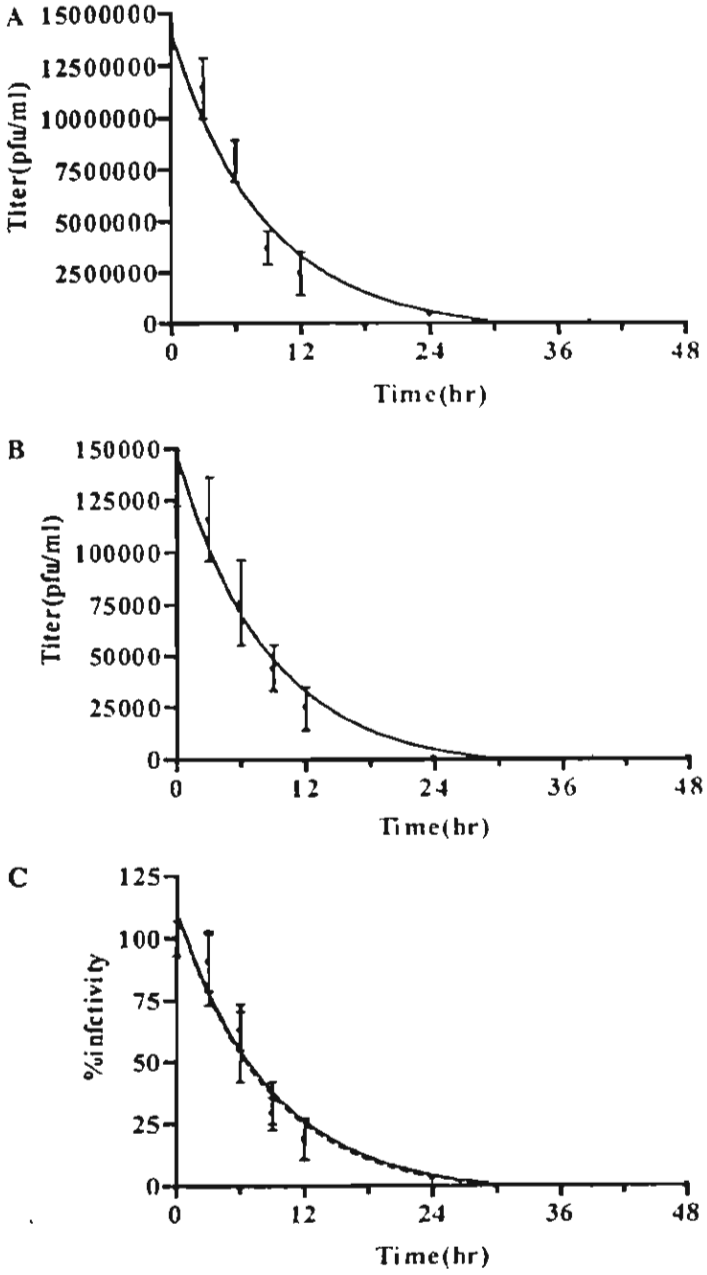

Fig. 3. Effect of starting concentration on loss of infectivity for dengue serotype 2, with starting concentrations of $1.25 \cdot 10^7$ pfu/ml (**Panel A**) and 1.25×10^6 pfu/ml (**Panel B**). Profiles are also shown as a percentage of starting value (**Panel C**: triangles 1.25×10^7; squares 1.25×10^6). Bars represent SEM of three independent experiments with duplicate assay of titer for each point.

HepG2 which originated from a human hepatoma. The half-life times (0.85, 1.35, 1.43, and 3.0 hr for DEN 1–4, respectively; Fig. 8) were determined to be significantly different from the times determined for virus propagated in Vero cells.

Immunoreactivity of the Dengue Virus

To determine whether the loss of virus viability was associated with a loss of immunoreactivity, the titration experiment was repeated, but instead of assaying by viral titer the samples were analyzed by Western dot blot analysis after drying the aliquots onto nitrocellulose membranes (Fig. 9). No loss of immunoreactivity was seen with any serotype.

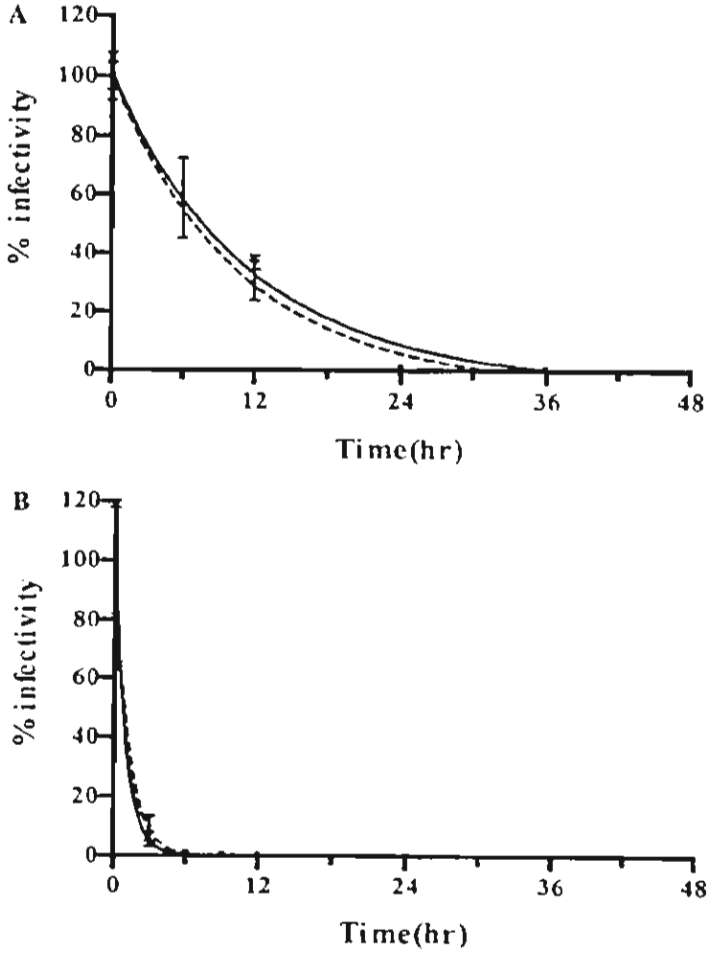

Fig. 4. Time course profile of loss of infectivity of dengue serotype 2 in (A) presence (dotted line) and absence (solid line) of RNase inhibitor and (B) presence (dotted line) and absence (solid line) of protease inhibitor cocktail. Error bars represent SEM.

Electron Microscopy Imaging of the Dengue Virus

While the Western dot blot analysis was able to show that even with a significant loss of virus titer there was no loss of immunoreactivity, it was still unclear as to whether the dengue virus remained intact but unable to infect cells, or had undergone a loss of structural integrity. We therefore examined solutions of the virus at two time-points (0 and 48 hr) by transmission EM (Fig. 10). The dengue virus is approximately 40–50 nm in diameter, and so while fine details are not discernable, the overall integrity of the virus may be established. As can be seen from Figure 10 at the 0 hr incubation time-point is it possible to discern discrete virions. However, after 48 hr incubation the dengue virus has aggregated and it is no longer possible to discern individual discrete dengue particles.

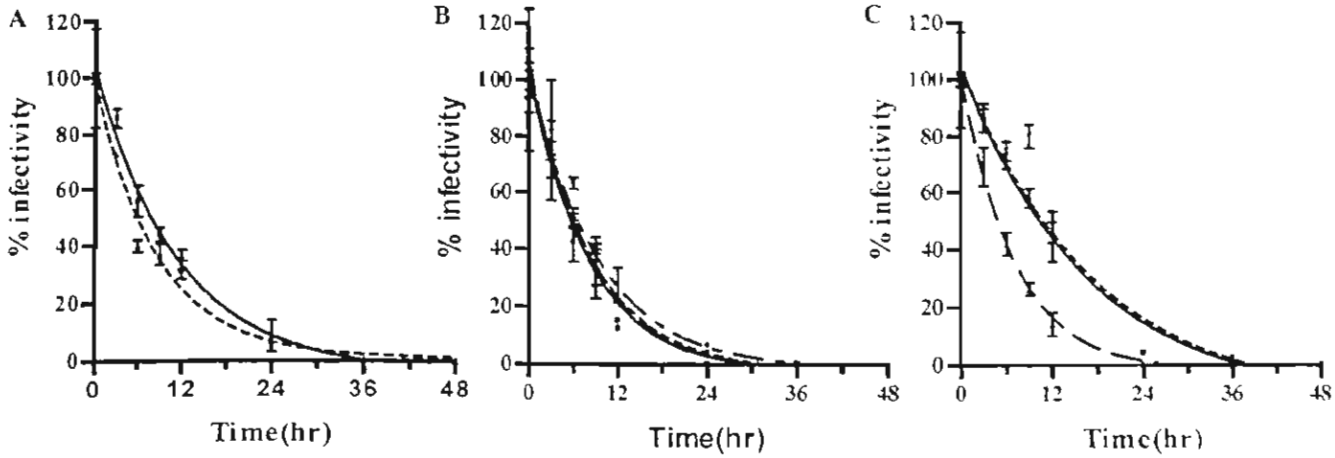

Fig. 5. Time course profile of loss of dengue serotype 2 infectivity in (A) presence (solid line) and absence (dotted line) of FBS; (B) absence (solid line) and presence of EDTA at 2.5 mM (dotted line), 25 mM (dashed line), and 250 mM (dots and dashes line), and (C) absence (solid line) and presence of Mg^{2+} ions at 10 mM (dotted line, and 100 mM (dashed line). Error bars represent SEM.

RT-PCR Analysis of Viral RNA

Given that the electron microscopy (EM) suggested that the virus was still intact, albeit non-infectious, after 48 hr incubation, we next sought to determine if the dengue genomic RNA molecule was also intact. Dengue virus solutions after 0, 24, and 48 hr incubation were subjected to RT-PCR using primers directed against the NS3 protease region of the dengue virus, along with a cloned NS2B-NS3 positive PCR control [Khumthong et al., 2002]. A no template negative RT-PCR control was also included. Results (Fig. 11) show a clear signal of the expected 602 base pairs (bp) detectable even after 48 hr. While this RT-PCR methodology is not quantitative, signal intensities between the 0, 24, and 48 hr incubation samples are roughly equal. This result suggests that there is no detectable loss or degradation of the RNA genome of the virus, and suggests that the viral coat is intact.

DISCUSSION

In this study we have shown that the dengue virus in solution loses infectivity over time in a concentration independent manner, and that the rate of loss of infectivity is primarily determined by temperature and pH of the solution. EM data shows that the dengue virus tends to aggregate over time and it is evident that the aggregated virus is no longer able to be internalized into cells, as evidenced by the decline in virus titer over time, and the aggregation of the virus is apparently non-reversible as all samples are placed into the same solution at the plaque assay stage. Furthermore, the virus apparently retains structural integrity, given that the RNA genome is apparently intact as determined by RT-PCR. At this point, it is unclear why the dengue virus should be so susceptible to aggregation. However, in the best studied member of the *Flaviviridae* family, Tick Borne Encephalitis (TBE) virus, it is known that low pH

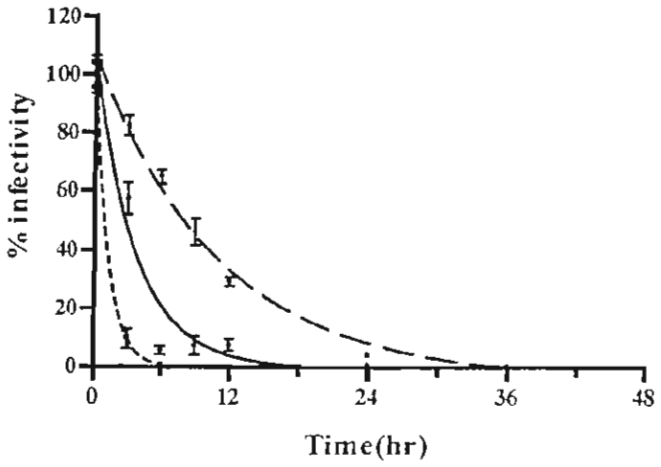

Fig. 6. Time course profile of loss of dengue serotype 2 infectivity at pH 6.4 (dotted line), pH 8.4 (solid line), and pH 9.0 (dashed line). Error bars represent SEM.

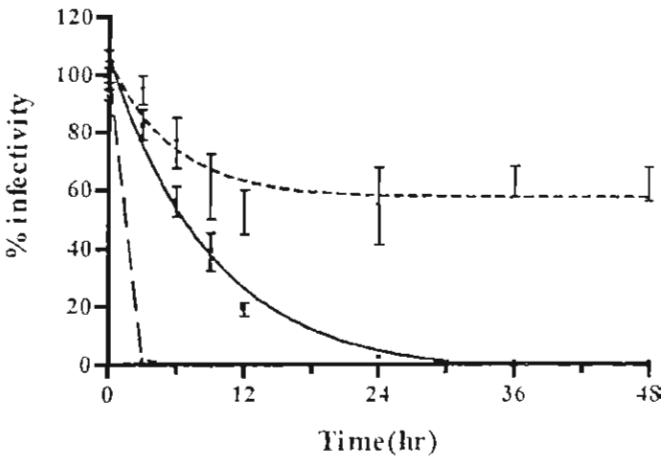

Fig. 7. Time course profile of loss of dengue serotype 2 infectivity at 4°C (dotted line), 37°C (solid line), and 42°C (dashed line)

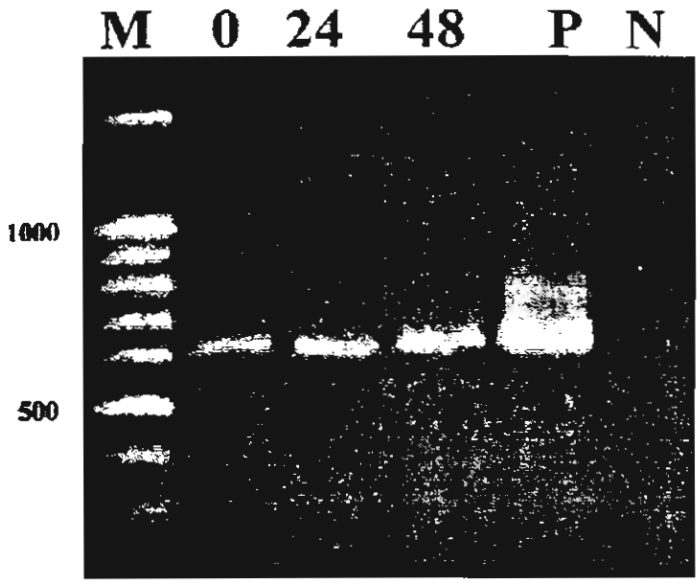

Fig. 11. RT-PCR of dengue virus serotype 2 incubated in standard medium for 0, 24, and 48 hr along with a positive PCR control (P) and negative RT-PCR control (no template: N). M: 100 bp DNA marker ladder. Positions of 500 and 1,000 bp marker bands are shown. The expected product is 602 bp.

to the interesting possibility that any individual infected with the dengue virus may produce a spectrum of dengue viruses with different half-lives dependent upon the tissues involved in the infection.

Our study investigated single strains of the four dengue serotypes, and as such we are unable to determine at this point whether the loss of infectivity is serotype or strain type specific and we are currently attempting to obtain other strains to address this issue. Whether the loss of infectivity is strain type or serotype specific is not really germaine to the issue of loss of viral infectivity of the dengue virus over time, at this point. Our results have clearly demonstrated that the solution environment of the dengue virus is a determinant of the behavior of the dengue virus, and in the context of the development of live attenuated vaccines may have important implications.

In this study, we have addressed the issue of the loss of infectivity of the dengue virus in clearly defined medium. Preliminary results in our laboratory have determined that the dengue virus shows a similar loss of infectivity over time in both serum and plasma of normal health volunteers and that a single virus strain shows a range of half-lives in different individuals. The issue of loss of infectivity in human samples is vastly more complex, given the complicated relationship between the dengue virus and the immune system [Sierra et al., 2002]. However, recent data showing that the dengue virus is cleared more slowly in defervescent dengue hemorrhagic patients as opposed to defervescent dengue fever patients [Wang et al., 2003] indicate that the issue of loss of infectivity of the dengue virus over time may also have clinical significance. However, the study by Wang et al. [2003] assessed the viral load by quantitative reverse transcription-polymerase chain reaction (RT-PCR), and, as we have shown here, there is a decoupling between the presence of the RNA genome as detected by RT-PCR and the infectivity of the virus. Given that many studies use RT-PCR methodologies to assess actual virus load [Wang et al., 2000; Chen et al., 2001; Sudiro et al., 2001; Libraty et al., 2002], this issue clearly requires further investigation.

ACKNOWLEDGMENTS

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Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2

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Summary. This study sought to identify receptor elements for dengue virus serotype 2 on human liver cells (HepG2) using the viral overlay protein binding assay (VOPBA) technique and Mass Spectrometry fingerprinting. A single major and several minor virus binding bands were observed, and mass spectrometry identified a candidate binding protein for the major binding band as GRP 78 (BiP). GRP78 expression on the cell surface was confirmed, and antibodies directed against both the N and C-terminus of GRP 78 (BiP) altered both the binding of the virus to the cell surface as well as the infectivity profile of HepG2 cells in response to dengue serotype 2 infection. GRP 78 (BiP), which has previously been identified as a co-receptor protein for coxsackievirus A9, is the first non-Fc receptor protein identified for the dengue virus, although GRP78 probably functions as part of a receptor complex.

Introduction

Despite being a critical determinant of tissue tropism and thus of pathogenicity, the nature of the dengue virus (family *Flaviviridae*, genus *Flavivirus*, species *Dengue virus*) cellular receptor or receptors remains largely unknown. It is known that in cases of secondary dengue infection, where the virus becomes complexed with cross reacting but non-neutralizing antibodies from a primary infection with a different serotype the Fc receptor is used by the virus to gain entry to monocytes [12]. However the identification of proteins used in primary infections of the dengue virus has been less successful, although several groups have identified potential virus binding proteins using the virus overlay protein binding assay (VOPBA) technique [22, 23], and recently glycosaminoglycans [8], an LPS/CD14-associated binding protein [9] and DC-SIGN [27] have all been

implicated as elements mediating dengue virus entry into cells. It is clear however that receptor usage is both cell type and serotype specific [2]. While infection with one of the four dengue serotypes usually results in no symptoms or a relatively mild, self limiting febrile disease [7], in a number of cases the disease can progress to a severe form with significant hemorrhage and significant mortality may result [18]. In these cases the liver is frequently involved in the disease [24], and the virus has been recovered from the livers of fatal cases of dengue infection [25]. Given that the liver is an established target organ, we sought to identify any protein or proteins that may be acting as a receptor on liver cells (HepG2) by a combination of VOPBA and mass spectrometry fingerprinting.

Materials and methods

Virus and cells

Dengue serotype 2 (strain 16681) was a kind gift of Siritorn Butrapet (Center for Vaccine Development, Mahidol University, Thailand). Virus was propagated in Vero (African Green Monkey Kidney) cells. Vero cells were cultured at 37 °C, 2% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, Utah, U.S.A.) supplemented with 5% heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, U.S.A.) and 100 units/ml of penicillin and 100 μg/ml streptomycin. Virus was purified through a 30/60% sucrose step gradient in TNE buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA). The human hepatoma cell line HepG2 was cultured at 37 °C, 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah, U.S.A.) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, U.S.A.) and 100 units of penicillin and 100 μg streptomycin.

Dengue virus production in HepG2 cells

HepG2 cells were grown in 75 cm^2 tissue culture flasks containing 20 ml of growth medium. Prior to confluence, when the cell number reached 1.0×10^7 the culture media was discarded and replaced with 3 ml of BA-1 (1 × Medium 199/Earle's balanced salts; 0.05 M Tris-HCl pH 7.6, 1% BSA Fraction V, 7.5% $NaHCO_3$, 100 units penicillin-streptomycin per ml) containing the dengue virus at a MOI of 1. Viral absorption was allowed to proceed for 90 min at 37 °C with constant agitation. After this period cell/virus mixture was treated with acid glycine at pH 3.0 for 1 min to inactivate any uninternalized viruses [19] and cells washed three times with PBS. Fresh culture media was added to the cells and the cells incubated under standard conditions. Aliquots of growth media were taken at hourly intervals between 10 and 24 h and analyzed for viral titer in duplicate. Experiment was undertaken in duplicate. Data was analyzed using the GraphPad Prism program (GraphPad Software Inc., San Diego, Ca, U.S.A.).

Cell membrane preparation

Cell membrane preparations were prepared essentially as described by Martinez-Barragan and del Angel [22] although with minor modifications. Briefly, exponentially growing HepG2 cells were harvested by scraping from 100 mm × 20 mm culture plates in TBS (50 mM Tris HCl, pH 7.6, 150 mM NaCl) and pelleted by centrifugation at 600 × g for 3 min followed by resuspension in 5 volumes ice cold buffer M (100 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF). Cells were lysed by vortexing and solution was centrifuged at 600 × g to remove nuclei and cell debris. The supernatant was then centrifuged

at 6000 × g to remove membranous organelles and then at 20,000 × g to pellet the membrane proteins. The pellet was resuspended in buffer M, and protein concentration determined by the Bradford method [6].

Western blotting and Viral overlay protein binding assay (VOPBA)

Membrane proteins (80 μg) were separated by electrophoresis through an 8% SDS-PAGE gel and transferred to nitrocellulose membranes using the WetBlot technique (Bio-Rad, Richmond, CA, U.S.A.). After transfer was complete the membrane was blocked with 5% skim milk in TBS at room temperature of 1 h with constant agitation. For western blots the membranes were incubated with a 1:500 dilution of either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) or anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) for 2 h at room temperature. Following rinsing three times with TBS membranes were incubated with a 1:2000 dilution of either peroxidase conjugated anti-goat IgG or peroxidase conjugated anti-rabbit IgG (Pierce, Rockford, IL, U.S.A.) for 1 h at room temperature. Signal was developed using the ECL Plus Western Blotting Analysis kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). For viral overlay the membranes were incubated with 1×10^7 pfu dengue serotype 2 virus in 1% skim milk in TBS. Hybridization was undertaken at 4 °C for 2 h followed by washing three times with TBS at room temperature. Immunoblotting was undertaken with a pan specific anti-dengue virus monoclonal antibody produced by hybridoma cell line HB-114, (a kind gift of Siritorn Butrapet) at a dilution of 1:100 in 5% skim milk in TBS. Incubation was undertaken at room temperature for 2 h followed by washing three times with TBS. Following this, membranes were incubated at room temperature for 1 h with HRP-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., St Louis, MO, U.S.A.) at a dilution of 1:3000 in 5% skim milk in TBS. Signal was developed as for western blotting.

Mass spectrometry

Mass spectrometry was undertaken commercially by the Australian Proteome Analysis Facility (APAF). Bands were subjected to tryptic digestion for 16 h followed by Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry was performed with a Micromass Maldi Time of Flight (MALDI-TOF) Mass Spectrometer. Spectra were acquired in the mass range 750–3500 Da. Spectra were searched against Human using ProteinLynx on MassLynx by APAF.

Inhibition of infection

HepG2 cells (1×10^6) were pre-incubated with either anti-human GRP 78 goat polyclonal antibody N-20 (Santa Cruz Biotechnology Inc.), anti-human GRP 78 rabbit polyclonal antibody H-129 (Santa Cruz Biotechnology Inc.) or anti-human cytochrome C mouse monoclonal antibody A-8 (Santa Cruz Biotechnology Inc.) for 1 h at 37 °C in 1 ml of serum-free medium. The cells were then washed twice with PBS and incubated with dengue serotype 2 at a multiplicity of infection (MOI) of 0.1 for 90 min at 37 °C. Cells were treated with acid glycine pH 3.0 to inactivate un-internalized viruses [19], then allowed to grow under standard conditions for 24 h before viral titer was assayed by plaque titration on Vero cells.

Immunohistochemistry for cell surface expression of GRP78

HepG2 cells were grown on glass slides for 1 day and subsequently fixed with cold acetone for 10 min. Endogenous peroxidases were quenched by incubation with 5% H_2O_2 for 5 min at room temperature. Cells were blocked with PBS containing 0.02% calf serum for 5 min

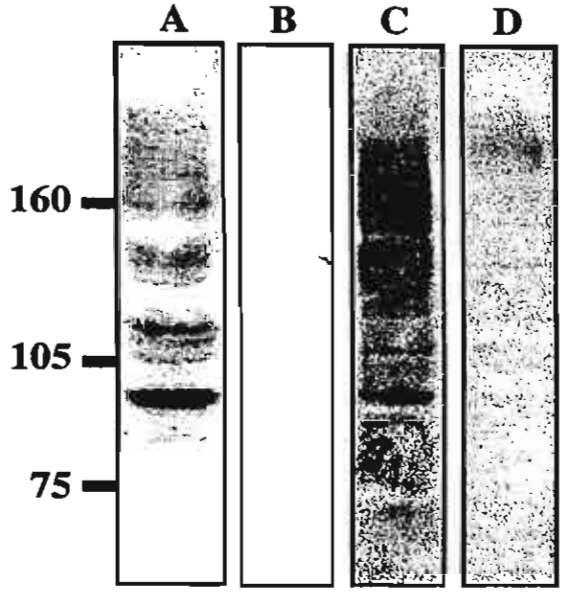

Fig. 1. VOPBA analysis of dengue virus serotype 2 on HepG2 cell membranes. Cell membranes were prepared from HepG2 and separated by SDS-PAGE. Proteins were transferred to solid matrix support and incubated with dengue virus serotype 2. Position of binding of dengue serotype 2 was detected by using a pan-specific monoclonal antibody directed against the dengue virus E protein (VOPBA). Panels **A** and **C** are independent experiments. Negative control (**B**) was obtained by incubating filter with no virus. The major binding band of approximately 90 kDa was established to be trypsin sensitive by pre-treating intact HepG2 cells with trypsin prior to membrane extraction (**D**). Position of protein marker bands are shown in kDa

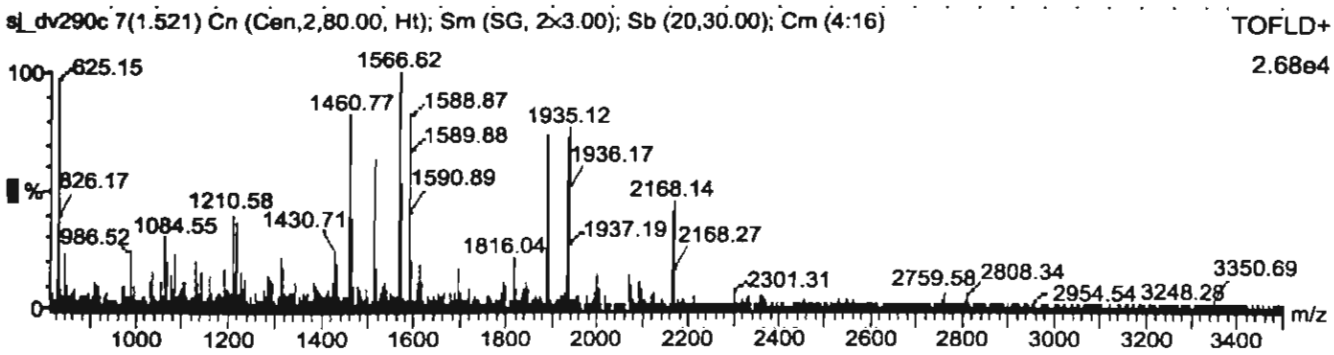

Fig. 2. Spectra received from APAF (see Materials and methods) of MALDI-TOF analysis of dengue virus binding band from VOPBA experiment

Given that GRP 78 (BiP) has been primarily characterized as an endoplasmic reticulum resident chaperonin [21] we sought to confirm the expression of GRP 78 (BiP) on the surface of HepG2 cells via immunohistochemistry. Two polyclonal antibodies directed against human GRP 78 (BiP) were evaluated, one directed

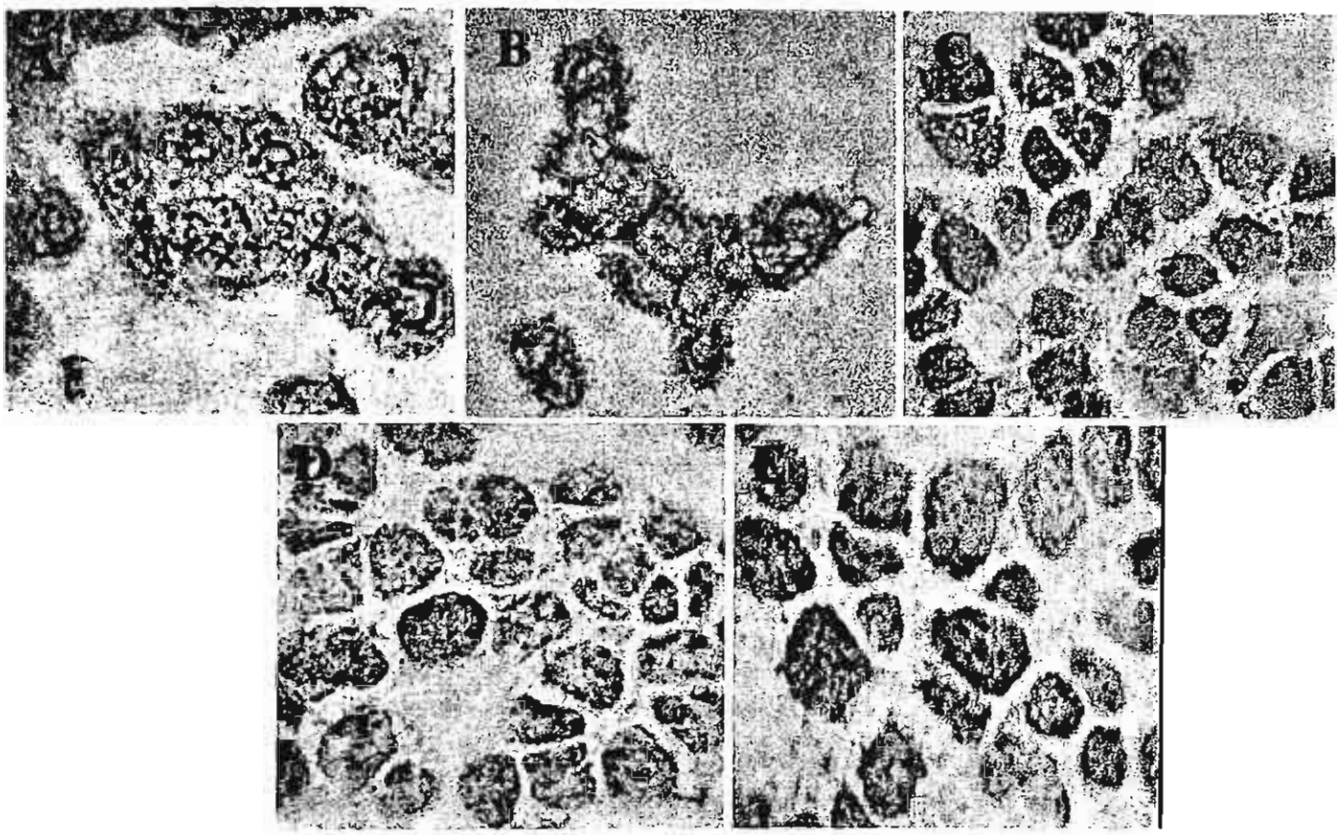

Fig. 3. Expression of GRP78 on the cell surface of HepG2 cells. HepG2 cells were grown on glass slides and reacted with polyclonal antibodies against the N-terminus (**A** and no primary antibody control, **D**) or C-terminus (**B** and no primary antibody control, **E**) of GRP 78 (BiP) as well as an irrelevant antibody (against human cytochrome C, **C**). Positive signal stains brown. Original magnification ×400

against an N-terminal epitope (GRP 78 N-20) and one against a C-terminal epitope (GRP 78 H-129). Both antibodies showed significant levels of expression on the surface of HepG2 cells (Fig. 3) in contrast to an irrelevant antibody directed against human cytochrome C (Fig. 3).

To investigate the position of the major VOPBA binding band as compared to the position of GRP78 (BiP) in the gel system used, we undertook parallel western blotting and VOPBA with the two antibodies. A total of two major and one minor band were identified with the two antibodies in the western blots, suggesting that the membrane associated GRP78 (BiP) may have different forms (Fig. 4). The VOPBA binding band was co-incident with the upper, minor band of GRP78 (BiP) detected with the C-terminal epitope antibody. A corresponding band was not observed with the N-terminal epitope antibody, but this may be below the sensitivity of this antibody.

To investigate the binding of the dengue virus onto HepG2 cells in the presence of anti-GRP78 (BiP) antibodies, cells were pre-incubated with both

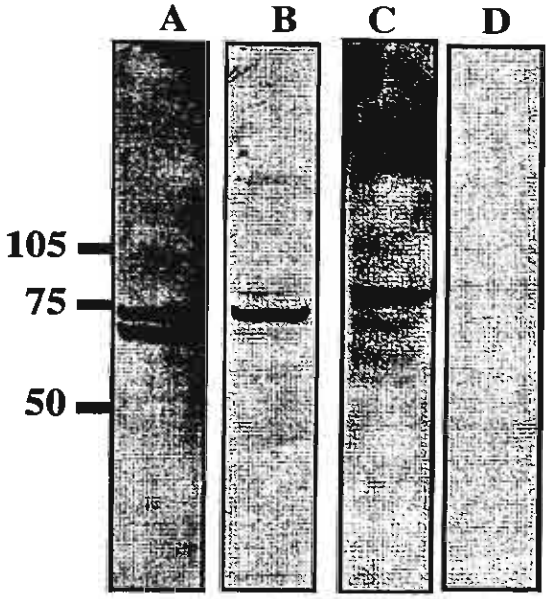

Fig. 4. Western blot and VOPBA analysis. HepG2 cell membrane proteins were extracted, separated by SDS-PAGE and transferred to solid matrix support. Filters were probed with polyclonal antibodies directed against either the N-terminus (**A**) or C-terminus (**B**) of GRP 78 (BiP), or incubated with dengue serotype 2 followed by detection of virus binding by incubation with a pan-specific monoclonal antibody directed against the dengue virus E protein (VOPBA; **C**). Negative control for VOPBA was obtained by incubating the filter with no virus (**D**). Position of protein marker bands are shown in kDa

anti-GRP 78 (BiP) antibodies, as well as an irrelevant control antibody prior to incubation with dengue virus serotype 2. Subsequent to incubation with the dengue virus, cells were incubated with a pan-specific anti-dengue monoclonal antibody and a fluorescent secondary antibody. Visualization under a fluorescent microscope (Fig. 5) showed a significant reduction from the levels of fluorescence seen with either with no antibody pre-incubation or pre-incubation with an irrelevant antibody for the pre-incubation with the antibody directed against the N-terminus of GRP78 (BiP), and a more equivocal picture with pre-incubation with the antibody directed against the C-terminal of GRP78 (BiP).

Functional confirmation of a candidate protein as a viral receptor can be achieved through a plaque reduction assay [29]. However, as there is no validated plaque assay for HepG2 cells which do not grow in monolayer, we sought to evaluate the effect of pre-incubation of HepG2 cells with antibodies directed against the putative receptor by determining the level of mature infectious virus particles produced. To avoid complications arising through multiple rounds of infection we first determined a virus production profile for dengue serotype 2 (Fig. 6) which determined that infectious viruses were first detectable 17 h after infection. Hence viral levels detected at 24 h after infection would result from a single non-amplified round of viral production. Antibody mediated inhibition

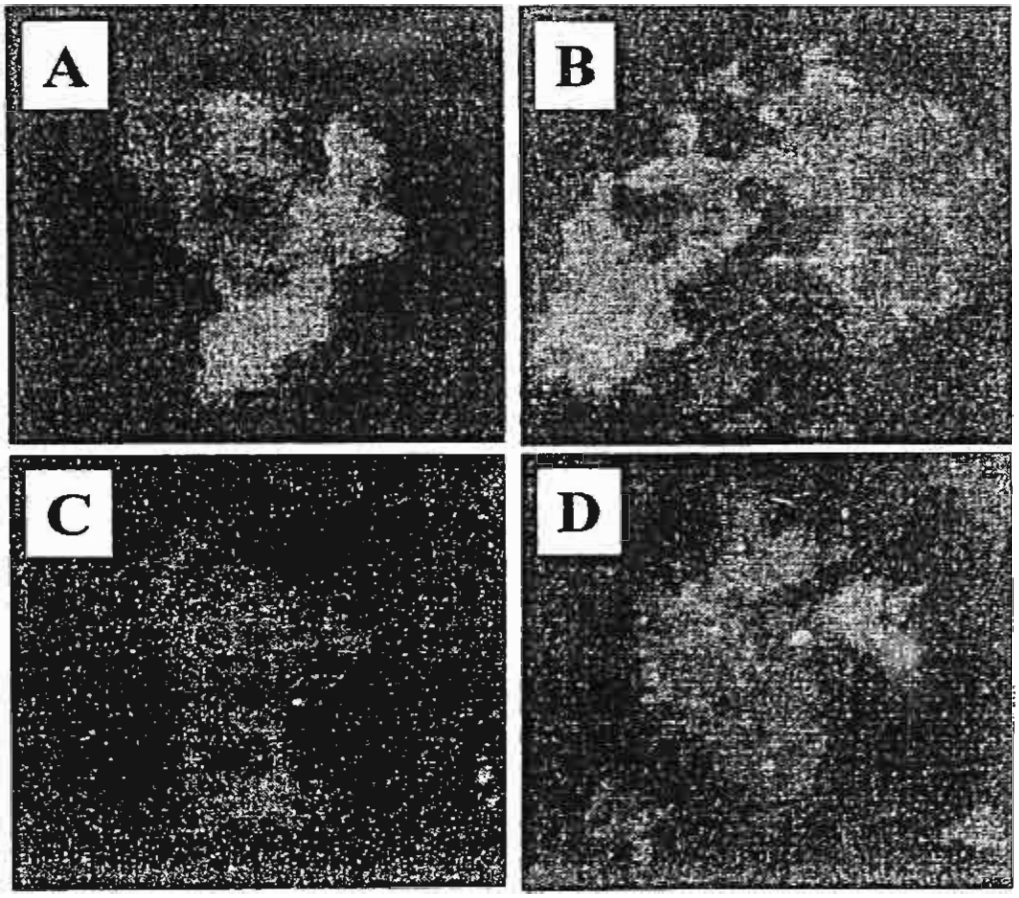

Fig. 5. Fluorescence microscopy of dengue virus serotype 2 binding to HepG2 cells. HepG2 cells were grown on glass slides and incubated with the dengue virus after pre-incubation with antibodies directed against the N-terminus (**C**) or C-terminus (**D**) of GRP78 (BiP) or an irrelevant antibody (**B**) or no antibody (**A**). Binding of the dengue virus was visualized using a pan-specific monoclonal antibody directed against the dengue virus E protein and a FITC-conjugated secondary antibody. Samples were viewed under a fluorescent microscope Original magnification ×400

of infection was carried out using 10 μg/per reaction of the two anti-GRP 78 polyclonal antibodies as well as 10 μg of an irrelevant antibody (directed against human cytochrome C). Experiment was undertaken twice, once in triplicate with three assays of each virus titer, and once in triplicate with six assays of each virus titer. Results (Fig. 7) show an inhibition to about 60% of control levels with pre-incubation of the cells with the antibody directed against the N-terminus of GRP 78 (BiP) while pre-incubation of HepG2 cells with an antibody directed against the C-terminus of GRP 78 (BiP) shows an enhancement to approximately 180% of control levels. The enhancement of infection with the antibody directed against the C-terminus of GRP 78 (BiP) was shown to be concentration dependent (Fig. 7).

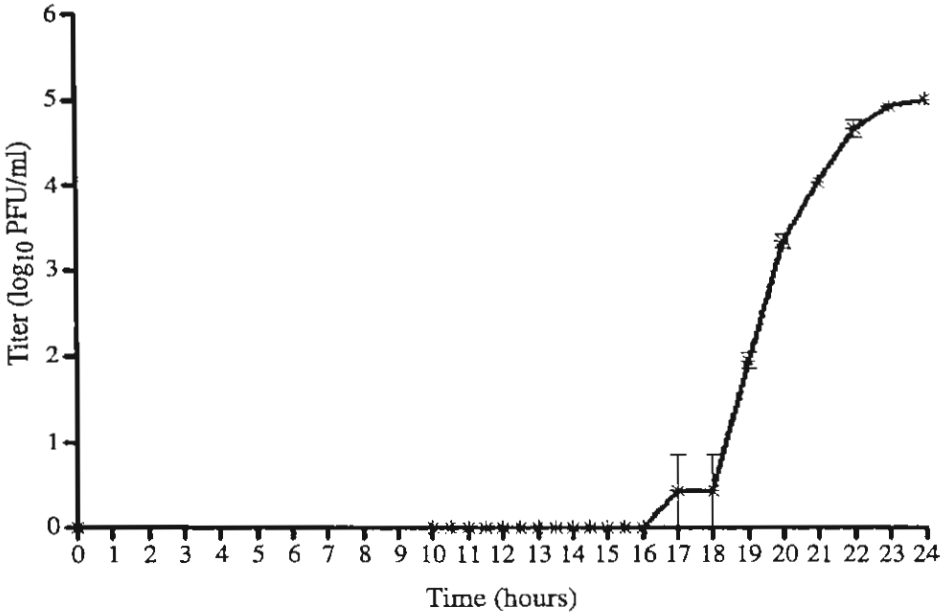

Fig. 6. Virus production curve of dengue serotype 2 in HepG2 cells. HepG2 cells were infected with dengue serotype 2 at a multiplicity of infection of 1 and un-internalized viruses inactivated with acid glycine at pH 3.0. Virus titer of the growth medium was determined hourly by plaque assay between 10 h and 24 h post infection

Discussion

Glucose regulated protein 78 is known to be primarily resident in the endoplasmic reticulum [16] where it is believed to function as a chaperonin involved in the folding an assembly of MHC class 1 antigens [21]. GRP 78 was first identified as an immunoglobulin heavy chain binding protein termed BiP [17] and it was shown to bind to immunoglobulin heavy chains in the absence of light chains in pre-B lymphocytes. Recently, several studies have shown GRP78 (BiP) to be expressed on the cell surface [13, 26, 28, 30] a finding confirmed by this study. In the most recent of these studies [26] global profiling on five different cell lines showed an abundance of ER chaperone proteins (including GRP78, GRP75, HSP70, HSP60, HSP54 and HSP27) on the cell surface, confirming several studies that had previously identified HSP70 on the surface of a number of cell types [1, 3–5, 14, 20]. While the functionality of these proteins on the cell surface remains to be elucidated, it is interesting to note that GRP78 (BiP) has previously been identified as a co-receptor protein for the non-enveloped Coxsackievirus A9 [29]. In this system, GRP 78 (BiP) acts as a binding protein for the Coxsackievirus A9, but internalization of the virus requires the expression of major histocompatibility complex class I molecules [28].

In this report we have demonstrated that GRP78 (BiP) functions as a receptor element on liver cells for dengue virus serotype 2. Interestingly, antibodies directed against the N terminus of GRP 78 (BiP) inhibit both binding and infection, while those directed against the C-terminus of the protein enhance infection (but not no-

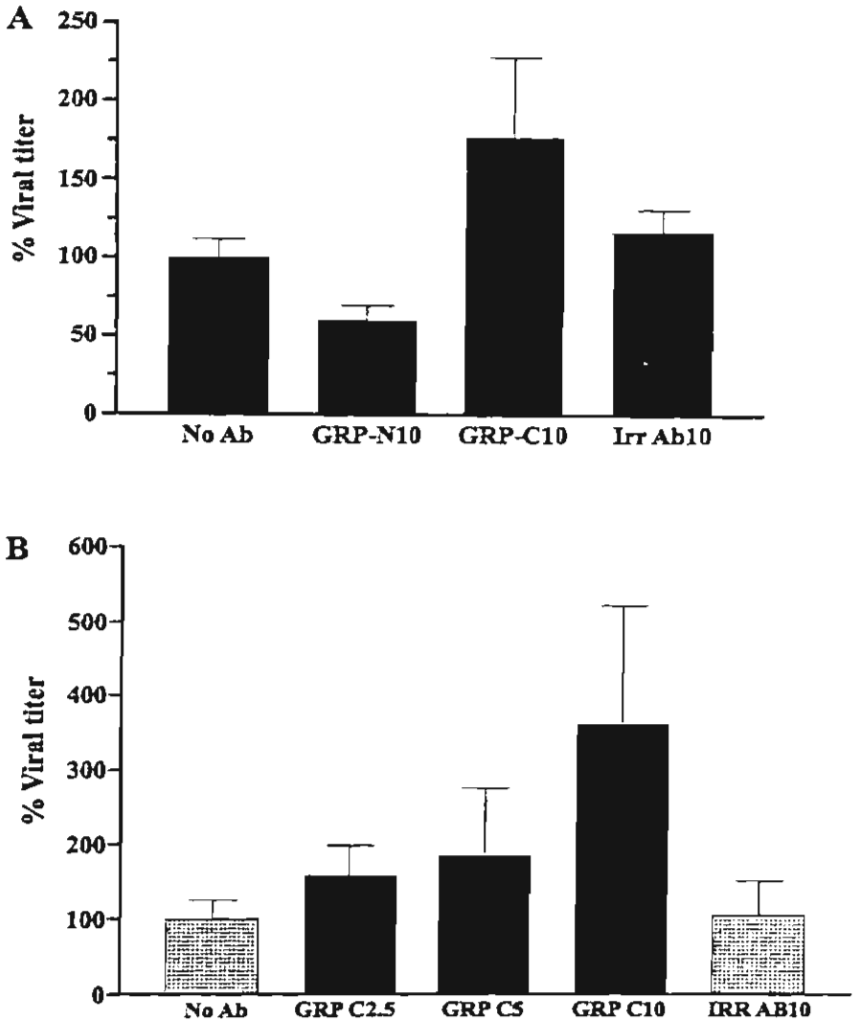

Fig. 7. **A** Antibody inhibition test. HepG2 cells were pre-incubated with either no antibody (No Ab), 10 μg of a polyclonal antibody directed against the N-terminus (GRP-N10) or C-terminus (GRP-C10) of GRP 78 (BiP) or 10 μg of an irrelevant monoclonal antibody (Irr Ab10) and subsequently incubated with dengue serotype 2 at a multiplicity of infection of 0.1. After 24 h the level of virus in the medium was assayed by plaque titer on Vero cells. Results are normalized against level of virus produced with no antibody pre-incubation and are the sum of two independent experiments carried out in triplicate with treble or six fold assay of titer. Error bars represent SEM. **B** Antibody inhibition test. HepG2 cells were pre-incubated with either no antibody (No Ab), 2.5 μg (GRP C2.5), 5 μg (GRP C5) or 10 μg (GRP C10) of a polyclonal antibody directed against the C-terminus of GRP 78 (BiP) or 10 μg of an irrelevant monoclonal antibody (IRR AB10) and subsequently incubated with dengue virus serotype 2 at a multiplicity of 0.1. After 24 h the level of virus in the medium was assayed by plaque titer on Vero cells. Results are normalized against level of virus produced with no antibody pre-incubation and are the sum of one experiment undertaken in triplicate with treble assay of titer. Error bars represent SEM

ticeably binding of the virus), albeit by approximately the same magnitude. While binding of proteins to GRP 78 (BiP) is believed to occur at the C-terminus of the protein, regulatory domains governing binding and acting through conformational changes in GRP 78 (BiP) are located at the N-terminus [10 and references therein], and as such it is likely that conformational changes induced through the binding of C-terminal antibodies may serve to enhance the ability of the virus to bind to the receptor, although it is possible that binding of the C-terminal antibody to GRP 78 (BiP) serves to activate the cell leading to enhanced viral replication.

Evidence from the western blot would suggest that it is actually a variant form of GRP 78 (BiP) that predominantly binds the dengue virus, although whether this is a translational or post-translational modification remains to be established, although post-translationally modified forms of GRP 78 (BiP) have been reported [15]. The immunoglobulin heavy chain binding function of GRP 78 (BiP) may be particularly significant in its interaction with the dengue virus as evidence has suggested that the functional binding motif of the dengue virus E protein resides in domain III and is characterized by an immunoglobulin like structure [11]. While this study has provided functional evidence that GRP78 (BiP) acts as a receptor element for dengue serotype 2 on liver cells by a virus reduction assay, further validation will require the transfection of a GRP78 (BiP) clone into a non-susceptible cell line to demonstrate the conversion of the cell line to a susceptible one. Given the dearth of non-susceptible cell lines, this may prove somewhat problematic. Finally, the presence of additional (albeit weaker) bands on the VOPBA analysis, coupled with only partial inhibition seen with the antibody directed against the N-terminus of GRP 78 (BiP) suggests that additional protein elements play a role in facilitating the entry of the virus into liver cells.

Acknowledgements

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Adaptation of the plaque assay methodology for dengue virus infected HepG2 cells

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Abstract

The HepG2 cell line is a useful tool for studying dengue virus–cell interactions but as it grows in clumps rather than monolayers, it does not readily adapt itself to the standard plaque assay technique. We therefore sought to develop an indirect plaque assay methodology. Initially HepG2 cells were infected with dengue virus serotype 2 and post-infection incubated for between 0 and 16 h before being treated with trypsin to separate the cells, followed by dilution and plating onto pre-grown monolayers of Vero cells in six well plates. After 7 days incubation and crystal violet staining, plaques were observed at all time points, although there was a relationship between number of plaques and post-infection incubation time, with the longest post-infection incubation time giving the highest number of plaques. To validate the assay with respect to virus input, the experiment was repeated at both the 0 and 16 h post-infection incubation times with different virus: cell levels. At both post-infection incubation times the response of input virus to plaque number was linear. This is a useful adaptation of the plaque assay methodology and one that may be applicable to other virus/cell line combinations.

Keywords: Flavivirus; Hepatocyte; Infection; Liver; Vero

Cell lines that are used commonly in plaque assays in dengue virus research include Vero (African Green monkey kidney) cells (Rao, 1976; Way et al., 1976; Matsumura et al., 1971), LLC-MK2 (Rhesus monkey kidney) cells (Morens et al., 1985; Kraiselburd et al., 1985) and BHK-21 (baby hamster kidney) cells (Malewicz and Jenkin, 1979). While these are useful cell lines for determining the levels of viruses, they are arguably less useful in studies investigating the mechanisms of entry of dengue viruses into cells given that they may not represent true target tissues, and as such their appropriateness may be somewhat limited. In dengue fever, the involvement of the liver in the pathogenesis of the disease is well documented (Kuo et al., 1992; Edelman et al., 1975; Bhamarapravati et al., 1967), and the dengue virus has been recovered from the liver of fatal cases of dengue fever (Rosen et al., 1999, 1989). As such, liver cell lines such as HepG2 which are derived from human hepatomas, probably more accurately represent a true target tissue. However, HepG2, while a useful cell line to study dengue virus: cell interactions has the disadvantage of growing in clumps rather than in a monolayers. As such, studies to detect the virus binding and internalization in this cell line are severely hampered due to an inability to maintain a concordance between an infected cell and a countable output, such as a plaque. We therefore sought to develop an indirect plaque assay by plating infected HepG2 cells onto monolayers of cells used commonly in the plaque assay technique, namely Vero cells.

We have established previously that it takes 18 h post-infection for mature infectious dengue serotype 2 viruses to be produced in HepG2 cells (Thepparit et al., Internalization and propagation of the dengue virus in human hepatoma (HepG2) cells, in press). Hence, if a dengue virus infected HepG2 cell was plated onto pre-grown monolayers of Vero cells, and the HepG2 cell was able to survive for 18 h a plaque should form. However, given that the conditions for growth under the overlay and under CO_2 levels required for maintenance of the Vero cells, it is possible that the HepG2 cells would not survive sufficiently long to produce mature viruses, or that this would be very in-

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significant effects upon the cells as a result of removal of most extracellular proteins. However, as stated earlier, this technique will have applications mainly in investigating the early mechanisms of binding and intenalization of the dengue virus. As such, the trypsin stage is post-internalization and will not affect this process. Other types of studies however may have to take this into account.

Overall the sensitivity of this methodology is relatively low, and calculations suggest that less that 1% of input viruses result in a plaque. However, provided that a constant post-infection incubation time is selected, the results, in respect of input virus, is linear and as such this method will have several applications in investigating the relationship between the dengue virus and liver cells, and may possibly be applicable to other virus/cell line combinations.

Acknowledgements

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INTERNALIZATION AND PROPAGATION OF THE DENGUE VIRUS IN HUMAN HEPATOMA (HEPG2) CELLS

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Key words: ***Flavivirus*, glycosaminoglycans, infection, liver**

Running Title : dengue virus in liver cells

ABSTRACT

Objectives: This study sought to undertake a comparative analysis of the internalization and propagation of all four dengue serotypes in a single cell line of human liver origin, HepG2.

Methods: Virus production after infection was determined by the plaque assay technique. Internalization profiles were determined by incubating virus and cells on ice and then raising the temperature for various times. The contribution of extracellular matrix components to internalization was determined by pretreatment of cells with either trypsin or Heparinase III.

Results: HepG2 cells were able to support the propagation of all four serotypes with mature viruses being produced by 12 hours for dengue serotype 4 and by 17 to 18 hours for the remaining serotypes. Virus internalization showed a plateau for serotypes 1, 2 and 4 entry while serotype 3 showed a constant increase in internalization for up to 5 hours. Pre-treatment of HepG2 cells with Heparinase III or trypsin both resulted in a reduction in viral production, with the smallest effect being noted for dengue serotype 3.

Conclusion: These results suggest that the interaction between the dengue virus and liver cells is a complex one that requires both protein and non-protein elements, and has a significant serotype/strain element.

and midzonal areas of the liver with Councilman bodies resembling those found in yellow fever cases have been reported [16], which are believed to be hepatocytes that have undergone apoptosis in response to dengue infection [11, 17]. The involvement of the liver in DHF and DSS is a characteristic sign that the disease will be fatal [18], and dengue virions have been recovered from liver biopsies of children with fatal dengue [13].

Despite the clinical evidence that the liver is an important target organ in DHF and DSS, few studies have addressed the nature of the interaction between the dengue virus and liver cells. Information regarding the binding, internalization and propagation of the dengue virus in liver cells is scattered and often fragmentary, especially given that most studies investigate only a single dengue serotype. Given that both serotype specific and cell specific factors have been implicated in the interaction between host cells and dengue viruses [19], comparisons between studies become problematic.

Given the absence of comprehensive studies on the basic parameters of dengue virus - liver cell interactions, this study sought to define the internalization and propagation of all four dengue serotypes to cells of a liver hepatoma origin, HepG2.

MATERIALS AND METHODS

Cells and viruses

The human hepatoma cell line HepG2 was cultured at 37°C, 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) and 100 units of penicillin and 100µg streptomycin. The African green monkey kidney cell line Vero was cultured at 37°C, 5% CO_2 in DMEM supplemented with 5% v/v FBS and antibiotics as for HepG2. Dengue serotypes 1 (strain 16007), 2 (strain 16681), 3 (strain 16562) and 4 (strain 1036) were a kind gift of Siritorn Butrapet (Center for Vaccine Development, Mahidol University). Dengue viruses were titered by ten fold serial dilutions on Vero cells followed by agarose overlay. After incubation for 7 days, plaques were determined by crystal violet staining.

Dengue virus production in HepG2 cells

HepG2 cells were grown in 75cm^2 tissue culture flasks containing 20ml of growth medium. Prior to confluence, when the cell number reached 1.0 x 10^7 the culture media was discarded and replaced with 3mls of BA-1 (1 x Medium 199 Earle's balanced salts; 0.05M Tris-HCl pH 7.6, 1% BSA Fraction V, 7.5% $NaHCO_3$, 100 units penicillin-streptomycin per ml) containing the dengue virus at a MOI of 1. Viral absorption was allowed to proceed for 90 minutes at 37°C with constant agitation. After this period cells were washed three times with PBS to remove unabsorbed viruses. Fresh culture media was added to the cells and the cells incubated under standard conditions for 7 days. Aliquots of the growth medium were taken daily for 7 days and analyzed for viral titer in duplicate. Experiment was

undertaken in duplicate. Data was analyzed using the GraphPad Prism program (GraphPad Software Inc, San Diego, Ca, USA).

To more precisely determine the early kinetic of viral production the above experiment was repeated with minor modifications. After the period of viral absorption, cells were treated with acid glycine (pH 3.0) [20, 21] for 1 minute to inactivate any un-internalized extra cellular viruses followed by washing in PBS. Aliquots of growth media were taken at hourly intervals between 10 and 24 hours and analyzed for viral titer in duplicate. The experiment was also undertaken in duplicate.

Viral Internalization

Sub-confluent HepG2 cells grown in a 5cm diameter dish were inoculated with dengue serotypes 1 to 4 (individually) at a MOI of 1 in BA-1 buffer at 4°C with gentle agitation for 2 hours to allow attachment of the virus to the cells surface to occur. Cells/virus mixes were then shifted to 37°C. At various time points the infected cells were washed once with PBS and then treated for 1 minute with acid glycine buffer (pH 3.0) at room temperature to inactivate any uninternalized viruses [20, 21]. Following acid glycine treatment cells were again washed with PBS. After washing normal growth media was added and cells incubated under standard conditions for 3 days. After three days the media was analyzed for viral titer in duplicate. Experiment was undertaken in duplicate.

Pre-treatment of cells with trypsin

HepG2 cells were washed with PBS and trypsinized with 0.25% trypsin-EDTA at 37°C for 5 minutes followed by the addition of fresh culture medium to inactivate trypsin. The cell suspension was distributed to 15-ml tubes ($1.08x10^6$ cells per tube) and cells infected with dengue virus at a MOI of 1 at 37°C. At times of 0, 20, 40, 60, 90, 120, and 150 minutes uninternalized extracellular viruses were removed by washing infected cells with PBS and treatment with acid glycine pH 3 [20, 21] followed by a further wash with PBS. After each washing step the infected cells were collected by centrifugation at 1,000 xg for 5 min. The infected cells were finally plated into 60 mm tissue culture dishes. After three days of growth culture medium was collected and assayed in duplicate for level of virus. Control infections were undertaken identically, with the exception that cells were grown to subconfluence directly in 60mm culture dishes and infected when number reached 1.08×10^6. Experiment was undertaken in duplicate.

Pre-treatment of cells with Heparinase III

HepG2 cells (5×10^5) were seeded into 48 well culture plates and grown for 24 hours after which time cells (1×10^6) were washed once with DMEM medium. Cells were then treated with 1 unit/ml Heparinase III (Sigma Chemical Co., St Louis, MO, USA) in DMEM at 37°C for 1 hour. Treated cells were then washed twice with BA-1 buffer before incubating with the dengue virus at a MOI of 1 for 90 minutes, following which cells were washed with PBS and extra cellular viruses inactivated with acid glycine for 1 minute at room temperature [20, 21]. Cells were again washed once with PBS followed by once with DMEM. Subsequently DMEM supplemented with 10% FBS was added and cells incubated at 37°C/10% CO_2 for 4 days at which point

levels of virus in the media were assayed by standard plaque assay in duplicate. Control samples were run in parallel and incubated with only DMEM instead of DMEM/heparinase III. Experiment was undertaken independently in triplicate.